The effect of single nucleotide polymorphisms of oxidative stress genes and low grade inflammation upon pulse wave contour analysis, a useful non invasive, intermediate vascular phenotype.

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1.1 Synopsis of Thesis

Cardiovascular disease remains a major cause of mortality and morbidity and is underpinned by Oxidative stress, within which, inactivation of nitric oxide (NO) by superoxide (SO) and other reactive oxygen species is characteristic. Two major enzyme systems are implicated within oxidative stress; NAD(P)H oxidase and endothelial nitric oxide synthase (eNOS). eNOS generates NO while at the same time, and within the same cells, NAD(P)H plays a powerful role in the generation of SO. Evidence is accumulating that polymorphisms of the genes encoding these enzyme systems may play an important role in the pathophysiology of CAD. Additionally there has been much recent interest in both biochemical markers of oxidative stress and low grade chronic inflammation as well as a non invasive vascular phenotype, pulse wave analysis. This thesis reports a series of studies (utilising the techniques described in chapter 2) which aimed to ascertain:-

The reproducibility of pulse contour analysis as a non invasive intermediate cardiovascular phenotype (Chapter 3).

Whether common single nucleotide polymorphisms of the p22phox gene *CYBA* and the endothelial nitric oxide synthase gene, *NOS3,* have an effect upon arterial compliance in patients with coronary artery disease (Chapters 4,5 and 6).

In healthy volunteers, free of cardiovascular disease whether a relationship existed between markers of low grade inflammation and arterial stiffness (Chapter 7).

Chapter 3: The reproducibility of diastolic pulse wave contour analysis and its relation to systolic pulse contour analysis.

This clinical study demonstrated that both large (C1) and small artery (C2) compliance values were reproducible and that there was a significant correlation between both Augmentation Index (AIx) and C1and AIx and C2 in healthy volunteers and though there was no association

between AIx and C1 in patients with coronary artery disease AIx did correlate with C2 in this population.

Chapter 4: The effect of the G894T SNP of the NOS3 gene upon arterial stiffness in patients with coronary artery disease.

There was no association observed between this polymorphism and blood pressure or large artery compliance however ANOVA revealed a statistically significant association for TT homozygosity and small artery compliance. The highest small artery compliance was seen in the patients homozygous for the G allele, an intermediate value observed in heterozygotes and the lowest value demonstrated in patients homozygous for the T allele. Multiple regression analysis, examining the possible contribution of confounders showed that only small artery compliance was significant when *NOS3* G894T genotype was assigned as the dependent variable.

Chapter 5: The C242T single nucleotide polymorphism of the CYBA gene and blood pressure and arterial compliance in patients with coronary artery disease.

We sought to examine the influence of the C242T SNP of *CYBA* upon vascular compliance and blood pressure using the dominant allele model. The presence of the 242T allele was associated with significantly higher systolic blood pressure. Patients homozygous for the C allele had lower systolic blood pressure than heterozygotes and patients homozygous for the T allele. There was no statistically significant effect upon diastolic blood pressure but there was however a significant association observed between the 242T allele and pulse pressure.

Chapter 6: Combined analysis of NOS3 G894T and CYBA C242T genotypes upon arterial stiffness.

In order to contrast the arterial stiffness between the favourable versus the non-favourable genotypes patients homozygous for the *NOS3* G allele and homozygous for the *CYBA* C allele were compared with those homozygous for the *NOS3*T allele and possessing the *CYBA* 242T allele. The former displayed higher large and small artery compliance than the latter group. Multiple regression analysis, examining the possible contribution of confounders showed that only the large and small artery compliance values contributed significantly when genotype was assigned as the dependent variable.

Chapter 7 Chronic low grade inflammation and insulin resistance and arterial compliance in healthy volunteers.

Within healthy volunteers multiple regression analysis showed that small artery compliance was significantly associated with IL 6, CRP and ICAM. Augmentation index showed only an association with ICAM1. There was no significant correlation between Adiponectin levels and either of the arterial stiffness parameters studied.

Conclusions

Diastolic pulse wave contour analysis is a reproducible assessment of arterial stiffness with the potential to represent a high fidelity non invasive vascular phenotype.

Small artery compliance is correlated with Augmentation Index and although the measurements are not analogous they both represent useful means of acquiring quantitative data concerning arterial stiffness.

The 242T allele of the p22phox gene, *CYBA*, is associated with decreased large but not small artery compliance and increased systolic and pulse pressure.

Homozygosity for a common *NOS3* polymorphism (894 G→T) was associated with decreased small artery compliance but not with large artery compliance or blood pressure.

The markers of chronic inflammation Interleukin 6, ICAM and hsCRP but not Adiponectin, a marker of Insulin resistance, predict small artery compliance in healthy individuals apparently free of vascular disease.

1.2 Declaration

I declare that this thesis has been composed entirely by myself and is a record of work performed by myself. It has not been previously submitted for any other degree. The work described in this thesis was carried out under the supervision of Professor Anna F Dominiczak in the Division of Cardiovascular and Medical Sciences at the Western Infirmary, Glasgow.

1.3 Acknowledgements

I am greatly indebted to Professor Anna Dominiczak my supervisor who guided me throughout these studies from conception to completion. Her enthusiasm, energy and attention to detail made the process an enjoyable one. I am also grateful to both Professor John Reid who gave me the opportunity to pursue a higher degree within the Division of Cardiovascular and Medical Sciences and to Professor John Connell who provided excellent support during the period of research within the metabolic and vascular research group meeting.

I am most grateful to Drs' Wai K Lee, Nick Brain and Mrs Koh-Tan who performed the genotyping described in chapters 4 and 5. Dr John Mclure provided statistical advice at each stage and guided me through the regression analysis. Professor Naveed Sattar and Dr Lynne Cherry performed the analysis of the parameters associated with low grade chronic inflammation in chapter 7.

The members of the British Heart Foundation Glasgow Cardiovascular Research Centre over the study duration all provided useful discussion during the weekly laboratory meetings, especially with regards to presentations as well as continual tuition in genetics and molecular biology. The subjects who volunteered for the study deserve special mention. The patients who attended the week prior to Coronary Artery Bypass Graft operation gave of their time willingly as did the healthy volunteers with no financial recompense.

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1.6 List of Publications, abstracts, oral and poster presentations.

Publications

Strategies to reduce oxidative stress in cardiovascular disease.

Hamilton CA, Miller WH, Brosnan MJ, **Drummond RS**, McBride MW & Dominiczak AF.

Clinical Science106(3):219-34, 2004.

Abstracts

Single nucleotide polymorphisms (SNP's) within oxidative stress genes affect arterial compliance in patients with coronary artery disease: gene-function relationships.

Drummond RS, Brosnan MJ, Kirk A, Hamilton MJ, Connell JMC & Dominiczak AF.

Association of Physicians of Great Britain and Ireland, Dublin 2004.

Quarterly Journal of Medicine 2004; 97: 615-629.

A single nucleotide polymorphism in the p22 phox gene affects arterial compliance.

Drummond RS, Brosnan MJ, Tan C, Lee WK, Al-Benna S, Kirk A, Hamilton CA & Dominiczak AF.

Hypertension 2003 42(3) 388-448.

A single nucleotide polymorphism in the p22phox gene affects arterial compliance in coronary artery disease.

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Journal of Hypertension vol 21(suppl 4) June 2003. S317.

A single nucleotide polymorphism in the p22phox gene affects arterial compliance in coronary artery disease.

Drummond RS, Brosnan MJ, Lee WK, Kirk A, Hamilton CA & Dominiczak AF.

British Endocrine Society National conference, Glasgow, March 2003.

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Oral Presentations to learned societies

Single nucleotide polymorphisms (SNP's) within oxidative stress genes affect arterial compliance in patients with coronary artery disease: gene-function relationships.

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Association of physicians of Great Britain and Ireland, Dublin 2004.

A single nucleotide polymorphism in the p22 phox gene affects arterial compliance.

Drummond RS, Brosnan MJ, Tan C, Lee WK, Al-Benna S, Kirk A, Hamilton CA & Dominiczak AF.

57th Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research in association with the Council on Kidney in Cardiovascular Disease, Washington DC Sept 2003.

Single nucleotide polymorphisms in oxidative stress genes and vascular compliance.

Drummond RS, Brosnan MJ, Tan C, Lee WK, Al-Benna S, Kirk A, Connell JMC, Hamilton CA & Dominiczak AF.

Scottish Society of Experimental Medicine, Aberdeen May 2003.

Winner of Sir James Black Young Investigator Prize.

Oxidative Stress Genes and Vascular Compliance in Coronary Artery Disease.

Drummond RS, Brosnan MJ, Tan C, Lee WK, Al-Benna S, Kirk A, Connell JMC, Hamilton CA & Dominiczak AF.

British Hypertension and Vascular Research Group, Turnberry, April 2003.

A single nucleotide polymorphism in the p22phox gene affects arterial compliance in coronary artery disease.

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American Diabetes Association, Chicago 2007.

The variable number of tandem repeat polymorphism in the *NOS3* gene is associated with arterial compliance and insulin resistance in coronary artery disease.

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Drummond RS, Brosnan MJ, Lee WK, Kirk A, Hamilton CA & Dominiczak AF.
Scottish Society of Experimental Medicine Edinburgh 2002.

1.7 List of Abbreviations

AIx	Augmentation Index
ANOVA	Analysis of variance
C1	Large Artery Compliance
C2	Small Artery Compliance
CHD	Coronary Heart Disease
CRP	C Reactive Protein
CVD	Cardiovascular disease
DBP	Diastolic Blood Pressure
DPCA	Diastolic Pulse Contour Analysis
eNOS	Endothelial Nitric Oxide Synthase
HDL	High Density Lipoprotein
IL-6	Interleukin 6
L-NMMA	L-N^G-monomethyl arginine
LDL	Low Density Lipoprotein
MAP	Mean Arterial Pressure
NADH/NAD(P)H	Nicotinamide Adenine Dinucleotide (phosphate) Oxidases
NO	Nitric Oxide
$\bullet O2^-$	Superoxide
PP	Pulse Pressure
PWA	Pulse Wave Analysis
PWV	Pulse Wave Velocity
SBP	Systolic Blood Pressure
sICAM-1	Soluble Intracellular Adhesion Molecule 1.

SD	Standard Deviation
SEM	Standard Error of the Mean
SNP	Single Nucleotide Polymorphism
SPCA	Systolic Pulse Contour Analysis
UK	United Kingdom
VNTR	Variable Number of Tandem repeats

1 Introduction

Cardiovascular disease (CVD) is a designation for multiple pathologies underscored by atherosclerosis of which there are multiple clinical phenotypes. CVD is the main cause of death in the United Kingdom (UK) accounting for approximately 238,000 deaths per year: 39% of all deaths (BHF statistics at www.heartstats.org). The principle forms of CVD are Coronary Heart Disease (CHD) and stroke. CHD itself causes over 117,000 deaths per year in the UK: approximately 1 in 5 deaths in men and 1 in 6 deaths in women. It is the most common cause of premature death in the UK: 22% of premature deaths in men and 13% of premature deaths in women are from CHD. Over 1.2 million people in the UK have had a myocardial infarction and it is estimated that 2 million people are suffering from angina (www.heartstats.org). The aim of this thesis is to investigate non invasive intermediate vascular phenotypes that have a potential to be useful in the assessment of cardiovascular disease; namely pulse wave contour analysis and biochemical markers of inflammation.

Conventionally risk stratification has rested upon traditional, established risk factors including smoking, poor diet, lack of physical activity, obesity, hypertension, hypercholesterolemia and diabetes mellitus. Genetic factors are increasingly recognised to predispose to CVD from studies that have provided long standing evidence that CVD clusters in families (Rose 1964). The advent of pulse wave analysis (PWA) as a non invasive vascular phenotype and increasing understanding of the importance of new surrogate markers of cardiovascular disease has introduced the capability of new ways of risk stratification and new methods of investigating the effect of genotype in CVD. It had previously been calculated that traditional risk factors *per se* may only explain approximately between 50%(Manson 1992) of the CHD risk but more recently this figure has been more accurately calculated as 80-90% (Khot 2003). A portion of

risk however remains unexplained and it is timely to examine the association between genetic factors, biochemical surrogate markers as well as non invasive vascular phenotypes with CHD.

The purpose of this thesis is to describe the relationship of genotypes of common oxidative stress gene polymorphisms upon these new vascular phenotypes. The introduction will seek to depict PWA as a useful non invasive cardiovascular phenotype, describe the common oxidative stress gene single nucleotide polymorphisms and their role in cardiovascular physiology and pathophysiology and clarify the potential function of biochemical markers of low grade inflammation and insulin resistance in CVD.

1.1 Pulse Wave Analysis and Pulse Wave Velocity: Non-invasive vascular intermediate phenotypes.

1.1.1 Introduction and historical perspective.

Cardiovascular research has long searched for the holy grail of a non-invasive, high fidelity, reproducible technique to function as an intermediate phenotype facilitating mechanistic basic physiology, intervention and outcome studies. Returning to techniques first utilised over 100 years ago has led to the development of methodology which may end this search.

The arterial pulse waveform has been interesting clinical scientists since the end of the 19th century and the assessment of the arterial pulse remains a bedrock of basic clinical examination. Following the invention of the sphygmograph by Marey (Roy 1880) clinicians like Osler and Mohamed relied upon it heavily as a diagnostic tool (Sharpey 1866). Ultimately however the sphygmograph was superseded by the sphygmomanometer which was invented for recording blood pressure and used widely thereafter for clinical purposes. Technological

advances now allow non-invasive acquisition of pressure pulse waveforms in a repeatable and reproducible manner. Early and consistent changes in the pulse contour occur with aging and cardiovascular risk factors and therefore descriptive analysis of the pulse contour may ultimately hold the potential to refine cardiovascular risk stratification and guide therapeutic interventions (McVeigh 2003).

1.1.2 Why move beyond blood pressure?

The Framingham Heart Study has generated important data on how systolic blood pressure (SBP) diastolic blood pressure (DBP) and pulse pressure (PP, the difference between SBP and DBP) change with advancing age (Franklin 1997). DBP is determined classically by peripheral arterial resistance and increases until middle age when it then tends to fall. SBP and PP, however, are influenced by the stiffness of large arteries, the peripheral pulse wave reflection and the pattern of left ventricular ejection and increase persistently with age. Changes in large artery (i.e. aorta or its major branches) stiffness accounts for the changes in SBP, DBP and PP observed from 50 years of age. The link between blood pressure and cardiovascular disease has been clearly established (Goldberg 1996), and SBP has been identified as having a greater predictive value for CHD than DBP in those aged over 60years (Kannel 1971, Franklin 2001a). Isolated systolic hypertension (ISH) where SBP is >140mmHG 140mmHg and DBP is <90 mmHg is the most commonly observed form of hypertension in the elderly (Franklin 2001b) and is a major risk factor for stroke (Nielson 1995), coronary heart disease (Franklin 2001a) and total cardiovascular mortality (Antikainen 1998). Also recently the brachial artery PP, the chief determinant of which in the elderly is large artery stiffness, has emerged, albeit controversially (Prospective studies collaboration Lancet 2002), as an even more powerful predictor of CHD (Franklin 1999).

Hence with the integral part that altering arterial stiffness plays, not only in the ageing process but also, as will be discussed, with other risk factors for cardiovascular disease there is a growing appreciation that a better understanding of the mechanisms involved in generation of arterial stiffness will improve the treatment of hypertension and cardiovascular disease than that offered by simply measuring brachial BP. The ability to measure and monitor arterial stiffness would confer not only a useful research tool but also a clinical tool that will aid all levels of practitioners and physicians in diagnosis, treatment and possibly risk stratification.

1.1.3 Arterial properties and arterial stiffness.

Risk factors for cardiovascular disease mediate their effects by altering the structure, properties, and function of wall and endothelial components of arterial blood vessels (Gibbons 1994).The ability to detect and monitor change in the physical properties of arteries, representative of the cumulative and integrated influence of heamodynamic, metabolic and inflammatory stimuli in impairing arterial wall integrity, holds potential to intervene at a preclinical stage to prevent or attenuate disease progression (McVeigh 2003).

The elasticity of the arteries is not uniform and varies throughout different sites within the arterial tree. The elasticity of the proximal large arteries is the result of the high elastin to collagen ratio in their walls which steadily diminishes toward the periphery. The increase in arterial stiffness that occurs with increasing age (Hallock 1937) is largely due to progressive elastic fibre degeneration (Avolio 1998). The elasticity of a given arterial segment is not constant but depends on the distending pressure (Greenfield 1962). As distending pressure increases, there is greater recruitment of relatively inelastic collagen fibres (Roach 1957, Apter

1967, Bank 1996) and consequently, a reduction in elasticity. The background level of distending pressure in the circulation is determined by mean arterial pressure (Oliver 2003).

Elasticity is not only dependent on collagen and elastin but the endothelium (Kinlay 2001, McVeigh 2001, Wilkinson 2002a) and arterial wall smooth muscle bulk and tone (Bank 1996, Bank 1999) also contribute to elasticity. The latter is under control from the endothelium and the balance between the vascular smooth muscle cell derived free radical and superoxide (SO) and the endothelium derived nitric oxide (NO) will be discussed later in more detail.

1.1.4 Genetics and arterial stiffness.

Polymorphisms within genes that generate the components that define arterial structure and function have been related to arterial stiffness. Polymorphic variation in the fibrillin (Medley 2002), angiotensin II type I receptor (Lajemi 2001a), and endothelin receptor genes (Lajemi 2001b) have been associated with altered arterial stiffness. Additionally the angiotensin converting enzyme (ACE) I/D polymorphism has been associated, albeit inconsistently with vascular stiffness (Lajemi 2001a, Balkestein 2001). Thus putatively as eluded to above and to be discussed in more detail later in the introduction logically it could be hypothesised that allelic polymorphisms amongst genes involved in the oxidative stress pathway, whose products influence the relative bioactivity of NO and consequently vascular tone, may also influence vascular stiffness.

1.1.5 Generation of the arterial pulse wave and pulse pressure amplification.

Left ventricular ejection pumps blood into the arterial tree but simultaneously creates a pulse pressure wave that travels in the arterial wall in an anterograde fashion until it reaches areas of bifurcation or other areas of impedance mismatch, mainly at high resistance arterioles, where a retrograde (reflected) waveform is initiated (Nichols & O'Rourke 1998). The shapes of the arterial pulse waveforms vary at different sites, attributable to both varying elastic qualities along the arterial tree and to wave reflection. In healthy, young subjects without vascular disease mean arterial pressure (MAP) declines in the peripheral circulation but SBP and PP are amplified (Kroeker 1955), amplification which is accentuated during exercise and diminishes with increasing age (Rowell 1968, Wilkinson 2001). The wave form at the proximal aorta is critical as it is the BP profile here rather than the typically measured peripheral BP which determines left ventricular afterload and coronary blood flow. The contour and amplitude of the pressure waveform are influenced by large artery pulse wave velocity (PWV) as pressure waves of higher velocity arrive and are reflected from the peripheral circulation earlier. With compliant arteries and slow PWV, reflected waves return to the central aorta in diastole, augmenting DBP and subsequently coronary blood flow which occurs in diastole. On the contrary when arteries are less complaint and PWV fast, the reflected waves return earlier and augment central SBP, increasing left ventricular workload and compromising coronary blood flow (Bogren 1989, Ohtsuka 1994, Oliver 2003).

1.1.6 Definitions of parameters used to describe the static and dynamic qualities of the arterial tree.

One of the problems in studying or discussing arterial stiffness is that it is a term which is imprecise and has been applied to a wide variety of different vascular parameters. These include arterial compliance, arterial distensibility (change in area or diameter of an artery resultant from a given change in pressure), elastic modulus (the change in stress for a given change in strain of the wall materials), volume elastic modulus, Young's modulus, PWV, characteristic impedance (the ratio of pressure and flow in an artery when pressure and flow waves are not influenced by wave reflection), stiffness index (β), capacitive compliance and oscillatory compliance as measures representative of the mechanical properties of arteries (O'Rourke 1999, McVeigh 2002). Table 1.1 outlines definitions of commonly used indices that are used to generate quantitative estimates of arterial wall properties.

Table .1.1 Definitions of the commonly measured indices of arterial stiffness.

Parameter	**Definition**	**Formula & Units**
Arterial Compliance	Absolute diameter or area change for a given pressure step at fixed vessel length.	$\Delta D/ \Delta P$(cm/mmHg or cm^2/mmHg)
Arterial Distensibility	Relative diameter or area change for a given pressure increment; the inverse of elastic modulus.	$\Delta D/ \Delta P \times D$ ($mmHg^{-1}$)
Volume Elastic Modulus	Pressure step required for a theoretical 100% increase in volume where there is no change in length.	$\Delta P/(\Delta V/V)$ (mmHg)= $\Delta P(\Delta D/D)$(mmHg)
Elastic Modulus	Pressure step required for a theoretical 100% increase in stretch from resting diameter at fixed vessel length.	$(\Delta P \times D/\Delta D)$ (mmHg)·
Young's Modulus	Elastic modulus per unit area; the pressure step required per square centimetre for a theoretical 100% stretch from resting length (accounts for wall thickness).	$\Delta P \times D/(\Delta D \times h)$ (mmHg/cm)·
Pulse Wave Velocity	The speed at which the pulse wave travels along a defined arterial segment.	Distance/Δt (m/second)
Pressure Augmentation	Increase in aortic or carotid pressure after the peak of blood flow in the vessel.	mmHg or % of pulse pressure
Characteristic Impedance.	Relationship between pressure change and flow velocity in the absence of wave reflections.	$(\Delta P/\Delta V)$[(mmHg/cm)s]
Stiffness Index. B	Ratio of logarithm (systolic/diastolic pressures) to (relative change in diameter).	B = In (Ps/Pd)/ [(Ds-Dd)/Ds] (non dimensional)
C1 Large Artery Elasticity Index/ Capacitative.	Relationship between the decline in pressure and decline in volume in the arterial tree during the exponential component of diastolic pressure decay.	$(\Delta V/\Delta P)$ ml/mmHg x 10
C2 Small Artery Elasticity Index/ Oscillatory or Reflective.	Relationship between oscillating pressure change and oscillating volume change around the exponential component of diastolic pressure decay.	$(\Delta P/\Delta V)$ ml/mmHg x 100

P = pressure, D = diameter, V = volume, h = wall thickness, t = time, s = systolic, d = diastolic.

Adapted from Oliver 2003 & O'Rourke 2002.

1.2 Applanation tonometry, pulse wave velocity and pulse contour analysis.

The technique of applanation tonometry detects the pressure pulse wave at a distinct anatomical site, for example the radial artery at the wrist by using a micromanometer tipped probe. The artery is compressed between the sensor and the underlying structures, and therefore the intra-arterial pulse pressure is transmitted through the arterial wall to the sensor. The recorded pressure waveform is then digitalized such that it can then be viewed upon a computer screen. The waveform that is recorded as stated above varies in different vessels in the same individual and is dependent on

1) The viscoelastic properties of the artery.
2) The viscosity of the blood.
3) Wave reflection.
4) Wave dispersion.

As will be discussed below the computer software then utilises the recorded peripheral wave to either analyse the recorded pulse pressure wave in terms of a modified Windkessel model to generate arbitrarily labelled large and small artery compliance values (Diastolic Pulse Contour Analysis (DPCA)) or to use a mathematical generalised transfer function to construct the corresponding central aortic pressure and waveforms (Systolic Pulse Contour Analysis (SPCA)).

1.2.1 Pulse Wave Velocity

Pulse Wave Velocity is simply a function of distance and time.

Velocity = Distance (m)/Time (seconds)

To calculate the PWV, the time delay between the pulse pressure waves at two distinct sites has to be calculated by either placing probes simultaneously over two sites or by recording the waveforms at two sites independently and comparing the time delay at both sites against a simultaneously recorded QRS complex. Examples of sites where PWV is commonly recorded include carotid and radial arteries, or carotid and femoral arteries. Distance is measured simply with a suitable device. Calculating the time from the foot of the pressure wave at the first point to the foot of the pressure wave as it arrives at the next point is more complicated as it can be difficult to ascertain where the foot of the wave is, in particular identifying and defining the carotid pulse wave shape can be technically very difficult (Schram 2004). There are four recognised methods for calculating the location of the foot of the wave (Davies 2003a). The method using the point at which the second derivative of the pressure wave is maximal and the method looking at the point yielded by the intersection of a line tangent to the initial systolic upstroke of the pressure tracing and a horizontal line through the minimal point are the most reproducible. These are known as the second derivative method and the intersecting tangent method respectively (Chiu 1991). Comparison of studies utilising PWV as the means of assessing the arterial tree is must take into account the anatomy measured as there is no uniformity to the arterial tree segment that is being studied (i.e. carotid-femoral or carotid-radial) and there are differences in arterial elasticity and tendency to atherosclerosis at different sites.

The strength of this technique is that it is simple to perform, no mathematical transformation is required and there is good published inter- and intra-operator reproducibility (Wilkinson 1998). Liang and colleagues report good repeatability of measurements preformed on two separate occasions (Liang 1998). Another group, however, reported a 16% variation in PWV on separate study days (Chiu 1991) which they suggest could be caused by changes in blood pressure, respiratory changes in arterial pressure or movement of the transducer on the skin overlying the arterial measuring site.

PWV is sensitive to changes in heart rate and blood pressure (Quick 1998, Mitchell 1997, McVeigh 2002a) and small changes in arterial wall properties may not be detected between individuals, as data generated can often show considerable scatter for a given age range (Avolio 1983). Stewart *et al* (Stewart 2003) have demonstrated that inhibiting basal NO release caused an in increase in PWV, due to changes in MAP.

Probably the most important facet of the literature pertaining to PWV is that it has been shown to be an independent predictor of outcome in high risk populations (Blacher 1999a, Blacher 1999b, Blacher 2003, Guerin 2001). The utility of further refining risk stratification in patients already designated as being high risk, who should be receiving optimal therapy for risk reduction, has been suggested to be of limited clinical value (McVeigh 2002a).

1.2.2 Systolic pulse contour analysis.

SPCA is generated from pulse pressure waveforms recorded by applanation tonometry. When recorded at the radial artery at the wrist the waveform is calibrated to the brachial BP measured conventionally by arm/cuff oscillometric means. This has recently come under scrutiny and the

inaccuracy of oscillometric cuff method for measuring arm blood pressure was identified as the limiting factor in predicting potentially clinically useful, non-invasive aortic pressures (Smulyan 2003).

SPCA uses a generalised transfer function to derive central aortic waveforms from those acquired from the peripherally acquired arterial waveform – most commonly from the radial artery. From the generated central aortic waveform central BP values and the augmentation index (AIx) can then be calculated. The AIx is the proportion of central PP that results from arterial reflection and is a commonly, and successfully, used measure of arterial stiffness (Figure 1.1).

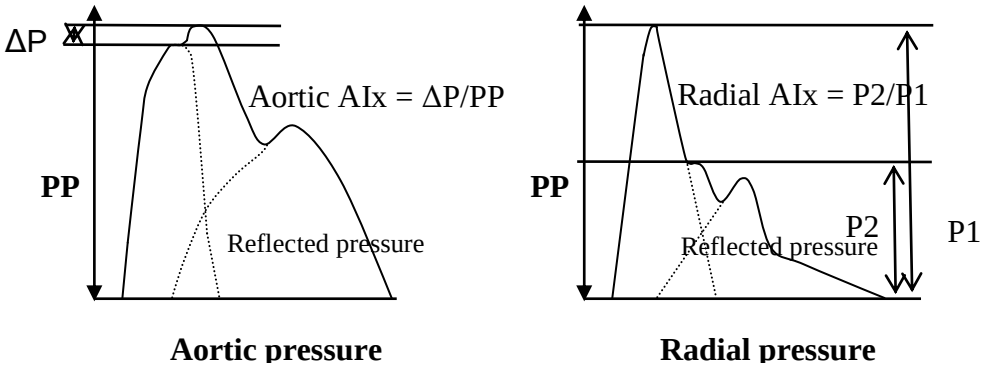


Figure 1.1: Augmentation Index.

Aix, the ratio of pressure augmentation to local pulse pressure (PP), can be defined for both the aortic and radial pulse waveforms. It depends on the relative amplitude and timing of the direct and reflected waves that summate to produce the overall waveform (from Oliver 2003).

The timing of arrival of the reflected wave at the proximal aorta is determined chiefly by large artery PWV. AIx is not, however simply a surrogate or a different way of measuring PWV. Simultaneous measurement of AIx using the generalised transfer function and PWV, used as an

estimate of arterial stiffness revealed a positive (r=0.29) but modest correlation (Yasmin 1999). In this study only 9% of the variation of PWV was explained or accounted by variation in AIx.

The Aortic AIx increases with age and blood pressure (Nichols & O'Rourke 1998) and is elevated in subjects with other risk factors for cardiovascular disease namely diabetes (Wilkinson 2000) and hypercholesterolemia (Wilkinson 2002b). In patients with end stage renal failure (a group with high cardiovascular mortality) AIx is a highly predicative indicator of cardiovascular mortality (London 2001). As with the other vascular parameters derived using applanation tonometry AIx is sensitive to modulations of NO bioactivity. Inhibition of basal NO synthesis, with intra-arterial infusion of L-N^G-monomethyl arginine (L-NMMA), has been shown to lead to a dose dependent increase in MAP, peripheral vascular resistance, and aortic and systemic arterial stiffness (Wilkinson 2002a).

There have been a number of criticisms levelled at the use of a generalised rather than an individualised transfer function and moreover at the use of any transfer function whatsoever. The use of a generalised transfer function can show bias and a significant amount of the variation in predicting the centrally obtained AIx (Glasser 1997, Chen 1997, Stergioplus 1998). SPCA has recently been used increasingly in healthy volunteer studies and it merits stressing that the generalized transfer function has never been validated in young healthy volunteers (Oliver 2003). Millasseau and colleagues have recently suggested that similar information on central pressure wave reflection can be obtained directly from the radial pulse and a radial AIx without use of a generalised transfer function (Millasseau 2003).

The unequivocal association of increased Aix with arterial stiffness in recent publications (Wilkinson 2000, Wilkinson 1999, Brooks 1999) has been criticised (McVeigh 2002a).

Although in some circumstances this may indeed be the scenario this supposition is to some degree speculative as it is based solely on a descriptive change in waveform morphology.

The prognostic value of AIx has been shown in that carotid AIx is an independent predictor of cardiac ischaemic threshold during exercise in patients with CHD (Kingwell 2002). Additionally Aix is also a predictor of all cause and cardiovascular mortality in patients with end stage renal failure, even in those with normal PWV (London 2001).

1.2.3 Diastolic pulse contour analysis.

Cohn and McVeigh have pioneered DCPA as a useful research methodology for the early detection of vascular disease. Again the technology utilises applanation tonometry of the radial artery at the wrist and measures a waveform that is calibrated against the brachial BP.

The modified Windkessel model (figure 1.2) is employed to interpret the consistent and predictable changes in the pulse pressure wave shape in diastole in terms of compliance, inertance and resistance in the arterial system (McVeigh 2002a). The Windkessel concept represents a lumped parameter non-propagative approach to interpret changes in the arterial mechanical properties that doesn't account for wave travel in the arterial system.

The Windkessel model was first described, in 1769, by Stephen Hales. The Windkessel was the air filled dome in early fire engines which acted as a cushion to absorb fluctuations in water pressure delivered directly to the engine reservoir, hence allowing a smooth delivery of water to the fire hose. (O'Rourke 1992). The physiological analogy is obvious with the pulsatile pump representing the heart, the Windkessel reservoir the central elastic arteries into which the

heart pumps and the fire hose as the peripheral resistance (i.e. relatively non –elastic conduits of the peripheral arteries. The problem with this model is the assumption of uniformity throughout the arterial tree and as arteries differ in compliance and elasticity to smaller, and more muscular arteries such as the brachial and radial vessels. Additionally compliance varies throughout the length of each artery around junctions, curves and bifurcations (Kawasaki 1987, Van Merode 1991). This model cannot therefore account for wave amplification or the secondary diastolic wave that results from wave reflection in the arterial system.

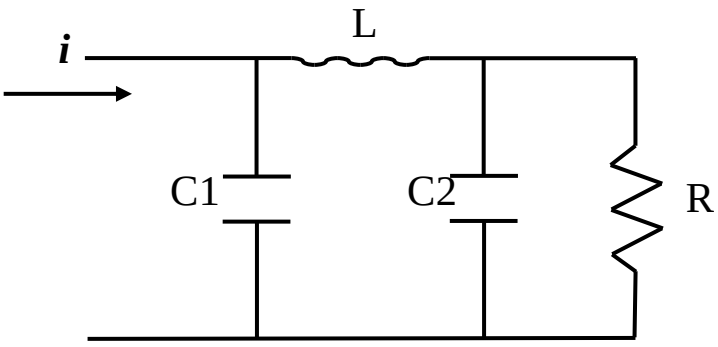


Figure 1.2: The modified Windkessel model of the arterial system. C1, proximal compliance; C2, Distal compliance; I, systemic inflow; L, inertance; R systemic vascular resistance.

The energy contracts between all the elements in a modified Windkessel model can account for distortions in pressure-pulse contour produced by wave reflection, interpreting these changes in terms of altered compliance, resistance and inertance elements in the model (McVeigh 2002a). This non-invasive approach generates the cardiac output from an algorithm incorporating the radial pulse pressure waveform (McVeigh 1999). Two components of the diastolic waveform

are distinguished in DCPA. An exponential decay curve represents large artery compliance (C1). Oscillatory or reflective compliance (C2) consists of peripheral wave reflections that are superimposed upon the basic waveform and provide a measure of small artery compliance (Watt 1976).

Values obtained by this non-invasive technique have been compared with those obtained from waveforms generated invasively (Cohn 1995). Non-invasively derived waveforms from the tonometer were found to underestimate, but were tightly correlated to, pressure wave forms obtained invasively. Compliance values calculated from invasive and non-invasive methods were correlated statistically, though more strongly for C1 than for C2, with non-invasive measures tending to overestimate.

Again, as with SPCA, the derived model based parameters change in a consistent and predictable manner with ageing and with disease states associated with vascular disease (Cohn 1995, Finkelstein 1982, McVeigh 1999, Watt 1976).

Utilising both brachial artery waveforms obtained invasively and radial artery waveforms obtained non-invasively with tonometry increased age was associated with lower C1 and C2, whether assessed invasively or non-invasively (McVeigh 1999). SBP was associated, in this study with C1 but not C2, which was not associated with any BP parameter. In an earlier study McVeigh (McVeigh 1991) demonstrated reduced C2 in patients with hypertension compared to age matched controls. In this study, moreover, C2 was reduced to a greater extent in young hypertensives than C1, whereas C2 was reduced to a similar extent in older hypertensives and healthy controls. C2 but not C1 was also reduced in postmenopausal women with CAD (Cohn

1995) and smoking and diabetes have also been associated with lower C2 than C1 (McVeigh 1997, McVeigh 1993).

Additionally inhibition of endothelial nitric oxide synthesis with L-NAME was associated with progressive diminution in the amplitude and frequency of the oscillatory diastolic waveform, identified as a decrease in C2 in the model analysis, with resolution to baseline with the addition of L-Arginine. This shows that, dynamically, the C2 is a sensitive means of identifying NO modulation of blood vessel tone (McVeigh 2001).

Thus reduction in the small artery compliance value (C2), which reflects a diminished amplitude, frequency and duration of the secondary diastolic wave has been presented as a putative marker for the early detection of vascular disease.

As for SPCA there has been criticism of this method and in particular debate as to whether SPCA or DPCA represents the most useful non invasive vascular phenotype. Rietzschel *et al* (Rietzschel 2001) preformed the first comparative study showing that, within 100 healthy individuals, the coefficients of variation were 32.8% for C1, 33.3% for C2 and 6.7% for Aix suggesting that in terms of reproducibility at least, SCPA was more robust. In this study, however, C2 was significantly and inversely correlated to AIx (r=-0.487). This was corroborated by a further study by Segers (r=-0.36) (Segers 2001). There is still some dubiety as to what C2 actually represents in terms of an anatomical site and though theoretically, the site measured should not affect measurements of overall proximal and distal compliance there was no correlation between values of C1 and C2 measured at the radial artery as compared to those measured at the posterior tibial artery (Manning 2002). Moreover in this study, despite

good quality tonometry recordings, application of the model yielded non interpretable results in some subjects (Manning 2002).

1.3 Oxidative stress, the NAD(P)H oxidase system and & vascular disease.

The principal pathological lesion of coronary artery disease is atherosclerosis. The nidus for the lesion would appear to be transfer of oxidized low density lipoprotein (LDL) across the endothelium to the artery wall (Navab 1996). The transfer is reported to be due either directly to the oxidized LDL or physical, chemical or infective stimulation (Ross 1999). Endothelial cells, vascular smooth muscle cells, and macrophages are the sources of oxidants for the oxidative modification of phospholipids. Oxidised LDL may damage endothelial cells which subsequently produce adhesion molecules and chemotactic factors that induce monocyte and T lymphocytes recruitment (McEver 1992,Madamanchi 2005). Thereafter monocytes migrate to the sub endothelial space and, ingesting lipoproteins, become macrophages which generate reactive oxygen species which convert oxidized LDL to highly oxidized LDL. Upon taking up highly oxidized LDL the macrophages become foam cells which combine with leucocytes to become the fatty streak. Foam cells themselves generate growth factors that facilitate smooth muscle cell migration into the intima. As the process persists and progresses the fatty streaks become more complex lesions which protrude into the vessel lumen and may undergo fibrosis and calcification. Ultimately the rupture of this complex with release of thrombi may lead to vessel occlusion and the acute coronary syndrome (Madamanchi 2005).

1.3.1 Introduction to oxidative stress

Oxidative stress describes the injury caused to cells by the oxidising of macromolecules resulting from increased formation of reactive oxygen species and/or diminished anti oxidant reserves (Zalba 2001b). Recent work has demonstrated that all types of vascular cells generate reactive oxygen species and a growing number of reports have depicted a pivotal role for oxidative stress in the pathogenesis of cardiovascular disease (Cahilly 2000). Almost all cardiovascular disease states including hypertension, hyperlipidaemia, diabetes, arteriosclerosis, unstable angina, vasculitis and myocarditis, restenosis as well as ischaemia/reperfusion have been linked to an enhanced generation of oxygen derived free radicals (Kojda 1999). Oxidative stress has a prognostic role for cardiovascular morbidity and mortality. Coupled with endothelial dysfunction increased oxidative stress has been demonstrated to predict the risk of cardiovascular disease in patients with coronary artery disease (Heitzer 2001).

The molecule oxygen is usually quiescent as although a radical, it is sparingly reactive as its two unpaired electrons are situated in different molecular orbitals and demonstrate parallel spins. Molecular oxygen thus undergoes univalent reduction to form $\bullet O^{2-}$ by means of several enzyme systems; nicotinamide adenine dinucleotide (phosphate) (NADH/NAD(P) oxidases, xanthine oxidase and endothelial nitric oxide synthase (eNOS)in the absence of sufficient co factor or substrate. The perilous balance between oxidation and reduction is maintained by a series of pro and anti oxidant enzymes and molecules. The best studied of these is the NAD(P)H oxidase system.

1.3.2 NAD(P)H Oxidase

NAD(P)H oxidase is the major inducible source of $\bullet O^{2-}$ within phagocytes for which it plays a bactericidal role and produces $\bullet O^{2-}$ in response to pathogens (Cross 1991). The oxidase is a membrane bound enzyme that catalyses a single electron reduction of molecular oxygen to form $\bullet O^{2-}$. The components of this enzyme system are highly conserved across mammalian species (Hitt 1996, Davis 1998). The enzyme system comprises several components:- a membrane bound portion the cytochrome b_{558}, two cytosolic components p^{47}phox and p^{67}phox as well as the small GTPase protein rac2 (Leusen 1996, DeLeo 1996). The membrane bound heterodimeric protein referred to as the flavocytochrome b_{558} is the final electron transporter from NAD(P)H to molecular oxygen and consists of the larger gp91phox and the smaller p22phox (Parkos 1987). It is regarded as the redox centre of the NAD(P)H oxidase (Knoller 1991). The NAD(P)H oxidase is ubiquitous to all vascular cells and is the chief source of vascular $\bullet O^{2-}$ (Greindling 2000).

1.3.3 NAD(P)H Oxidase associated $\bullet O^{2-}$ and atherosclerotic vascular disease.

The oxidative stress hypothesis of atherosclerosis posits that it is an inflammatory disease triggered, as described above, by subendothelial accumulation of LDL particles modulated by reactive oxygen species (Sorescu 2002). Furthermore reactive oxygen species mediate several additional pathological processes in the vessel wall including endothelial dysfunction and smooth muscle cell migration, growth and apoptosis (Griendling 2000).

The evidence base linking $\bullet O^{2-}$with vascular disease is substantial. Levels of NAD(P)H stimulated $\bullet O^{2-}$ have been reported to be elevated in hyperinsulineamic rats (Kashiwagi 1999),

hypercholesterolaemic rabbits (Warnholtz 1999) as well as being positively correlated with endothelial dysfunction and clinical risk factors for atherosclerosis (Guzik 2000a). Hypertension and aging have been shown to be associated with increased $\bullet O^{2-}$ levels and consequently diminished NO levels (Hamilton 2001). Furthermore increased $\bullet O^{2-}$ production has been illustrated in coronary arteries from rats with methionine diet induced hyperhomocysteinemia, an independent risk factor for coronary artery disease (Ungvari 2003). $\bullet O^{2-}$ has also been implicated in the pathogenesis of vein graft intimal hyperplasia (West 2001).

1.3.4 p22phox

As described above the p22phox molecule is the smaller component of the flavo cytochrome b_{558} moiety of the NAD(P)H oxidase system. It has been implicated in the pathophysiology of vascular disease including atherosclerosis, diabetes and hypertension.

The first investigation of p22phox and its difference in expression between non atherosclerotic and atherosclerotic human coronary arteries emanated from Azumi and co workers (Azumi 1999). Utilising autopsied cases they demonstrated that in non atherosclerotic arteries p22phox was weakly expressed mainly in the adventitia. In contrast in atherosclerotic human arteries p22phox expression was more pronounced and was present in adventitial fibroblasts, smooth muscle cells, macrophages in the neointima and media and endothelial cells. As atherosclerosis progressed the expression of p22phox increased through the vessel wall (Azumi 1999). In their study examining O_2^-production in experimental venous bypass graft intimal hyperplasia West demonstrated increased p22phox protein expression in vein grafts as compared with control jugular veins (West 2001). In coronary arteries from 20 patients undergoing heart transplantation Sorescu and colleagues sought to localize the cellular sources of intracellular O_2^- production in atherosclerotic and non atherosclerotic human coronary arteries and

characterise the cellular distribution of the Nox proteins –novel gp91phox homologues (Sorescu 2002). They found, in accordance with work by others detailed above, that O_2^- was produced by all cell types in the vessel wall but specifically that O_2^- was especially high in the shoulder regions of the plaque. Moreover gp91phox and Nox 4 were abundant in human coronary arteries, whereas Nox 1 expression is very low. p22phox co-localized with gp91phox and additionally the severity of atherosclerosis correlates with NAD(P)H oxidase subunit mRNA expression (Sorescu 2002).

The p22phox protein was found to be significantly increased in diabetic arteries and veins compared to non diabetic patients undergoing coronary artery bypass graft surgery (Guzik 2002a). Further association of the pathological role of this molecule in diabetes mellitus was recently produced which found that in decompensated type 2 diabetes p22phox expression was increased in circulating monocytes (Avogaro 2003). In terms of a role of the p22phox protein in hypertension the p22phox mRNA is elevated in spontaneously hypertensive rats (Fukui 1997), can be induced in rats with a ligated renal artery (Ushio-Fukai 1996) and recently increased expression of p22phox has been documented in hypertensive lymphoblasts (Pettit 2002).

	Endothelial cells	Fibroblasts	Vascular Smooth Muscle Cells
p22phox	+	+	+
gp91phox	+	+	-
p67phox	+	+	-
p47phox	+	+	+
Rac	+	+	+

Table 1.2: Expression of NAD(P)H oxidase Components in vascular cells (From Zalba 2001 b).

1.3.5 Modulation of vascular NAD(P)H oxidase.

The best characterized stimulus for NAD(P)H oxidase activation and induction is angiotensin II from studies in animals and man. Animal studies have documented that not only is the NAD(P)H oxidase system modulated by Angiotensin II but, in turn the $\bullet O^{2-}$ anion may play a role in the physiological and pathophysiological actions of this peptide. Angiotensin II increases vascular smooth muscle cell $\bullet O^{2-}$ production by activation of a membrane bound NAD(P)H oxidase (Griendling 1994). Berry *et al* characterized cellular and enzymatic sources of $\bullet O^{2-}$ production in human blood vessels showing that $\bullet O^{2-}$ production was greater in human internal mammary arteries than in saphenous veins and that the prime sources were the NAD(P)H oxidase and xanthine oxidase enzyme systems (Berry 2000). Jacobson *et al* reported that inhibition of vascular NAD(P)H oxidases, using the chimeric peptide inhibitor, gp91ds-tat, suppressed angioplasty induced neointimal hyperplasia in the carotid artery of the rat (Jacobson 2003). This finding therefore linked NAD(P)H oxidase dependent free radical production and neointimal formation. Within human blood vessels NAD(P)H oxidase inhibition has been shown to improve endothelial function (Hamilton 2002). The reduction in NO bioactivity is thus not related to diminished NO production *per se* but to increased $\bullet O^{2-}$ production.

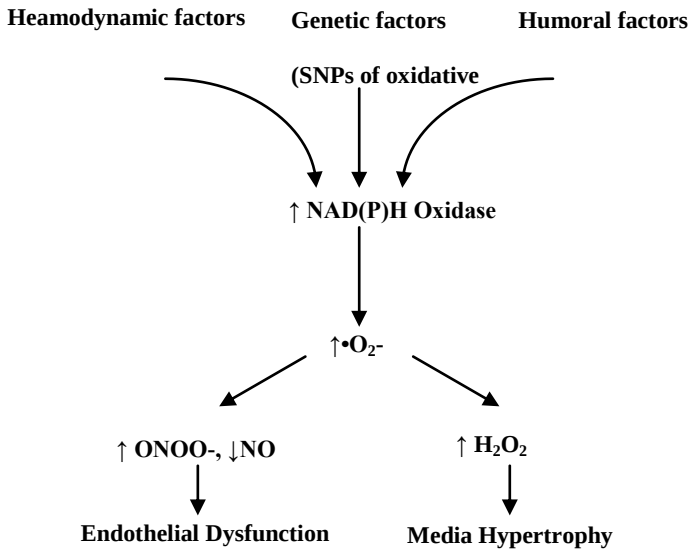


Figure 1.3: The functional consequence of NAD(P)H oxidase activation in hypertension. Ang II = Angiotensin II, PDGF platelet derived growth factor, TNF α Tumour Necrosis Factor α. Adapted from Zalba 2001 b.

1.3.6 The p22 phox gene, CYBA.

The gene coding for the p22phox gene, *CYBA*, is located on chromosome 16q24 (Cahilly 2000) and has several allelic variants (Cahilly 2000, Gardemann 1999, San José 2004). The association of three of these and vascular pathophysiology, C242T, A640G and -930 A/G, shall be discussed in detail.

1.4 The *CYBA* C242T single nucleotide polymorphism and vascular disease.

The *CYBA* C242T SNP within exon 4 of the gene induces a replacement of histidine by tyrosine at amino acid position 72 which is a potential heme binding site (Parkos 1988). This is

the best investigated of the three allelic variants of the *CYBA* and hence will be discussed in the most detail.

The mechanism by which this SNP is functional is contentious. Most authors speculate that the C242T SNP may alter the structure of the heme binding site of the NAD(P)H oxidase and thereby lead to a reduced activity of the enzyme and thus modulate oxidative stress within the vasculature (Guzik 2000b). However, spectral analysis of the membrane fractions from transfected COS7 cell lines, expressing either gp91phox alone or gp91 and p22phox, indicated that the gp91 phox is the sole heme-binding component of flavocytochrome b558 (Yu 1998). The link with atherosclerosis and NAD(P)H oxidase mediated oxidative stress is well established as oxidation of LDL, a key step in the genesis of atherosclerotic lesions, is associated with the NAD(P)H oxidase system. Aviram and co workers established that NAD(P)H-cytochrome P450 reductase is able to oxidize LDL *in vitro* (Aviram 1999).

1.4.1 Coronary Artery Disease.

The first series of data associating this SNP with coronary atherosclerosis was generated by a number of case control studies. Inoue et al compared the distribution of C242T genotypes in 201 Japanese patients with CAD compared to 201 controls (Inoue 1998). At odds with findings from other, and notably European and American populations (Cai 1999, Cahilly 2000) they found that the presence of the T allele was more prevalent in controls rather than patients thus suggesting that the T allele conferred a protection against atherosclerosis. The presence of the T allele has been reported, within an Italian study of 237 patients with coronary stenosis, to be associated with a reduced recurrence of cardiovascular deaths, non fatal myocardial infarction and new revascularization procedures (Arca 2008). In contrast Cai and co workers documented

that the T allele was a modest risk factor for the development of CAD in younger patients (less than 45 years) but not in the overall population (Cai 1999). Opposed to both these studies was an American study which failed to find a difference in prevalence of the C242T allele in 140 patients with angiographically documented CAD or 103 patients with normal coronary arteries (Li 1999). Neither did they elicit any significant difference in coronary epicardial or microvascular responses to intracoronary acetylcholine or sodium nitroprusside (Li 1999). Further negative studies emanated from Saha and co workers who did not find that the C242T SNP was associated with coronary heart disease risk in Asian Indians and Chinese (Saha 1999) and Zafari *et al* who, in American population of 216 patients referred for coronary angiography did not find any association of this SNP with CAD (Zafari 2002). Additionally a study by Stanger *et al* in 108 male Caucasians with coronary artery disease and 65 controls failed to associate any relation of this SNP with the generation of malondialdehyde, a sensitive and specific marker of lipid peroxidation as biochemical end-product (Stanger 2001).

In contrast to these reports a prospective study was done by Cahilly and co workers who determined the association of the C242T variants with the severity, progression and regression of CAD with serial quantitave coronary angiography (Cahilly 2000). They elicited that the T allele was associated with progression of CAD. In detail they documented that the presence of the T allele was linked with greater losses in mean lumen diameter and greater lesion specific mean lumen diameter and was associated with progression and also less regression of angiographically documented lesions over the 2.5 years of follow up (Cahilly 2000). There was no difference in characteristics at baseline suggesting self selection but the distribution of the genotypes was according to that expected under Hardy Weinberg equilibrium hence suggesting otherwise. A Polish case control study of 172 patients with angiographically documented

coronary artery disease versus 169 healthy controls found a cumulative effect of the 242T allele carrier state and cigarette smoking and hypercholesterolemia (Niemiec 2007).

Schächinger and colleagues examined whether this SNP was associated with differences in coronary endothelial vasodilator function (Schächinger 2001). In accordance with Inoue they found that the CC homozygote was deleterious and was associated with a significantly blunted endothelium dependent dilator response. Guzik *et al*, in an elegant study measuring $\bullet O2^-$ with lucigenin-enhanced chemiluminescence in human saphenous veins from patients undergoing coronary artery bypass graft surgery found that the presence of the T allele was associated with decreased $\bullet O2^-$ production (Guzik 2000b). Not all results have been in parallel and most recently the 242T allele has been found to be a predictor of lower risk of cardiovascular events in high risk patients (Arca 2008).

1.4.2 Cerberovascular Disease.

Ito *et al* studied 226 Japanese patients with ischaemic (rather than cardioembolic or haemorrhagic) stroke and 301 control subjects from Tokyo (Ito 2000). They found that the T allele was associated with ischemic stroke with an odds ratio of 1.81. The T allele was also recently associated with an enhanced risk for ischaemic stroke in a German population based case control study of patients below the age of 50 (Genius 2008)

1.4.3 Diabetic Nephropathy.

Hodgkinson *et al* found that there was a marked increase of the TT homozygous genotype in patients with type 1 diabetes mellitus and nephropathy compared with long term uncomplicated subjects and those with retinopathy alone (Hodgkinson 2003). This suggested that the T allele contributes to the susceptibility to diabetic nephropathy. The further findings from this study are discussed below with the A640G SNP.

1.4.4 Carotid Atherosclerosis.

The p22phox protein is significantly increased in human diabetic veins and arteries (Guzik 2002a). Within a Japanese population of 200 subjects with type 2 diabetes mellitus and 215 healthy non-diabetic controls the effect of this SNP with carotid intima media thickness was studied (Hayaishi-Okano 2003). In accordance with other studies by Inoue (Inoue 1998), Schächinger (Schächinger 2001) and also Guzik (Guzik 2000b) they found that the presence of the T allele conferred upon patients a protective, anti atherogenic effect with a smaller intima media thickness observed in this group (Hayaishi-Okano 2003).

1.4.5 Peripheral Vascular Disease.

Given the association of this SNP with CAD, Renner and co workers attempted to document whether there was any similar relationship with peripheral atherosclerosis (Renner 2000). In 341 patients with peripheral vascular disease ranging from intermittent claudication to

gangrene compared to 295 control subjects they failed to ascertain any association with the C242T SNP with this phenotype.

1.4.6 Pre Eclampsia.

As the p22phox gene variants had been linked to other oxidative stress implicated disease phenotypes Raijmakers *et al*, within a Dutch population, compared normotensive pregnancies with pregnancies complicated by pre-eclampsia or the haemolysis elevated liver enzymes and low platelets syndrome (Raijmakers 2002). They found no difference in prevalence of C242T alleles between cases or controls concluding that the C242T SNP of *CYBA* was not associated with pre-eclampsia.

1.4.7 Endothelial Function.

There is some evidence that this SNP is implicated in endothelial function. Fan and co-workers noted that CC homozygotes had a lower flow mediated vasodilatation at the brachial artery than TT homozygotes – a relationship evident in subjects who smoked or were obese (Fan 2007). Schneider *et al* sought to ascertain whether this SNP was associated with endothelial function as measured by endothelium dependent vasodilatation of the forearm vasculature utilising forearm plethysmography in 90 subjects with elevated cholesterol levels (Schneider 2003). They found no association of the *CYBA* C242T and endothelial function.

1.4.8 Insulin Resistance.

A link with insulin resistance was documented by Hayaishi-Okano within the non diabetic control group of the study into carotid atherogenesis (Hayaishi-Okano 2003). They found that fasting insulin levels and the insulin resistance index of homeostasis model assessment was significantly lower in patients with the T allele (Hayaishi-Okano 2003).

In conclusion there have been multiple studies that have identified associations between CYBA gene polymorphism and cardiovascular diseases although there have not been consistent results. This is perhaps because these variants may identify a particular high-oxidative-stress risk subgroup within the healthy population and the population exhibiting cardiovascular risk factors, including hypertension and diabetes. Hence this points toward the importance of multiple polymorphism assessment in functional and association studies of complex disease traits (San José 2008).

1.5 The CYBA A640G and CYBA 930A/G single nucleotide polymorphisms and vascular disease.

The A640G SNP is situated within the 3' untranslated region of *CYBA*. This gene variation may modify messenger RNA processing and stability and therefore modulate p22phox protein biosynthesis (Gardemann 1999). The first study examining this SNP was in the context of CAD in a Japanese population (Inoue 1998). In the 402 participants analysed the T allele of the C242T gene polymorphism but neither the A allele nor the G allele of the *CYBA* A640G gene variation was associated with a reduced or an increased risk of coronary artery disease (Inoue 1998). This association was further studied in a German population of 2205 male caucasians

(Gardemann 1999). On the contrary they found that the G allele of the A640G SNP was significantly more frequent in controls without CAD than in patients with CAD. Correspondingly the AA genotype of the A640G SNP was preferentially found in patients with CAD. They also found that the association of the A640G gene variation with the presence and extent of CAD was not only evident in the entire population but was even stronger in high risk (e.g. those with hypertension),younger individuals (Gardemann 1999). They found no relation with CAD when assessing potential influence of the C242T SNP. In contrast no association was found by Zafari et al when examining the effect of the A640G SNP in an American population (Zafari 2002).

Hodgkinson *et al* found that there was not only a contribution of the C242T SNP upon the contribution to risk of nephropathy in patients with type 1 diabetes but also demonstrated that the entire *CYBA* haplotype – including the G640 allele -was important and that there was also a significant contribution of the aldose reductase (*AKR1B1)* gene (Hodgkinson 2003).

The 930A/G SNP, within the promoter of *CYBA* was recently described by Moreno *et al* (Moreno 2003). They screened the promoter, which they found to contain TATA and CCAC boxes as well as Sp1, γ interferon and NFκB binding sites for new mutations. Moreno and co workers identified this SNP, localised at position -930 from the ATG codon, which was associated with hypertension in a Spanish population (Moreno 2003). In comparing 88 patients with hypertension and 68 normotensive controls they found that the presence of the G allele was associated with hypertension. Mutagenesis experiments suggested that the G allele had a higher promoter activity than the A allele by approximately 30% thus signifying the potential functional nature of this SNP. In a multi-factorial chronic disease such as hypertension a discrepancy in activity of 30% may well be significant. The same group went on to show that

hypertensive GG homozygotes displayed a variety of phenotypes linked with oxidative stress, i.e. higher NAD(P)H oxidase activity as assessed by chemiluminescence, higher levels of p^{22}phox mRNA and protein expression as well as lower NO production as assessed by quantification of NO metabolites (San José 2004). Both these studies found functionality that was confined to hypertensives only. Why this should be is not clear but the presence of the G allele may modulate the transcription of the p22phox gene. Furthermore the -930 polymorphic site lies on a potential binding site for C/EBP transcription factors which may be relevant to the pathophysiology of hypertension (San José 2004). The G allele of this SNP has also been implicated within insulin resistance (Ochoa 2008).

1.6 Nitric Oxide

Initially NO was considered to be a noxious pollutant in exhaust fumes, cigarette smoke and responsible for acid rain and degradation of the ozone layer. Since the endothelium derived relaxing factor, proposed by Furchgott and Zawadski in 1980 (Furchgott 1980), was identified by Palmer and co workers (Palmer 1987) to represent NO there has been considerable attention to the multiple functions of this ubiquitous messenger molecule in human physiology and pathology. Under normal conditions eNOS generates NO which is multifunctional and in addition to its vasorelaxant role also inhibits platelet aggregation, leukocyte adhesion to the endothelium, vascular smooth muscle cell migration and growth and also the oxidation of low-density lipoprotein (Cooke 1997). As stated earlier the latter is a key stage in the evolution of atherosclerosis, the means of a structural and functional change within the arterial wall, potentially identified by PWA. NO is synthesised from L-arginine by the NOS family of oxidoreductases via the L-arginine nitric oxide pathway (Palmer 1988). There are three known

isoforms of NOS which are the products of three distinct genes and summarised in table 1.3 (Hecker 1990, Bredt 1991, Xie 1992).

Gene	Enzyme	Calcium dependent?	Chromosome Location	Protein (kDa)
NOS1	NOSI (neuronal)	Yes	12q24.2-24.31	155
NOS2	NOSII (inducible)	No	17cen-q11.2	125-135
NOS3	NOSIII (endothelial)	Yes	7q35-36	135

Table 1.3: The characteristics of the three human nitric oxide synthase isoforms.

1.6.1 Endothelial nitric oxide synthase

This constitutively expressed 135 KDa protein is predominantly associated with the particulated specific structures in the plasmalemmal membrane, caveolae, of vascular endothelial cells (McDonald 1997, Sakoda 1995). Endothelial nitric oxide synthase is responsible for the conversion of L-arginine to L-citruline and NO in the endothelium (Moncada 1991) and loss of endothelium derived nitric oxide is plays a pivotal role in atherogenesis (Ross 1993). Moreover experimental inhibition of NO synthesis is associated with acceleration of formation of early atherosclerotic lesions (Cayette 1994).

1.6.2 The eNOS gene, NOS3: position and background.

The human eNOS gene, *NOS3*, is situated on the long arm of chromosome 7(7q 35-26) and comprises a total of 26 exons spanning 21 kb (Marsden 1993). Due to the importance of eNOS in terms of cardiovascular function in man many investigators have sought to elucidate whether mutations or polymorphisms of the *NOS3* gene correlate with either an altered physiological response or increased risk of cardiovascular disease.

1.8 The NOS3 G894T single nucleotide polymorphism and human physiology.

Given the centrality of NO in the regulation of numerous essential physiological processes including maintenance of vascular tone, inhibiting platelet and leukocyte adhesion to vascular endothelium, inhibiting vascular smooth muscle cell migration and growth as well as anti-apoptotic and antioxidant functions SNPs within the *NOS3* gene have been the subject of several studies which have associated common allelic polymorphism of this gene with human physiology (Loscalzo 1995a, Hingorani 1999, Vanhoute 1997, Lowenstein 1994, Karvonen 2002).

The importance of these studies is in that while the studies detailed in subsequent sections detail the importance of genetic variation within established disease phenotype these studies are preformed in the preclinical stage, prior to the potentially confounding lifetime of environmental risk and also when future strategies may seek to modify the initiation and progression of atherosclerosis. The G894T SNP has been examined in most detailed and further studies within are central to this thesis hence although other SNPs will be discussed the

importance of the G894T SNP in both human physiology, and later, in human pathology will be discussed in detail.

1.8.1 Endothelial function: Interaction with environmental and dietary factors.

Utilising flow-mediated arterial dilation, a nitric oxide dependent endothelial response, Leeson and co workers (Leeson 2002) investigated the influence of this polymorphism upon environmental risk factors, namely pro-atherogenic cigarette smoking and anti-atherogenic n-3 fatty acid intake, in a young cohort (aged 20-28). They found, even at this age, within males the number of aspartate^{298} alleles dictated the degree as to which endothelial function was impaired by smoking and, within both sexes the degree upon n-3 fatty acid status augmented endothelial function. They postulated that the steady state of the eNOS enzyme may be lower in the asp^{298} variant with a consequent diminution in NO production which, though sufficient to maintain vascular homeostasis in the absence of risk factors, is more amenable to further attenuation in the presence of risk factors (Leeson 2002). A lager study generated conflicting results. Kathiresan and co workers took advantage of the Framing Heart Study Offspring cohort which has almost 2500 subjects that have had vascular phenotyping with brachial artery flow-mediated vasodilatation or hyperaemic flow velocity measurements (Kathiresan 2005). They found no association of the G894T SNP and either of these NO mediated phenotypes. Recently however analysis of this SNP within the Prospective Study of the Vasculature in Uppsala Seniors Study of 959 subjects aged 70 found that of 23 SNPs analysed only this SNP was related to flow mediated vasodilatation (Ingelsson 2008). Furthermore recent work from Dundee has shown that within 68 healthy volunteers aged 18-44 the T allele was associated with blunted endothelial dependent vasodilatation assessed by forearm venous occlusion plethysmography (Godfrey 2007).

1.8.2 Maternal vascular adaptation to normal healthy pregnancy.

Flow mediated dilatation was also employed to study 139 women at 12 weeks gestation during an early, complication free, singleton pregnancy (Savvidou 2001). At this point in pregnancy it is recognised that the eNOS enzyme is physiologically up regulated (Weiner 1994). There was an inverse and highly statistically significant relation between brachial artery flow mediated dilation and the number of maternal eNOS asp^{298} alleles (Savvidou 2001). Not only is this a fascinating insight into the pathophysiology of normal healthy pregnancy but moreover it implicates a role in genetic variation of the *NOS3* gene in modulating risk of pre-eclampsia.

1.8.3 Carotid intima-media thickness.

An Italian study undertook to examine the effect of this SNP as well as the $T^{-786}\rightarrow C$ SNP of the *NOS3* gene upon not only upon flow mediated dilatation but also carotid intima-media thickness, a marker of early atherosclerosis, in 118 subjects aged 21-45 (Pardossi 2004). They found that the TT genotype (asp^{298}/ asp^{298} variant) was significantly and independently associated with decreased brachial artery flow mediated dilatation and increased carotid intima-media thickness in this group of young healthy individuals free of traditional cardiovascular risk factors. On the contrary they found no evidence of an effect of the $T^{-786}\rightarrow C$ SNP on this measurement of endothelial function or upon carotid thickening. This parallels the findings of Rossi *et al* who found that the $T^{-786}\rightarrow C$ altered forearm blood flow in patients who were hypertensive but not normotensive (Rossi 2003b). The extrapolation of this being that this

is a ‘disease-modifying allele’ and in itself insufficient to account for enhanced susceptibility to vascular dysfunction.

1.8.4 Baseline production of nitric oxide.

Veldman et al examined the differences in basal NO production between the different *NOS3* G894T genotypes but quantifying the vasoconstrictor response to eNOS inhibition by infusion of N^G-monmethyl-L- arginine (L-NMMA) (Veldman 2002). The absolute and relative decrease in forearm blood flow during infusion of L-NMMA was attenuated in the asp^{298} variant concluding that basal NO production was attenuated in healthy subjects heterozygous or homozygous for the T allele of this substitution.

1.8.5 Blood pressure response to endurance training.

Four hundred and eighty four subjects were studied by Rankinen et al to elicit the association between this polymorphism and endurance training in previously sedentary normotensive white subjects (Rankinen 2000). They found that the TT homozygote had a blunted responsiveness of sub maximal exercise diastolic blood pressure and rate pressure product (an index of myocardial workload) than heterozygotes or GG homozygotes.

1.8.6 Heamodynamic reactivity to stress.

Malhotra and colleagues examined the effect of the G894T SNP upon heamodynamic response to stress whilst also taking into account possible confounding factors such as adipocity and ethnic background (Malhotra 2004). They showed that European Americans exhibited lower

diastolic blood pressure reactivity unless they were obese Asp allele carriers. African American non obese asp carriers exhibited the greatest total peripheral resistance reactivity. Obese asp allele carriers exhibited the greatest increases in cardiac output and the largest decrease in NO metabolites to a stress response.

1.8.7 Inflammatory and oxidative stress markers.

The association of the G894T SNP and elevated levels of inflammatory and oxidative stress markers comes from a group from Greece who studied 595 from the greater Athens area (Chrysohoou 2004). They found that there was TT homozygotes had higher levels of oxidised low density lipoprotein, white cell count and fibrinogen. They found a non significant association with homocysteine (p=0.08) but Brown *et al*, looking at 2 different populations found that the TT homozygote was associated with significantly higher levels of homocysteine in non smokers with low folate levels (Brown 2003).

1.8.8 Post challenge insulin levels.

Finally, in terms of human physiology a recent study by Maruyama and colleagues from Japan found no difference in insulin sensitivity between 247 non diabetic controls but post standard 75g oral glucose tolerance elevated levels of Insulin were noted in carriers of the T allele as compared to GG homozygotes (Maruyama 2003).

1.9 The NOS3 G894T single nucleotide polymorphism and human pathology.

As detailed thus several studies have documented the effect of this SNP upon human physiology. Likewise, although some studies have been negative a substantial amount of the available literature links this functional polymorphism with an important role in human pathology.

1.9.1 Ischaemic heart disease.

The first report emanated from Japan in 1998 when Hibi and associates investigated the role of this SNP in 226 patients at first presentation of acute myocardial infarction with comparison to 482 control subjects (Hibi 1998). They found that those homozygous for the TT allele were at increased risk of acute myocardial infarction. Moreover they noted that lack of an association with the heterozygotes for this SNP suggested that the 894T allele was not dominant and that the increased risk posed by this SNP was confined to the TT homozygote. Evidence associating this SNP with coronary artery disease in a European population was subsequently published in 1999 by Hignorani and co workers (Hignorani 1999). They compared 249 white individuals with acute myocardial infarction with healthy control subjects from East Anglia. Again the increased risk was confined to individuals homozygous for the TT allele and they quantified the increased risk as lying between 2.5 to 4 times that of individuals homozygous for the GG allele. A subsequent study, the ECTIM study, genotyping patients from France and Northern Ireland failed to corroborate this finding failing to elicit a significant association between this SNP and Ischaemic heart disease. A further positive association came again from a Japanese population of 285 patients with an acute myocardial infarction and 607 age matched controls which demonstrated that the missense G894T *NOS3* variant (heterozygote and TT

homozygote) was associated with acute myocardial infarction (Shimasaki 1998). Guzik *et al* (Guzik 2001) did not show any association between this variant, however, and endothelium dependent vasorelaxation to different agonists in human saphenous veins from patients with coronary artery disease. Further negative studies between this polymorphism and ischaemic heart disease were generated by Jeeroburkhan in the United Kingdom and Schmoelzer from Austria (Jeeroburkhan 2001, Schmoelzer 2003).

The overwhelming balance of evidence does, however, implicate this SNP as being a risk factor for ischaemic heart disease. Further corroboratory evidence was provided by Gardemann from a German population of 2717 individuals undergoing coronary angiography who associated, in younger individuals, an association between T allele carriers and an increased risk of coronary artery disease or myocardial infarction and also by Colombo from an Italian population of 201 patients with coronary artery disease where the T allele was associated with not only the presence of coronary artery disease but also the extent and severity of the atherosclerotic lesions (Gardemann 2003, Colombo 2002).

Perhaps the most compelling evidence comes from a recent meta-analysis of 26 studies involving 23 028 subjects that sought to clarify the role of this SNP and the -786T/C SNP as well as the intron 4 SNP that are discussed below (Casas 2004). The strength of this study is that by sheer weight of numbers throughout a variety of populations they were able to overcome the potential criticism of smaller, possibly underpowered case-control allele association studies. This study concluded that the summary odds ratio under a fixed-effect model showed that individuals homozygous for the T allele were 1.31 times more likely to develop ischaemic heart disease. Even when the study with the largest influence upon this odds ratio was removed (Hignorani 1999), the overall estimate remained similar. Also interestingly

despite racial differences between the frequency of the 894T allele between Asian and non-Asian populations, a meta-regression analysis showed that ethnic background as well as smoking, age and gender were not significant sources of heterogeneity (Casas 2004). A subsequent larger meta-analysis however suggests that that a potentially deleterious effect of the T allele may infact be markedly reduced (Odds Ratio 1.17) (Casas 2006). Furthermore the association is not without contention as in a prospective multi centre study Andrikopoulos and co workers did not, within a Greek population, show any association of the Asp298 variant with risk of further myocardial infarction, extent of coronary artery disease or in hospital mortality after acute myocardial infarction (Andrikopoulos 2008).

1.9.2 Cerebrovascular disease

Following the finding that the G894T SNP had proven to be putatively a strong risk factor for atherosclerosis within the coronary circulation (Hignorani 1999) several investigators sought to elucidate as to whether this finding potentially translated to the cerebral circulation (Markus 1998, MacLeod 1999, Elbaz 2000, Akar 2000). Markus and co workers studied 361 consecutive white patients recruited to a London teaching hospital comparing the allelic frequencies with 236 normal controls. They found no relationship between this SNP in exon 7 of *NOS3* (Markus 1998). A larger study from Paris, by Elbaz et al on behalf of the GÉNIC investigators, recruited 460 cases and 460 controls that were recruited among individuals recruited at the same individuals hospitalized at the same institutions and individually matched on age, sex and centre (Elbaz 2000). They found, counter-intuitively that homozygosity for the G allele was associated with cerebral infarction, especially with lacunar stroke. Other studies from Aberdeen (MacLeod 1999) and Ankara, Turkey (Akar 2000) failed to find any significant association between the G894T SNP and ischaemic stroke.

1.9.3 Coronary in-stent stenosis

The genetic contribution to coronary in-stent restenosis was recently examined within London teaching hospitals (Gomma 2002). A total of 226 patients that underwent elective or emergency coronary artery stenting were recruited and carriers of the T allele exhibited a significantly higher frequency of restenosis (Odds Ratio 1.88) when compared to GG homozygotes (Gomma 2002). Moreover the -786T/C SNP was also found to have an association with the carriers of the -786C allele showing a higher risk of restenosis with an odds ratio of 2.06. These effects were found essentially to be additive and independent of other classical risk factors (Gomma 2002).

1.9.4 Survival in patients with congestive cardiac failure.

The role of the T allele variant of this common SNP was recently reported in a study from the USA (McNamara 2003). 469 patients with an ejection fraction of less than 45% were recruited and the investigators demonstrated that event free survival was influenced by the presence or absence of the T allele. Interestingly, in subset analysis the adverse impact of the T allele was evident primarily in patients with non ischaemic cardiomyopathy (McNamara 2003). This is surprising given the evidence linking this SNP with risk of ischaemic heart disease as listed above.

1.9.5 Coronary artery spasm and an enhanced vascular response to phenylephrine.

Philip *et al* (Philip 1999) injected the alpha adrenergic agonist, phenylephrine into patients undergoing cardiac surgery and found the enhanced response to alpha adrenergic stimulation in patients with the 894T allele. This finding highlights the potential modulation of a vascular response to vasoconstricting hormones by this SNP. This finding did not extend however to α_2 adrenoceptor-induced coronary vasoconstriction, which appeared exclusively associated to the 825T allele of *GNB3*, the gene which encodes the G protein β_3 subunit, previously associated with an enhanced coronary blood flow reduction in response to α_2 adrenoceptor activation (Naber 2003). Within a Japanese population where variant angina tends to be associated with diffuse coronary artery spasm the role of this polymorphism was examined (Chang 2003). They found, further implicating this SNP in cardiovascular pathophysiology, that the T allele was associated with diffuse, rather than focal spasm (usually associated with an area of atherosclerosis) of the coronary arteries (Chang 2003).

1.9.6 Renal Disease

Noiri *et al* compared 185 patients with end stage renal disease with 304 unrelated healthy individuals. They demonstrated an accumulation of T alleles, especially in patients with diabetes mellitus as the cause of their end stage renal disease (Noiri 2002). They postulated that diminished NO production in individuals with the T allele may augment the proinflammatory leukocyte adhesion to endothelial cells consequently decreasing the vasodilatory capability and further exacerbating atherosclerotic lesions in end stage renal disease.

1.9.7 Pre Eclampsia

Pre eclampsia, hypertension and proteinuria in pregnancy, is a transient endotheliosis in other wise healthy young women. Serrano and colleagues postulated that the *NOS3* gene was a candidate gene for pre eclampsia as physiologically there is considerable evidence that NO plays a role in pregnancy induced uterine vasodilatation (Serrano 2004). Acetylcholine is more potent and efficacious in producing dilatation of isolated uterine arteries from pregnant than from non-pregnant patients, an effect blocked by NOS inhibitors (Nelson 1995, Nelson 1998). Furthermore, in human uterine arteries there is a pregnancy associated increase in calcium dependent NOS activity and eNOS protein expression (Nelson 2000).

Therefore Serrano *et al* studied 322 pregnancies with pre-eclampsia with comparison to 522 controls (Serrano 2004). They analysed the effect of all 3 SNPs discussed in this introduction but found no increase in the risk of pre-eclampsia for the intron 4 or the -786T/C polymorphisms. They did however find a significant association in women homozygous for the T allele who had an adjusted odds ratio for pre-eclampsia of 4.6 (Serrano 2004). Furthermore after a multivariate analysis, carriage of the 894T-786C-intron 4b haplotype was associated with an increased risk of pre-eclampsia (odds ratio 2.11) compared to the 894G-786T-intron 4b haplotype. This corroborates earlier evidence associating the 894T allele with severe pre-eclampsia in a Japanese population (Yoshimura 2000).

1.9.8 Cognitive Function

A Spanish study recently reported on mild cognitive impairment and the G894T SNP (Solé-Padullés 2004). Though no direct association was observed between this *NOS3*gene variation and mild cognitive impairment in 62 subjects studied those carriers of the 894T allele with mild cognitive impairment preformed worse in the Mini Mental State Examination, Wechsler Memory Scale (Revised), long term visual memory and the phonetic verbal fluency tests (Solé-Padullés 2004). Thus the T allele represents a genetic risk factor for cognitive impairment in the elderly.

1.9.9 Hypertension

The first study linking this SNP and hypertension was published by Miyamoto and co workers (Miyamoto 1998). They examined the association of several SNPs of the *NOS* gene and hypertension including the G894T SNP and the VNTR Intron 4 SNP in 2 different geographic sites in Japan, comparing hypertensives and normotensive controls. The odds ratio was similar in both places (2.3%) associating the 894T allele, but not the Intron 4 SNP, with hypertension (Miyamoto 1998). Though these findings in an were not substantiated in Australian (Benjafield 2000) or a Scandinavian (Karvonen 2002) populations further evidence of an association of the 894T allele, and with essential hypertension resistant to conventional treatment, came from a Czech population (Jáchymová 2001).

Following the conflicting results generated from these association studies an elegant examination of the association of all three di-allelic SNPs (G894T, -786T/C and Intron 4

VNTR) and also another SNP, within Intron 13 of *NOS3*, was recently published by Persu (Persu 2005). They genotyped 110 dizygotic white twin pairs from Flanders, Belgium and examined blood pressure as a continuous trait using ambulatory blood pressure recorders. Although sib-pair analysis failed to show any significant association of any specific SNP with blood pressure, haplotype analysis disclosed a significant association between *NOS3* haplotypes and daytime systolic blood pressure (Persu 2005). Ultimately as this approach is more informative further studies, and specifically functional studies within a given phenotype would be most informative.

1.9.10 Insulin resistance

Hyperinsulinaemia and insulin resistance are cofactors in the pathophysiology of essential hypertension with the association of impaired endothelial with decreased nitric oxide production shared by insulin resistance and essential hypertension (Cleland 2000). Hence Chen and colleagues examined, in an American population, a community based sample of 1021 unrelated African American and white young adults aged 19 to 38 years (Chen 2001). They sought to obtain information on the combined effects of the *NOS3* G894T SNP and Insulin resistance status (using the homeostasis model assessment of insulin resistance utilising fasting insulin and glucose) on blood pressure. After adjusting for sex, age, body mass index, carriers of the T allele displayed higher systolic, diastolic and mean arterial blood pressure and was not race specific (Chen 2001). This association was modulated by insulin resistance status.

1.9.11 Aortic Stiffness: Pulse Wave Velocity.

Given the association described above between SNPs of the *NOS3* gene and hypertension Lacolley and colleagues sought to ascertain whether this SNP had an association with a surrogate marker of arterial stiffness, PWV (Lacolley 1998). They measured carotid-femoral pulse wave velocity in 309 untreated hypertensive and 123 normotensive controls. They failed to show any association with either blood pressure or pulse wave velocity in this French population (Lacolley 1998).

1.9.12 How may the G894T single nucleotide polymorphism of the *NOS3* gene be functional?

The G894T SNP of the *NOS3* gene has, therefore been widely implicated in both human physiology as well as the pathogenesis of vascular disease in man. The key question pertains as to if this SNP is itself functional and thus an important potentially modifiable target in the treatment of vascular disease or whether not this is merely a marker of another significant loci that heralds a functional effect that has so far eluded discovery.

Several groups have undertaken painstaking research upon the structure, localization and putative effects of this SNP upon the biology of NO. This exon 7 polymorphism (894G→T) which determines the location of either a glutamate or an aspartate residue at position 298 has been associated with altered vascular biology but with several reservations.

Firstly both glutamate and aspartate are conservative substitutions hence it is unlikely that they themselves produce functional affects. This SNP is of special interest as this conservative

amino acid substitution within the oxygenase domain of the eNOS may influence eNOS function. Additionally analysis of the crystal structure of eNOS indicates that residue 298 is situated externally, distant from the active catalytic site and cofactor binding domains, and thus aspartate substitution would theoretically have minimal effect upon enzymatic activity (Fischmann 1999).

Initial evidence was provided by Tesauro and co-workers who, utilising transfected cells, primary human endothelial cells and human hearts, demonstrated that eNOS with aspartate but not glutamate at position 298 was cleaved generating 100 KDa and 35 KDa products (Tesauro 1999). The altered cleavage was postulated to alter NO generation. Additionally they noted, using Chou-Fasman secondary structure predictions that this seemingly conservative replacement generated significant structural changes. This contention was ultimately challenged by Fairchild et al who showed that the intracellular cleavage discovered in cells harbouring the Asp^{298} *NOS3* substitution was an *in vivo* artefact due to the acidic pH used in the study (Fairchild 2001).

Gosler et al performed a detailed characterization of the G894T SNP with respect to its effect upon enzyme kinetic parameters, bound cofactors, uncoupled NADPH oxidase activity and binding affinities for calcium calmodulin and tetrahydrobiopterin. They showed no difference between the Glu/Glu or the Asp/Asp variants (Gosler 2003).

1.10 The NOS3 -786T/C SNP and human pathophysiology.

The -786T/C SNP of the *NOS3* gene has also been studied with respective to human pathophysiology. This variant results in a cytosine instead of thymidine substitution at nucleotide -786 (Nakayama 1999). This SNP has been shown to reduce the promoter activity (Nakayama 1999) and consequently reduce eNOS protein expression and eNOS activity (Wang 2000a).

1.10.1 Ischaemic heart Disease and coronary in-stent stenosis.

The first evidence of the putative role of this SNP in ischaemic heart disease was produced by Álvarez who not only found that the frequency of patients homozygous for the C allele were significantly increased in patients compared to controls but also observed a synergistic effect between the *NOS3* CC and the ACE DD genotypes in the risk of developing early coronary artery disease (Álvarez 2001). Colombo *et al* (Colombo 2003) in a study of 415 unrelated individuals from Italy with coronary artery disease described significant linkage disequilibrium between the G894T SNP and the -786T/C SNP. Both variants, they found, were significantly associated with the occurrence and severity of coronary artery disease and that the risk of CAD was increased amongst individuals homozygous for the C allele of the -786T/C SNP when compared to individuals homozygous for the T allele. Moreover individuals who were homozygous for the T allele of the G894T allele of the G894T SNP and who had at least one C allele of the -786T/C SNP were at greatest risk of coronary artery disease. As detailed above carriers of the -786C allele were found to be at higher risk of coronary instent stenosis (Gomma 2002). Not all studies have been congruous as Jeerooburkhan did not show any association between this SNP and risk of ischaemic heart disease in the UK (Jeeroburkhan 2001). In a

large study of 1225 individuals, however, Rossi *et al* for the GENICA study performed multiple logistic regression analysis for the effect of this SNP and the G894T SNP on two and three vessel coronary artery disease. They found that the -786T/C SNP, but not the G894T SNP, was associated with coronary artery disease with subjects harbouring a -786C allele having an increased risk of tow or three vessel coronary artery disease (Rossi 2003b).

Again, as with the G894T SNP, the meta analysis described above examined the role of the -786 T/C SNP upon risk of ischaemic heart disease (Casas 2004). While examining for both the dominant or recessive models of genetic association the authors failed to ascertain any association between this SNP and risk of ischaemic heart disease (Casas 2004). The subsequent updated meta analysis from the same group in 2006 also did not assocaite this SNP with coronary heart disease (Casas 2006).

1.10.2 Insulin resistance.

Altered eNOS activity has been associated not only with hypertension but also glucose homeostasis and insulin resistance (Duplain 2001). Ohtoshi *et al* examined the relationship between the G894T and -786T/C SNPs, common eNOS gene variants and insulin resistance (Ohtoshi 2002). While they observed no relationship with the former several fascinating findings were observed with the latter. Non diabetic subjects with the -786C allele had higher fasting glucose and homeostasis model assessment of insulin resistance than -786 TT homozygotes; diabetic subjects with the T allele had higher HbA_{1c} levels and utilising the euglyceamic hyperinsulineamic clamp diabetic patients with the C allele demonstrated a lower glucose infusion rate than those without (Ohtoshi 2002). Moreover diabetic patients with the C allele had lower levels of NO metabolites. Further association associating this SNP with insulin

resistance was produced by Fernandez *et al* (Fernandez 2004) who observed that the -786C homozygote was significantly more frequent in hypertensive patients with metabolic syndrome than in those without the syndrome. When they combined analysis of this SNP with the G894T SNP they identified the 786C894G as the risk haplotype for metabolic syndrome susceptibility. Lastly -786T/C genotype has been associated with AIx in children with type1 diabetes mellitus (Zineh 2007).

1.10.3 Coronary artery spasm.

Similar to the G894T mutation this SNP is associated with increased susceptibility for the risk of coronary artery spasm (Nakayama 1999). Subjects homozygous for the C allele were at a 3 times elevated risk for coronary artery spasm as compared to heterozygotes or TT homozygotes. Moreover this group subsequently showed that this polymorphism combines with smoking to increase the risk of coronary artery spasm (Nakayama 2003).

1.10.4 Internal carotid artery stenosis.

Further evidence of the role of this SNP in the atherosclerotic process was recently published by an Italian group who observed that patients homozygous for the C allele were at higher risk of moderate to severe internal carotid artery stenosis, especially ulcerative lesions (Ghilardi 2002).

1.10.5 Cerebral blood flow.

The effect of this SNP upon cerebral blood flow parallels that of coronary artery spasm in regards to there being no recognised association between cerebral blood flow within non smokers but in smokers CC homozygotes had a significant decrease in cerebral blood flow (Nasreen 2002).

1.10.6 Hypertension.

Hyndman and colleagues, in a Canadian population of 705 healthy individuals, examined the effect of this polymorphism upon hypertension and documented that subject homozygous for the C allele had significantly higher systolic blood pressure and approximately 2 times more likely to be hypertensive (Hyndman 2002).

1.11 The NOS3 Intron 4 variable number of tandem repeat and human pathophysiology.

Several variable number of tandem repeats (VNTR) have been described in the *NOS3* gene (Marsden 1993) including a polymorphism which represents 4 or 5 times the 27 base pair variable number of tandem repeats in intron 4 (*NOS3* 4a/b) (Wang 1996). Considerable interplay appears to exist between this SNP and the -786T/C SNP. Wang demonstrated that the 27-bp repeat from the *NOS3* intron 4 has a *cis*-regulating effect on the eNOS promoter (Wang 2002a). Furthermore this regulation appeared to be haplotype dependent on both the eNOS promoter and intron 4 DNA sequence variants.

1.11.1 Ischaemic heart disease.

The vast majority of the associations of this SNP with cardiovascular pathology centre upon ischaemic heart disease

The first report associating this SNP with ischaemic heart diseased was published in 1996 by Wang *et al* (Wang 1996). They associated homozygosity for the NOS3a allele as a risk factor for coronary artery disease only in smokers. A further study, larger study with 455 patients with ischaemic heart disease and 550 controls, also from Japan confirmed the findings of Wang (Wang 1996) but found that the association of homozygosity for the *NOS3* extended to both smokers and non-smokers (Ichihara 1998). Furthermore these results were reproduced within a Turkish population where Hatemi found that the presence of an intron 4a allele was observed more frequently in patients with myocardial infarction than in controls (Hatemi 2002).

Not all the literature is congruous, however, and Hibi et al, though they associated the TT homozygote of the G894T SNP with risk of acute myocardial infarction showed, within this Japanese population, no association between homozygosity of this SNP and ischaemic heart disease (Hibi 1998). Similar findings were elicited from a German population who, in 2717 individuals undergoing coronary angiography compared to 533 healthy controls, failed to find an association between this SNP and risk of coronary artery disease and acute myocardial infarction (Gardemann 2002). Again as with all 3 SNPs Jeerooburkhan failed to ascertain any relationship between *NOS3* genotype and either risk of ischaemic heart disease or plasma NO metabolites in a sample of middle aged white men in the United Kingdom.

In combining the 16 studies available examining the contribution of the intron 4a allele to ischaemic heart disease Casas concluded that, with a probable recessive genetic model of inheritance, the aa allele was associated with ischaemic heart disease (Casas 2004).

1.11.2 Hypertension and correlation between blood pressure and physical activity.

Kimura et al found a significant association between the intron4 VNTR genotype and physical activity level on systolic blood pressure (Kimura 2003). The association was only confined to those in the lowest tertile of physical activity level who had at least one a allele but nevertheless facilitates a better understanding of the mechanism of exercise to reduce hypertension and modulate cardiovascular risk.

1.11.3 Pre-Eclampsia

For the same reasons detailed above the Intron 4a/b had been investigated with regard to pre-eclampsia. Although as previously described the influence of this SNP was found in one study to only extend as part of the 894T-786C-intron 4b haplotype in multiple regression analysis (Serrano 2004) others have associated this SNP with Pre-eclampsia (Tempfer 2001). Indeed the latter study elucidated a striking association odds ratio of developing pre-eclampsia when one of the shorter a alleles was present, 6.5 (Tempfer 2001).

Other important reports that failed to find a significant association between this SNP and vascular disease includes negative studies pertaining to diabetic nephropathy (Rippin 2003)

1.12 Biochemical indices as markers of cardiovascular disease

Markers of Inflammation have received widespread attention within the medical literature over recent years as the link between low grade inflammation and cardiovascular disease gains rising precedence (de Maat 2004). Inflammation would appear to play a crucial role in all steps in the development of atherosclerosis from the nascent lesion to a full blown acute coronary syndrome (Libby 2002). Some of the markers extensively studied in relation to their putative roles are C-reactive protein (CRP), interleukin 6 (IL 6), Adiponectin and Intracellular adhesion Molecule 1 (ICAM1).

1.12.1 C-reactive protein.

CRP is a plasma protein which is produced by the liver and is an acute phase protein. It belongs to the pentraxin family of proteins and was initially discovered by Tillett and Francis in 1930 as a substance in the serum of patients with acute inflammation that reacted with the C polysaccharide of pneumococcus (Tillett 1930). CRP is a prototypic marker of inflammation and as will be described has been denoted to predict cardiovascular events in apparently healthy subjects, and a poor prognosis following acute coronary syndromes.

Basal levels of CRP are associated with an increased risk of diabetes, hypertension and cardiovascular disease. Raised levels of CRP indicate that atherosclerosis is a chronic inflammatory process (Hirschfield 2003). Moreover several large scale prospective epidemiological studies have shown that plasma levels of CRP are strong independent predictors of atherosclerotic events in apparently healthy women and men (Ridker 1997, Ridker 1998a). Elevated CRP is a surrogate marker for sub clinical atherosclerosis (Wang

2002b) and is involved in development and progression of atherosclerosis (Pasceri 2000). Elevated baseline CRP was found to portend heightened risk of 30 day death or myocardial infarction in 727 patients prior to percutaneous coronary intervention (Chew 2001). Increases in pulse pressure in a large stuffy of almost 10,000 were associated with elevated CRP levels amongst healthy adults from the United States, independent of SBP and DBP (Abramson 2002). Low grade inflammation has also been associated with the insulin resistance syndrome (Yudkin 1999, Festa 2000) and markers of low grade chronic inflammation have been shown prospectively to independently predict those at high risk for type 2 diabetes mellitus(Schmidt 2000, Freeman 2001). Leinonen and co workers demonstrated that fasting levels of sICAM-1, IL-6 as well as CRP were associated with Insulin resistance and adiposity in 239 female patients with type 2 diabetes (Leinonen 2003). Additionally women with poly cystic ovarian syndrome have significantly increased CRP levels compared to healthy controls (Kelly 2001).

There has been some dubiety regarding the strength of the association between CRP and CVD and a large study by Danesh and colleagues suggested that CRP may only be a modest risk factor for CVD (Danesh 2004). Furthermore Timpson and co workers examined associations between serum CRP concentrations and metabolic syndrome phenotypes in the British Women's Heart and Health Study then comparing these estimates with those derived from a randomized framework with common CRP gene haplotypes (Timpson 2005). While they found that CRP haplotypes were associated with plasma CRP concentration they noted disparity between estimates of the association between plasma CRP and phenotypes comprising the metabolic syndrome derived from conventional analysis and comparing those from a Mendelian randomization approach. Thus they suggested that there was no causal association between CRP and the metabolic syndrome phenotypes (Timpson 2005).

There thus remains contention as to whether CRP levels are merely a marker or whether there is a pathophysiological role for CRP within the vascular diseases described (de Maat 2004, Schunkert 2008). A statistical association is not analogous to clinical or pathophysiological causality (Schunkert 2008).

CRP has been associated with arterial stiffness. Yasmin *et al* found that, even after controlling for confounding factors such as blood pressure, age, gender and smoking, aortic and brachial PWV were both associated CRP in 427 healthy individuals (Yasmin 2004). This group did not find any association with Aix and CRP. On the contrary another group from Estonia, in a smaller cohort of healthy individuals (158) found significantly higher Aix in subjects with CRP levels above 1mg/ml and in multiple regression analysis Aix correlated positively with age, female gender, short stature, mean arterial pressure and CRP (Kampus 2004).

1.12.2 Interleukin 6

IL-6 is a cytokine with both pro inflammatory and anti inflammatory effects on many cell types, affecting both B cell immunoglobulin production and T cell cytotoxic activity (Barton 1996). IL-6 also modulates platelet function as well as endothelial function and is the only substance known to induce synthesis of all of the acute phase proteins by the liver (Lindmark 2001). Il-6 itself is produced by many cellular elements such as activated macrophages, lymphocytes, endothelial cells and vascular smooth muscle cells (Rattazzi 2004).

A series of studies have revealed the association of Il-6 and 'traditional' vascular risk factors. Il-6 levels rise with age and are significantly associated with high blood pressure, smoking and insulin sensitivity (Bermudez 2002, Fernandez- Real JM 2001,Ridker 2000). Furthermore

production of IL-6 increases at increasing levels of adiposity in healthy men and women (Mohammed-Ali 1997). A circadian variation exists with increased cytokine levels during the night exists (Sothern 1995).

Chae and co workers documented significant graded relationships between blood pressure and IL-6 and sICAM-1 in 508 apparently healthy men (Chae 2001). Il-6 plasma levels are elevated in patients with unstable angina compared to those with stable angina or healthy subjects (Biasucci 1996, Biasucci 1999). Large scale prospective studies have shown that IL-6 plasma levels in the upper quartile of the considered normal range are independently predictive of an increased risk of premature death or further myocardial infarction (Harris 1999, Ridker 2000). The idea that IL-6 is involved in progression of CAD is further substantiated by a Swedish study which identified circulating levels of IL-6 as a strong independent risk factor of increased mortality in unstable CAD and identifies patients, with high IL 6 levels (>5ng/l), who benefit most from a strategy of early invasive management (Lindmark 2001).

1.12.3 Adiponectin

Adiponectin is a protein hormone which regulates a variety of metabolic processes including glucose regulation and fatty acid catabolism. It is a 244 amino acid long polypeptide with 4 distinct regions (Whitehead 2006). The gene for Adiponectin has been identified and is situated on chromosome 3p27 and has been highlighted as affecting genetic susceptibility to obesity and type 2 diabetes mellitus (Ukkola 2005). It is solely secreted from adipocytes and plasma levels are inversely and paradoxically proportional to body mass index, unlike leptin, another adipose tissue specific secretory product which is known to increase with body mass index (Takahashi 1996). Hence patients who have the highest BMI and highest quantity of adipose

tissue have the lowest levels of Adiponectin. There is also a sexual dimorphism with females displaying higher levels than males as well as circadian (Gavrila 2003).

This hormone has been described as playing a key role in type 2 diabetes mellitus, obesity and atherosclerosis. Patients with diabetes have lower Adiponectin levels than control subjects and moreover those with macroangiopathy have lower levels than those without (Hotta 2000). Moreover plasma levels of Adiponectin were lower in Pima Indians, a unique cohort where the high prevalence of obesity aggregates with diabetes (Lindsay 2002). Additionally plasma levels of Adiponectin strongly correlate with insulin sensitivity (Stefan 2002). Thus Adiponectin has a key role in insulin action and low levels may result in insulin resistance and diabetes mellitus (Matsuzawa 2004). Adiponectin has also been found to predict Insulin resistance but not endothelial function in 294 adolescents (aged 13 to 16 years) (Singhal 2005). Patients with hypertension and ischemic heart disease have also been found to have lower levels of Adiponectin (Mallamaci 2002, Kumada 2003). Interestingly, whereas there is no adiponectin present in untreated normal vascular walls in rabbit models when balloon injury is introduced to vascular walls, a markedly positive immunohistochemical stain was found with anti adiponectin antibody (Okamoto 2000). Therefore the presence of adiponectin may be a key pathological event in the development of CAD. The importance of adiponectin as a potential risk factor in vascular disease is further corroborated by Kaplan-Meyer analysis of patients with chronic renal insufficiency which demonstrated that subjects with lower levels died of cardiac events more frequently during a 4 year observation period (Zoccali 2002). The high molecular weight form of adiponectin is more strongly associated with insulin sensitivity (Hara 2006), is more strongly associated with incident risk of diabetes (Nakashima 2006) and is also more strongly associated with protection against endothelial cell apoptosis (Kobayashi 2004) and hence is perhaps a more sensitive marker of coronary heart disease then Adiponectin *per*

se. It was not, however associated with incident coronary heart disease in women from the British Women's Health and Heart Study (Sattar 2008). It is unclear whether adiponectin is a key mediator or bystander in cardiovascular disease (Antoiniades 2009). It is therefore too early to nominate adiponectin as the origin of vascular disease.

1.12.4 Intracellular adhesion molecules

Cellular adhesion molecules in part mediate adhesion of circulating leucocytes to the endothelial cell (Springer1994, Adams 1994). Subsequent transendothelial migration is putatively an important step in the initiation of atherosclerosis (Ross 1993). ICAM1 is constitutively expressed on endothelial cells in most regional vascular beds (Granger 2004). Pathological studies have shown increase cellular adhesion molecule expression in several components of the atherosclerotic plaque (Poston 1992, O'Brien 1996). There is a potential role for adhesion molecules in acute atherothrombotic syndromes (Jang 1994). Furthermore plasma concentrations may be higher among patients with atherosclerosis (Peter 1997, Blann 1994) and dyslipidaemia (Hackman 1996). As noted above Chae *et al* described in healthy men a graded relationship between blood pressure and levels of ICAM 1 (Chae 2001). Ridker defined the importance of ICAM1 in CHD by showing in a large prospective cohort of apparently healthy men that increasing concentration of ICAM1 is associated with risk of future myocardial infarction (Ridker 1998b). The risk of future myocardial infarction was 80% higher in patients with baseline ICAM1 concentrations in the highest quartile (Ridker 1998b).

In summary inflammatory activation, in part mediated by the molecules detailed above, in reaction to an atherogenic stimuli may, in the arterial wall, cause alterations in vascular

stiffness. We therefore sought to examine the putative relationship between these inflammatory markers and arterial stiffness in healthy volunteers.

1.13 Diminished nitric oxide bioactivity: A potential link between non invasive vascular compliance as an intermediate phenotype and low grade inflammation.

As described earlier in sections 1.2.2 and 1.2.3 both augmentation index and small artery compliance are sensitive to pharmacological manipulation in NO bioactivity with LNMMA (Wilkinson 2002a. McVeigh 2001). A growing body of evidence suggests that in the arterial wall low grade inflammation *per se* parallels the presence of poor NO bioactivity.

eNOS is present within endothelial cells as stated the derived NO stimulates arterial vasodilatation and inhibits smooth muscle cell proliferation, LDL oxidation, platelet adhesion and aggregation and monocyte adhesion to the endothelium (Wever 1998, Loscalzo 1995b, Boger 1996). Endothelial dysfunction which is complicit with impaired NO bioactivity occurs early in atherosclerosis. CRP has been shown to cause a direct reduction in eNOS expression and bioactivity in human endothelial cells with a simultaneous decrease in cyclic GMP and an increase in adhesion to monocytes to the endothelium – an early stage in atherosclerosis, with a rise in sICAM-1 levels (Venugopal 2002). Furthermore patients with angiographically documented coronary artery disease with high levels of CRP had diminished endothelial vasoreactivity (r=-0.46, Fichtlscherer 2000) and Cleland *et al* have shown a relationship between CRP levels and percentage decrease in forearm blood flow during infusion of the eNOS inhibitor L-NMMA (Clelland 2000b).

Aims

The aims of the current study were:

1) To assess the reproducibility of diastolic pulse contour analysis as a non invasive intermediate cardiovascular phenotype.

2) To determine whether the C242T single nucleotide polymorphism of the p22phox gene, *CYBA*, has an effect upon arterial compliance in patients with coronary artery disease.

3) To determine whether the G894T (Glu298Asp) single nucleotide polymorphism of the endothelial nitric oxide synthase gene, *NOS3*, has an effect upon arterial compliance in patients with coronary artery disease.

4) To determine whether there is an interaction between the *NOS3* G894T single nucleotide polymorphism and the *CYBA* C242T single nucleotide polymorphism in patients with coronary artery disease.

5) To determine, in healthy volunteers, free of cardiovascular disease whether a relationship existed between markers of low grade inflammation and arterial stiffness.

Chapter 2 Methods.

2.0 Summary

This chapter provides a description of the general protocols of the clinical techniques used in the studies described in this thesis.

2.1 Healthy volunteers and patients.

All the studies described within this thesis were performed in the Clinical Investigation and research Unit (CIRU), British Heart Foundation Glasgow Cardiovascular Research Centre, Western Infirmary Glasgow.

Twenty four healthy volunteers (Chapter 3) were recruited for validation studies. Each subject attended twice, at the same time of day for non invasive vascular phenotyping as described below. Volunteers were all in good health with no past or current medical history, aged 19-65 and taking no regular mediation. Two were smokers. All subjects gave a full clinical history and underwent a full examination to confirm health prior to the study.

Fifty three healthy normotensive volunteers were recruited via advertisements within the University, Glasgow Herald newspaper offices and local newspapers for the work described in chapter 6. No subjects were taking any medication and all abstained from alcohol, nicotine, tobacco, food and strenuous activity overnight before the study day. Physical health was confirmed by screening with history, a full physical examination, ECG and supine blood pressure measurement in triplicate. Ninety volunteers were screened and those with raised blood pressure, an antecedent history of vascular disease or abnormal ECG excluded.

The studies comparing oxidative stress genotype upon vascular compliance described in chapters 4 and 5 were performed in 103 individuals attending the Western Infirmary, Glasgow for coronary artery bypass grafting. Each individual previously underwent coronary angiography which had documented coronary artery disease and attended the BHF Glasgow Cardiovascular Research Unit in the University of Glasgow the week prior to surgery fasted and abstinent from tobacco, alcohol, tea or coffee from midnight the previous night. Each patient had their height and weight recorded as described below and a detailed drug and past medical history taken as well as a full physical examination and electrocardiogram. We excluded those who had a history of endocrine, hepatic, renal or valvular heart disease or atrial fibrillation. The latter two in themselves can alter the pulse pressure wave shape. All participants were asked to defer taking their normal medication on the day of the study until after the protocol was completed. 410 patients were screened and those withdrawn who did not manage to comply with fasting or due to an episode of anginal pain during the preceding 24 hours, or upon documented valvular heart lesion or audible murmur or arrhythmia discovered during clinical examination or electrocardiogram. Clinical details obtained from the patients were corroborated from case sheet examination. A history of current cigarette smoking, hypertension (defined as either current anti-hypertensive treatment or a blood pressure > 140/90 mmHg), diabetes mellitus and hypercholesterolaemia (plasma cholesterol >5.4 mmol/or on treatment) were considered as risk factors. Each patient brought with them an up to date repeat prescription sheet from their general practitioner to ensure that an accurate drug history was obtained.

2.2 General Clinical Protocol.

The study protocols were individually approved by the Ethics Committee of the West Glasgow Hospitals University NHS trust. All underwent the pre study procedure above following full informed consent. On each study day the healthy volunteers or patients with coronary artery disease were transported to the CIRU between 0700 hours and 0900 hours after an overnight fast from 2200hrs. After the clinical and morphometric measurements were taken the patients lay supine, at rest in a quiet room which was temperature controlled, at 24-25ºC, where the clinical studies were undertaken. Following each study a light meal was provided for subjects prior to transportation home.

2.3 Clinical and morphometric measurements.

2.3.1 Body Mass Index

Body weight and height were measured with subjects in light clothes and without shoes to the nearest 0.5kg of weight and the nearest 0.5cm of height. Exactly the same equipment was used throughout the studies, and the weighing scales (Seca, Germany) were calibrated regularly. The same observer preformed all the measurements (RSD). Body mass index (BMI, kg/m^2) was calculated as:

$$BMI = \text{Body weight (kg)} / (\text{height (m)})^2$$

2.3.2 Blood Pressure and heart rate.

Throughout each study the technique utilised to record blood pressure and heart rate remained uniform. Systolic and diastolic blood pressure and heart rate were measured, after 1 hours supine rest, by an oscillometric technique using a Dinamap Critikon (Johnson and Johnson Professional Products Ltd., UK) semi automatic sphygmomanometer, maintained and calibrated at regular intervals by the Department of Physics, Western Infirmary.

2.3 Pulse Wave Analysis.

Systolic and diastolic pulse wave analysis was carried out by a single investigator (RSD) in a dedicated investigation room as described. Subjects were placed in the supine position and right radial artery waveforms acquired as detailed below.

2.3.1 Diastolic pulse contour analysis.

Subjects were placed in the supine position and right radial artery waveforms were acquired with the use of a calibrated proprietary tonometer (model CR 2000 Hypertension Diagnostics Inc) and used according to manufacturers specifications. The tonometer consists of a 1.27cm diameter stainless steel canister with a 0.15mm thick stainless steel diaphragm connected to a double plated ceramic piezoelectric element used to amplify the waveform signal. The subject's arm is stabilised in an angulated wrist support and radial artery waveforms analysed for a 30 second period. The CR 2000 device then utilises the 4 element modified Windkessel model to generate C1 and C2 as described previously (Cohn 1995, McVeigh 1991, McVeigh 1993, McVeigh 1999). A description of the mathematical model used within the Windkessel is

described in the introduction in section 1.2.3. Four recordings were taken and the mean used for analysis. The procedure was well tolerated by each subject.

2.3.2 Systolic pulse contour analysis

For systolic pulse contour analysis the SphygmoCor Arterial Tonometry system (Atcor Medical Inc) was utilised. Central pressure waveforms are derived and analysed as described by Wilkinson et al (Wilkinson 1998). A high fidelity micromanometer probe (SPC-301; Millar Instruments) was used by one observer (RSD). The probe is placed on the right radial artery at the wrist. The wrist is extended and held within a support to ensure that the artery is kept in one position and to facilitate reproducibility. The probe is placed upon the wrist with sufficient pressure to flatten but not occlude the radial artery at the wrist. The probe is connected to a lap top computer upon which data was collected directly. After a waveform recording was established 20 sequential waveforms are collected and software integral to the system generates an impression of an average peripheral waveform. A generalised transfer function, the methodology of which is described in the introduction in section 1.2.2, then constructs a derived central waveform. Recordings were excluded as per standard procedures of other groups which have utilised this technique (Wilkinson 2000). In this way recordings were excluded if the systolic or diastolic variability of the waveforms exceeded 5%, or the amplitude of the waveform, a measure of the quality of the tracing exceeded 100mV. The derived central waveform was then analysed, again using the integrated software to generate the AIx, the difference between the first and second peaks of the central pressure waveforms as described in section 1.2.2 and figure 1.1 .The AIx is expressed as a percentage of the pulse pressure and is a measure of systemic arterial stiffness and wave reflection. All measurements were made four times and again the mean values utilised.

2.4 Genotyping.

Following non-invasive vascular measurements a sample of blood was taken and DNA extracted using the Wizard Genomic DNA Purification kit (Promega). Genotyping of the *CYBA* C242T and *NOS3* G894T SNPs was performed by investigators Dr Nick Brain and Mrs Koh-Tan who were unaware of the patients' phenotypes.

Genotyping of the C242T polymorphism was performed by restriction enzyme treatment and agarose gel resolution. Primers for polymerase chain reaction (PCR) were designed around the polymorphism. Sequences of these were TGCTTGTGGGTAAACCAAGG (Fwd), GGAAAAACACTGAGGTAAGTG (Rev). PCR was performed with 1U of Taq DNA polymerase (Promega) in a 20 μl reaction volume containing 25 ng of genomic DNA; 1X PCR buffer; 1.5 mM Mg^{2+}; 200 μM each dNTP and 20 pmol of primer. PCRs were cycled on PCT-225 thermal cyclers (MJ Research) as follows: 95°C for 5 min; then 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; followed by 72°C for 10 min. PCRs were run on 1.5% Ultrapure Agarose gels (Invitrogen) to confirm amplification. Restriction reactions consisted of 20 U of RsaI, 16 μl of PCR product and 2 μl of buffer. Reactions were incubated at 37°C for 2 hours, before resolution on 2% Ultrapure Agarose gels. Genotype was determined from fragment patterns as follows: CC: 353 bp; TT: 160 bp and 193 bp; CT: 160, 193 and 353 bp (Ito 2000). Genotyping was checked by two individuals. Fourteen representative samples were sequenced in forward and reverse directions, using PCR primers, to confirm fidelity of restriction genotyping.

The G894T polymorphism was genotyped by sequencing. PCR primers were designed with the following sequences: AAGGCAGGAGACAGTGGATGGA (Fwd), CCCAGTCAATCCCTTTGGTGCTCA (Rev). PCR was performed with 0.2U of HotStar Taq (Qiagen) in a 20 μl reaction volume containing 25 ng of genomic DNA, 1X PCR buffer (with 1.5 mM Mg^{2+}), 200 μM of each dNTP and 20 pmol of primer, and cycled on PCT-225 thermal cyclers (MJ Research). The following program was used: 95°C for 15 min followed by 30 cycles of 94°C for 1 min; 58°C for 1 min; 72°C for 1 min; followed by 60°C for 30 min. PCR products were purified using Nucleofast-96 plates (Macherey-Nagel) and the manufacturer's protocol. Products were sequenced using BigDye Terminator v3.1 kits (Applied Biosystems) and a modified 20 μl reaction protocol such that 0.5 μl of Ready Reaction mix and 3.75 μl of 5x sequencing buffer were used per reaction. The reverse PCR primer was used for sequencing. Sequencing reaction products were purified using genCLEAN plates (Genetix) before being resolved on an ABI 3730 capillary sequencer (Applied Biosystems) using standard sequencing run conditions. Genotyping of the sequencing data was performed using SeqScape (Applied Biosystems) software and two individuals checked all genotypes. Concordance between the two individuals was 100%.

2.5 Laboratory methods

Upon completion of pulse wave contour analysis venous blood samples were withdrawn from the antecubital fossa and samples immediately placed within ice. Thereafter samples for the biochemical analysis described below underwent centrifugation (3000 rpm, 4 ºC) prior to decanting and storage at -20ºC.

For all measurements batch analysis was performed to minimize inter assay variability and samples preformed in duplicate. All reagents and samples were allowed to come to room temperature prior to use. The inter- and intra-assay coefficients of variation were <10% across the range of measured results. Intra-assay coefficients of variation were <7% for all analytes.

Cholesterol, HDL-C and tricylglycerol were measured using commercially available enzymatic assay kits from Roche Diagnostics Corporation (Indianapolis, IN, U.S.A.) and a Hitachi 917 analyser, and LDL-C (low-density lipoprotein cholesterol) was calculated from the Friedewald equation

2.5.1 Adiponectin

Adiponectin was analysed using the Quantikine® Human Adiponectin/Acrp30 Immunoassay (Catalog Number DRP300). This is a solid phase ELISA containing recombinant human adiponectin and can measure human adiponectin levels within cell culture supernatants, serum and plasma. The assay utilises the quantitative sandwich enzyme immunoassay technique. The monoclonal antibody specific for adiponectin is recoated onto the micro plate. Standards and samples are pipetted into the wells and any adiponectin present is bound by the immobilized antibody. Following washing the enzyme linked monoclonal antibody specific for Adiponectin is added to the wells. Thereafter following a further wash a substrate solution is added to the wells and colour develops in proportion to the amount of Adiponectin bound in the initial step. A Multiscan Ascent plate reader and software were used for calculating results

2.5.2 Interleukin 6.

IL-6 was analysed using the Quantikine® Human IL-6 Immunoassay (Catalogue Number D6050). This is a solid phase ELISA designed to measure IL6 within cell culture supernates, serum and plasma. It contains recombinant human IL-6. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Il-6 is precoated upon a microplate.

A dilution series was generated with concentrations of 0pg/ml, 1.56 pg/ml, 3.12 pg/ml, 6.25 pg/ml, 12.5 pg/ml, 25 pg/ml, 50 pg/ml and 100 pg/ml. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. Following washing an enzyme linked polyclonal antibody specific for IL-6 is added to the wells. After a further wash substrate solution is added to the wells which develops in intensity in proportion to the amount of Il6 bound in the initial step. The colour development is stopped and the intensity measured using a Multiscan Ascent plate reader and software were used for calculating results.

2.5.3 Soluble ICAM-1 immunoassay.

sICAM-1 was analysed using the Quantikine® Human sICAM-1 Immunoassay (Catalogue Number BBE 1B). The principle stages are as described for adiponectin and IL-6. The technique employed is the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sICAM-1 has been precoated onto a micro plate.

A dilution series was generated using 0 ng/ml, 1.86 ng/ml, 10.3 ng/ml, 19.1 ng/ml, 29.8 ng/ml and 41.9 ng/ml. Standards, samples, controls and conjugate are pipetted into the wells and any

sICAM-1 present is sandwiched by the immobilised antibody and the enzyme-linked-monoclonal antibody specific for sICAM-1. After washing a substrate solution is added top the wells and colour develops in proportion to the amount of bound sICAM-1. Again the colour development is then stopped and the intensity measured using a Multiscan Ascent plate reader and software for calculating results.

2.5.4 Highly sensitive C reactive protein.

CRP was measured using a sensitive double-antibody sandwich ELISA with rabbit anti-human CRP and peroxidase-conjugated rabbit anti-human CRP (Roche Diagnostics Corporation Indianapolis, IN, U.S.A., Catalog No 1972855). The assay is a particle enhanced immunoturbidimetric assay. Anti-CRP antibodies coupled to latex microparticles react with antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. A Hitachi 917 analyzer was used. The assay was linear up to 5 mg/l and logarithmic thereafter.

2.6 Statistics.

2.6.1 Reproducibility studies.

For the reproducibility studies we utilised the technique described by Bland and Altman (Bland 1996). Bland-Altman plots were derived for to calculate intra-observer bias for C1 and C2 (Minitab 14).

2.6.2 The effect of genotype upon arterial compliance in patients with coronary artery disease.

All data are presented as mean ± SEM unless otherwise stated. Power calculations were performed before sample acquisition and 100 subjects were projected to provide 80% power for primary analysis based on two sample *t*-tests to compare mean C2 values between the two homozygous groups at each locus. The Chi squared statistic was used to ensure that the observed allele frequencies did not differ from that expected under Hardy-Weinberg equilibrium.

A two tailed Student's *t*-test was used to analyse differences in phenotype between subjects with presence or absence of the T allele for the *CYBA* C242T SNP, as this has previously been reported as being the dominant allele (Cahilly 2000, Cai 1999). One-way ANOVA was used to examine the effect of genotype of the *NOS3* SNP on vascular parameters as with this SNP there has been no documented dominant allele (Hibi 1998). Furthermore a comparison using the Student's *t*-test was carried out between patients homozygous for the *NOS3*T allele and possessing the *CYBA* 242T allele and patients homozygous for the *NOS3* G allele and for the *CYBA* C allele. Thereafter multiple regression analysis was preformed to identify the variables that independently predicted the relationships. Where a potential confounder was discovered a generalised linear model was constructed to ascertain if, adjusting for this variable, the relationship remained significant. Statistical significance was defined as $p<0.05$. Minitab 14 for Windows (Minitab Inc) was used for all statistical analysis and PRISM 4 used to generate graphs (Graphpad).

2.6.2 The relationship between markers of low grade inflammation and Insulin resistance upon arterial compliance in healthy volunteers.

The Kolmogorov Smirnov test for normality was used to establish whether the data sets were normally distributed. The data sets for ICAM and adiponectin were normally distributed whereas those for IL-6 and CRP were not. For data that did not follow the Gaussian distribution, for statistical analysis, the data set was log transformed to create a normal distribution.

Simple regression was used to create a model with only one predictor utilizing the least squares estimation. Where the data was normally distributed the Pearson correlation was used (ICAM and Adiponectin). Where the data did not fit a Gaussian distribution Spearman Rank correlation was used, i.e. for CRP and IL 6. Thereafter multiple regression was used to describe the statistical relationship between a response and 2 or more predictors. Again this used the method of least squares, which determines the equation for the straight line that minimizes the sum of the vertical distances between the data points and the line. As before $p<0.05$ was considered significant.

Materials and Methods Appendix

Manufacturers and Suppliers

Multiscan Ascent Plate Reader and Software.

Thermo Life Sciences

Unit 5, The Ringway Centre

Edison Road, Basingstoke, Hampshire

RG21 6YN

Telephone (01256) 817282)

Fax (01256 817292)

www.thermo.com

Where samples required dilution a Hamilton dilutor was used.

Microlab 500 series.

Scientific laboratory supplies ltd.

Unit 17.

Coatbridge business centre,

204 Main Street

Coatbridge, Lanarkshire, ML5 3RB

Telephone (01236) 431857

Fax (01236) 431050

Adiponectin

Quantikine® Human Adiponectin/Acrp30 Immunoassay (Catalog Number DRP300).

Interleukin 6.

Quantikine® Human IL-6 Immunosassay (Catalogue Number D6050).

sICAM-1

Quantikine® Human sICAM-1 Immunosassay (Catalogue Number BBE 1B).

Manufactured and distributed by

R&D Systems Inc.

614 McKinley Place, NE.

Minneapolis, MN 55413.

United States of America

Tel (0800) 343-7475.

Fax (612) 379-2956

European Distributors.

R & D Systems Europe

19 Barton Lane, Abingdon Lane, Abingdon,

Oxon, OX14 2NB.

United Kingdom.

Cholesterol, HDL-C and tricylglycerol

Enzymatic assay kits from

Roche Diagnostics Corporation

Indianapolis

IN

U.S.A

Chapter 3. Baseline subject characteristics and the reproducibility and comparison of vascular compliance values.

3.0 Summary.

This chapter describes the clinical characteristics of the subjects recruited; healthy volunteers and patients with coronary artery disease. Thereafter, reproducibility studies examining small and large artery compliance values were conducted as there has been some suggestion in the medical literature that the reproducibility of DPCA is not optimal (Manning 2002, Rietzschel 2001, Segers 2001).

3.1 Subject and patient recruitment.

As detailed in section 2.1 the twenty four subjects for reproducibility studies were recruited from within the British Heart Foundation Glasgow Cardiovascular Research Centre. The 53 healthy volunteers for the study of arterial compliance and low grade inflammation were recruited from advertisements within Glasgow University and also from the offices of the Glasgow Herald. The 103 patients to ascertain the effect of genotype upon arterial stiffness in patients with coronary artery disease were recruited from the Coronary Artery Bypass Graft operation waiting lists.

3.2 Patient Characteristics.

Table 3.1 describes the baseline characteristics of the 103 patients with coronary artery disease. The mean age was 61.8 but range included patients as young as 36 and as old as 82. The mean BP was 130.94/70.9 mmHg, total cholesterol 4.29 mmol/L and LDL cholesterol 2.34 mmol/L. In terms of vascular measurements the mean AIx was 28.89%, mean C1 14.74 ml/mmHg x 10 and mean C2 4.63 ml/mmHg x 100mmHg. 39(37%) had never smoked cigarettes, 25 (25%) had previously been smokers (more than 6 months since their last cigarette) and 40 (38%) were current smokers. The cardiovascular medication that the patients were taking is detailed in table 3.2. As a group the patients were on the standard regimen with most on a HMG CoA Reductase inhibitor (85%), Beta Blocker (72%) and Aspirin (71%). 49% were on an Angiotensin converting enzyme inhibitor. 41% were using transdermal or oral nitrate.

3.3 Baseline characteristics of healthy controls.

The characteristics of the healthy controls recruited are detailed in table 3.3. The controls were younger with a mean age of 52.74, range 37-72. The mean BP was 124/62 and in terms of arterial compliance values the C1 and C2 were higher (15.2 ml/mmHg x 10 and 6.74 ml/mmHg x 100 respectively) and AIx lower (23.85%) indicating more compliant arteries. None of the controls were taking any medication, 4 were still smoking and 12 were ex smokers.

Table 3.1. Baseline Patient Characteristics (n=103).

Variable	Mean	Median	SD	SEM	95% CI	Range
Age (years)	61.84	63.00	8.98	0.86	60.13, 63.56	36.00 to 82.00
Height (cm)	166.22	168.00	9.42	0.91	164.42, 168.02	142.00 to 183.00
Weight (Kg)	81.87	81.00	15.00	1.44	79.01, 84.74	47.00 to 138.00
BMI	29.69	29.00	5.36	0.52	28.67, 30.72	19.33 to 58.90
SBP(mmHg)	130.94	129.75	17.30	1.67	127.64, 134.24	92.00 to 185.50
DBP(mmHg)	70.88	70.625	9.66	0.48	69.03, 72.73	47.25 to 95.00
PP(mmHg)	60.06	58.00	12.87	1.24	57.60, 62.51	40.50 to 113.50
AIx(%)	28.89	29.67	11.43	1.16	27.58, 32.20	-13.40 to 59.67
C1(ml/mmHgx10)	14.74	3.64	5.00	0.48	13.79, 15.70	3.70 to 35.20
C2(ml/mmHgx100)	4.63	3.64	2.82	0.27	4.09, 5.17	1.40 to 17.20
CHOL(mmol/l)	4.29	4.25	0.83	0.08	4.13, 4.54	2.71 to 6.94
LDL(mmol/l)	2.34	2.23	0.74	0.07	2.20, 2.49	1.01 to 4.88
HDL(mmol/l)	1.07	1.00	0.37	0.04	1.00 to 1.14	0.38 to 2.67
VLDL(mmol/l)	0.88	0.77	0.51	0.05	0.78, 0.98	0.14 to 3.31
TRIG(mmol/l)	1.93	1.69	1.12	0.11	1.72, 2.15	0.31 to 7.25
CRP(mg/l)	4.73	2.00	8.36	0.82	3.11, 6.35	0.17 to 61.55
Adiponectin(ng/ml)	5053.32	3611.5	4811.90	471.85	4116.40, 5990.30	224.06 to 28355.00
ICAM(ng/ml)	374.93	353.38	150.31	14.67	345.81, 404.05	164.77 to 1296.70
IL-6 (pg/ml)	4.60	3.45	5.05	0.51	3.59, 5.61	0.47 to 33.98

Table 3.2. Patient Medication.

Medication	n	%
Aspirin	75	71
Aspirin + Clopidogrel	12	11
Clopidogrel	10	9.5
Beta Blockers	76	72
Calcium Channel Blockers	55	52
HMG Co A Reductase Inhibitors	89	85
Nitroglycerin	43	41
Angiotensin converting enzyme inhibitors	51	49
Angiotensin II type-1 receptor antagonist	5	4.8

Table 3.3 Healthy Control baseline characteristics (n=53)

Variable	Mean	Median	SD	SEM	95%	Range
Age (years)	52.74	52	9.147	1.25	50.21, 55.26	37-72
Height (cm)	171.35	172	10.20	1.40	168.54, 174.16	149-194
Weight (Kg)	77.02	75	13.85	1.90	73.20, 80.84	448-113
BMI	26.14	25.82	3.34	0.46	25.22, 27.06	19.40-35.8
SBP(mmHg)	124.62	122.25	12.97	1.782	121.03, 128.19	106.50, 168
DBP(mmHg)	72.42	71.50	7.96	1.09	70.23, 74.62	58.25, 98.250
PP(mmHg)	52.19	51.00	7.78	1.07	50.05, 54.34	40.50, 81.25
AIx(%)	23.85	26.00	11.26	1.55	20.74, 26.85	-7.50, 43.50
C1(ml/mmHgx10)	15.20	15.33	4.14	0.58	14.04, 16.37	6.83, 23.98
C2(ml/mmHgx100)	6.74	6.85	2.94	0.41	5.91, 7.57	2.08, 14.00
CHOL(mmol/l)	4.75	4.83	0.78	0.11	4.53, 4.96	3.27, 7.25
LDL(mmol/l)	2.97	3.03	0.81	0.11	2.76, 3.21	0.89, 5.73
HDL(mmol/l)	1.31	1.32	0.30	0.04	1.23, 1.40	0.79, 1.99
VLDL(mmol/l)	0.45	0.38	0.23	0.03	0.38, 0.51	0.14, 1.25
TRIG(mmol/l)	0.98	0.84	0.50	0.07	0.84, 1.12	0.30, 2.73
CRP(mg/l)	2.11	0.85	7.06	0.97	0.16, 4.06	0.22, 51.74
Adiponectin(ng/ml)	6234.63	4687.2	4824.20	662.66	4903.8, 7565.5	159.30, 19921.00
ICAM(ng/ml)	298.25	276.11	84.82	11.65	274.85, 321.65	169.98, 703.11
IL-6 (pg/ml)	3.49	1.75	6.20	0.87	1.75, 5.24	0.06, 37.28

3.4 The reproducibility of the Windkessel derived large and small artery compliance values.

3.4.1 Background.

The diastolic pulse wave contour analysis method customarily used by Cohn (Cohn 1995) uses the 4- element Windkessel model conceptually introduced by Goldwyn and Watt in 1967 (Goldwyn 1967). McVeigh and Cohn in recent years have described that fitting Windkessel model parameters into the diastolic portion of the radial artery pulse waveform generating small and larger artery compliance values is useful clinically. In particular the small, or oscillatory, compliance value is reduced with age, hypertension and diabetes and is sensitive to altered NO bioactivity (McVeigh 1999, McVeigh 1991, McVeigh 1993 and McVeigh 2001). There has been criticism in some quarters of this technique. Validity and widespread potential clinical utility have been questioned by one study finding an absence of correlations between different sites (radial and posterior tibial artery sites) as well as frequent uninterpretable values in hypertensive subjects (Manning 2002). Rietzschel and colleagues found coefficients of variation of 32.8% for C1 and 33.3% for C2 but only of 6.7% for AIx (Rietzschel 2001). Before conducting larger experiments we wished to ensure that, in our hands, C1 and C2 were reproducible vascular measurements.

3.4.2 Methods.

For the initial reproducibility studies with the aim of producing Bland Altman plots we recruited twenty four healthy volunteers who attended twice, more than one week apart, fasted between 8am and 10am and having abstained from tea, coffee and nicotine from midnight the previous night. The 24 volunteers were all in good health and none had any hepatic, endocrine, or renal disease or were on any regular medication. Mean BP in this group was 118/72 mmHg.

All subjects gave informed consent prior to enrolment in the study for which ethical approval had been granted by the Ethics Committee of the West Glasgow Hospitals University NHS trust. Following 60 minutes supine rest non invasive vascular profiling were performed as described within sections 2.3.1 and 2.3.2.

Statistical analysis was preformed using Minitab14. Coefficients of variation were calculated as the SD of the difference between 2 measurements divided by the mean value of the mean of both measurements. Reproducibility data using the method described by Bland and Altman (Bland 1996) generates Bland Altman plots depicting differences between 2 measurements plotted against the mean of the 2 measurements.

3.4.3 Results Reproducibility: Bland Altman Plots.

Using Bland Altman plots the calculated intra-observer bias for C1 was -0.1(SD of bias was 0.36, 95% CI -0.8 to 0.6) (Figure 3.1). Likewise for C2 the observed bias was -0.04 (SD of bias was 0.20, 95% CI -0.44 to 0.36) (Figure 3.2).

Figure 3.1 Bland Altman plots for intra observer variation within large artery compliance in 24 healthy volunteers. For C1 the calculated intra-observer bias was -0.1 (SD of bias was 0.36, 95% CI -0.8 to 0.6).

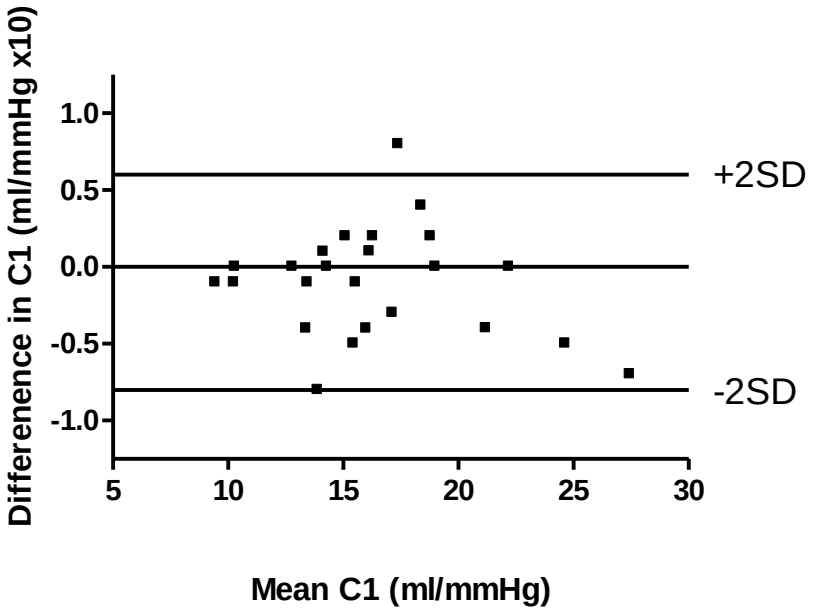
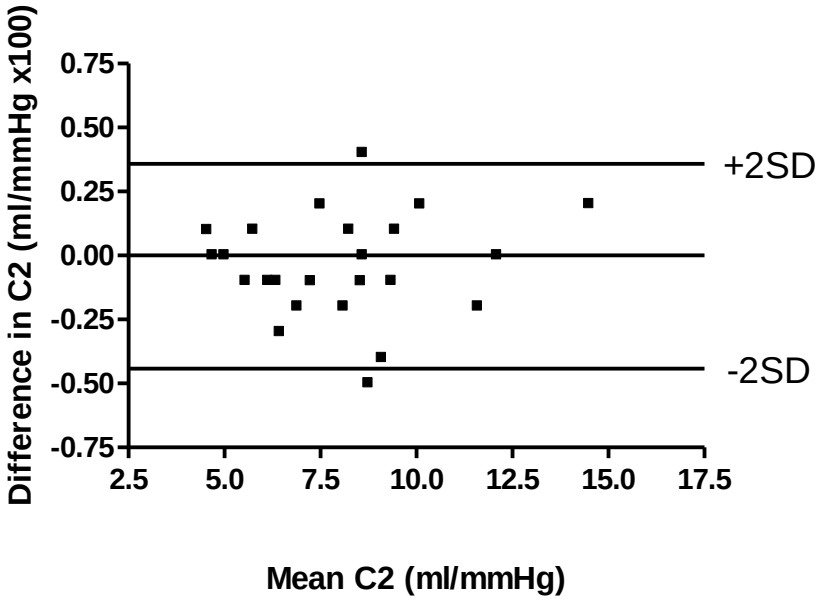


Figure 3.2 Bland Altman plots for intra observer variation within small artery compliance in 24 healthy volunteers. For C2 the observed bias was -0.04 (SD of bias was 0.20, 95% CI -0.44 to 0.36).



3.5 The comparison between systolic and diastolic pulse contour analysis.

3.5.1 Background

Both systolic and diastolic pulse contour analysis have been validated in different populations but only a few groups have sought to compare these values in healthy volunteers as well as in patients with established vascular disease. Segers *et al* analysed 45 human subjects, most of whom had coronary artery disease, simultaneously measuring aortic and radial pressure pulse waveforms (Segers 2001). They noted that C2 was inversely related to augmentation index (r=-0.36) (Segers 2001). Rietzschel and co workers measured AIx and C2 in 100 volunteers, 27 were taking anti hypertensive medication but all were free of atherothrombotic disease, and also found a significant inverse relation (r=-0.71) (Rietzschel 2001). A much smaller study was recently reported by Woodman *et al* examining 15 men with coronary artery disease and 15 healthy men. Their pooled correlation between AIx and C2 was -0.75 (Woodman 2005). The aim of this investigation was to compare the 2 methodologically different techniques focusing on either the systolic and diastolic portion of the arterial pulse waveform both in patients with coronary artery disease and in healthy controls.

3.5.2 Subjects and Methods.

The 53 healthy volunteers for the study of arterial compliance and low grade inflammation were recruited from advertisements within Glasgow University and also from the offices of the Glasgow Herald. The 103 patients to ascertain the effect of genotype upon arterial stiffness in patients with coronary artery disease were recruited from the Coronary Artery Bypass Graft operation waiting lists. A full description of recruitment, general clinical protocol and pulse wave contour analysis are described n sections 2.1, 2.2 and 2.3. In each case the volunteer provided informed consent and the procedure was tolerated well in each case. Minitab 14 was used as the statistical package to construct simple linear correlations. Statistical significance was defined as $p<0.05$.

3.5.3 Results: Correlations between large and small arterial compliance values in patients with coronary artery disease and healthy controls.

Scatter plots of large and small artery compliance values and augmentation index are displayed in Figures 3.3 – 3.6. There was a significant correlation between both AIx and C1($r=-0.42$, $p=0.002$) and AIx and C2 ($r=-0.60$, $p=<0.0001$) in healthy volunteers. While there was no association between AIx and C1 in patients with coronary artery disease ($r=-0.17$, $p=0.009$) AIx did correlate with C2 in this population ($r=-0.51$, $p=<0.001$).

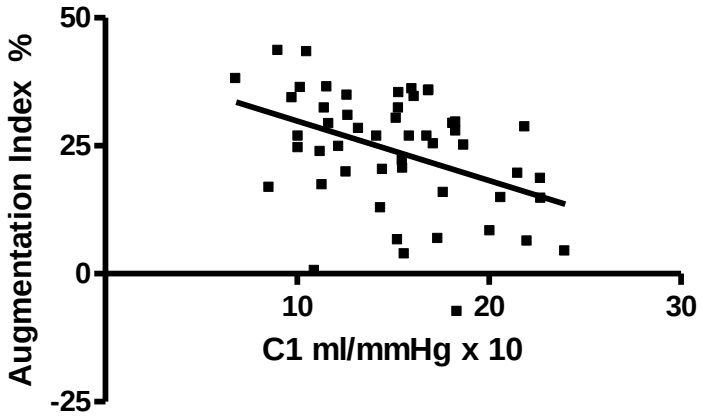


Figure 3.3 Scatter plots of large artery compliance (C1) and augmentation Index in healthy volunteers (n=53, r = -0.42, p=0.002).

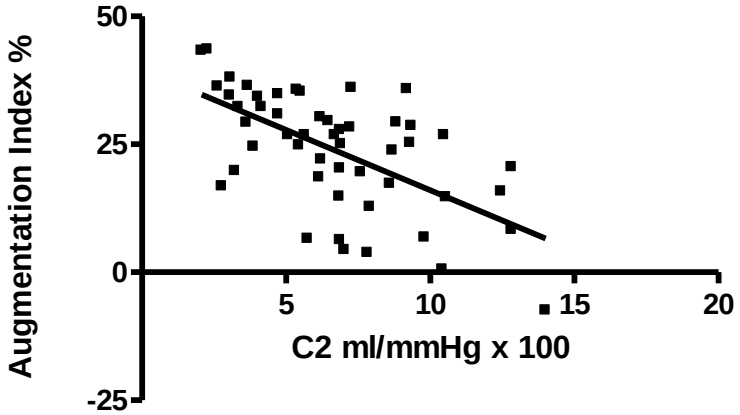


Figure 3.4 Scatter plots small artery compliance (C2) and Augmentation Index in 53 healthy volunteers. (r = -0.60, p<0.001).

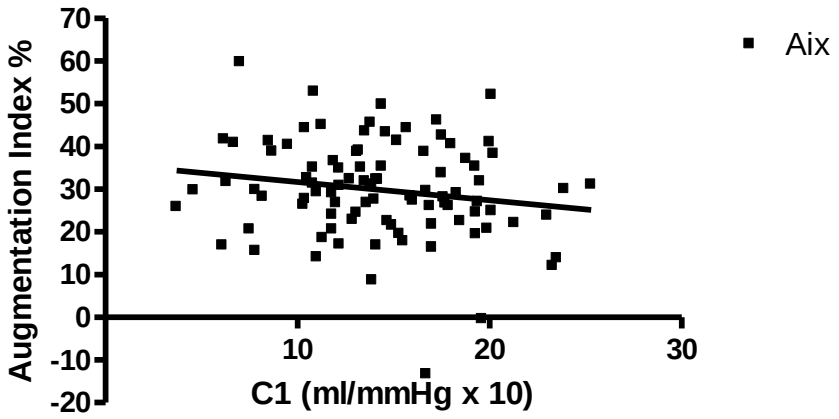


Figure 3.5 Scatter plots of large artery compliance (C1) and augmentation index in patients with coronary artery disease. (n=103, r = -0.17, p=0.09).

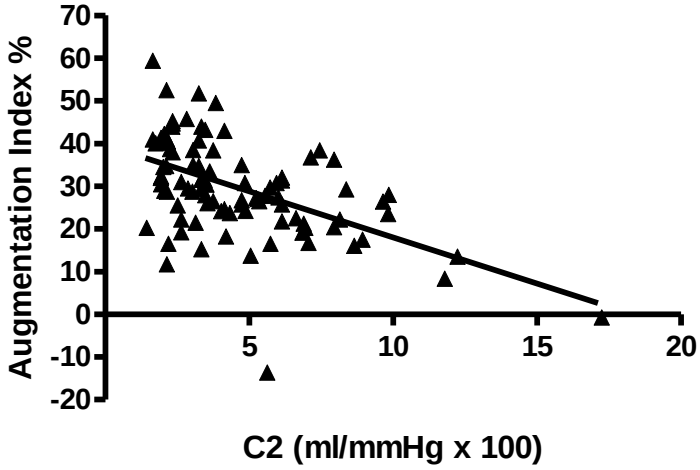


Figure 3.6 Scatter plots of small artery compliance (C2) and augmentation index in patients with coronary artery disease. (n=103, r = -0.51, p<0.001).

3.6 Discussion

The assessment of arterial stiffness is gathering growing recognition as a useful non invasive intermediate phenotype. Recently the Conduit Artery Function Evaluation (CAFÉ) study showed a SphygmoCor derived central SBP difference between the 2 arms of an Anglo-Scandinavian Cardiac Outcomes Trial substudy in spite of similar brachial blood pressure changes (Williams 2006). As there is a growing appreciation of the clinical utility of the assessment of arterial stiffness the assessment of reproducibility becomes of paramount importance.

In essence we have shown that the HDI/Pulsewave ^{TM} CR-2000 Research Cardiovascular Profiling system used to determine Windkessel based diastolic pulse contour arterial compliance measurements is reproducible. For C1 the calculated intra-observer bias was -0.1 (SD of bias was 0.36, 95% CI -0.8 to 0.6). Over 95% of the variability fell within 2 standard deviations. Furthermore for C2 the observed bias was -0.04 (SD of bias was 0.20, 95% CI -0.44 to 0.36). Again over 95% of the variability fell within 2 standard deviations.

The HDI/Pulsewave ^{TM} CR-2000 Research Cardiovascular Profiling System has previously been proven to be reproducible over both a short and intermediate period of observation by Prisant *et al* (Prisant 2002). This group studied 59 healthy volunteers of mean age 36.5 years a mean of 52 days apart finding no significant difference between the readings obtained. Previously the reproducibility of this technology had been brought

into question but whereas Rietzschel described coefficients of variation of 32.8% for C1, 33.3% for C2 and 6.7% for AIx (Rietzschel 2001) suggesting superiority of SPCA Woodman found different results with higher coefficient of variation for AIx (22.4%) than for C1 (11.3%) or C2 (15.6%) suggesting otherwise (Woodman 2005).

The extent of pulse pressure amplification from the aorta to brachial artery varies with age, posture and exercise. Pulse pressure amplification as measured by SPCA, but not PWV, has been shown to be heart rate dependent (via manipulation of heart rate by incremental pacing) both in a group of older subjects with probable left ventricular systolic dysfunction and in a younger group with normal unimpaired left ventricular function (Wilkinson 2000, Wilkinson 2002c). Hence studies including PWA require uniformity in terms of preceding overnight fast and abstinence from strenuous exercise, caffeine, nicotine and a period of supine rest. Consensus guidelines have recently been published with respect to uniform, optimal conditions such that conclusions obtained from studies incorporating PWA may be widely applicable (Laurent 2006).

Large and small artery compliance values, while they lack a discrete anatomical site, are associated with changes in arterial stiffness that occur in a predictable fashion with age and cardiovascular risk factors; similarly with AIx. We therefore anticipated that there would be a significant relationship between these two measurements of arterial stiffness as described previously within the medical literature (Segers 2001, Rietzschel 2001, Woodman 2005). The significant correlations of -0.60 and -0.51 in healthy controls and patients with coronary artery disease are approximate to those described by others (r=-

0.75 Woodman 2005, r=-0.48 Rietzschel 2001) but stronger than those from Segers (r=-0.36 Segers 2001). This study however does not pool healthy subjects with those with coronary artery disease or like those by Woodman and Rietzschel respectively (Woodman 2005, Rietzschel 2001) and has a larger number of subjects than that by Segers (Segers 2001). It is not clear why there was no correlation (or at best a borderline correlation p=0.09) between large artery compliance and AIx in patients with coronary artery disease while there was in healthy controls. It is possible that patients with increasing arterial stiffness, which is known to correlate with coronary artery plaque load (McLeod 2004) the established disease phenotype within the individuals studied with coronary artery disease may have precluded the observation of an association.

There is no clear consensus yet as to which means of assessing arterial stiffness may be the most appropriate and robust for widespread clinical use. It remains possible that a pharmacological challenge may provide more information. Albuterol has been shown to produce repeatable changes in the aortic waveform that are substantially inhibited by LNMMA and is therefore an endothelium dependent NO mediated vasodilator (Wilkinson 2002d).This technique is potentially a simple, repeatable, non invasive means of assessing endothelial function in vivo and was blunted in patients with hypercholesterolemia (Wilkinson 2002d). It requires validation in other groups of single and combined risk factors.

PWV is generally accepted as the most simple, non-invasive, robust and reproducible method to assess arterial stiffness and carotid-femoral PWV is currently endorsed as the

current gold standard (Laurent 2006). In healthy individuals a decline in endothelial function is associated with increased large artery stiffness, wave reflections and central pulse pressure (McEniery 2006). Within patients with coronary artery disease carotid radial PWV has been shown to correlate with the extent of coronary artery plaque volume (McLeod 2004). The Anglo Cardiff Collaborative Trial, which included 4001 healthy, normotensive individuals aged 18 to 90, however noted that while PWV is likely to be a better measure in older individuals AIx may be a more sensitive marker of arterial stiffness in younger individuals (McEniery 2005). The correlations observed within the groups examined suggest that Windkessel based modelling may still have a role although there remains, to date, no clinical outcome data utilising this technique.

Chapter 4 The NOS3 G894T genotype and arterial compliance in patients with coronary artery disease.

4.0 Summary

This chapter elucidates the effect of the G894T single nucleotide polymorphism of the *NOS3* gene upon arterial compliance. We found that there was a significant association between the number of T alleles and the small artery compliance value as assessed by Windkessel based diastolic pulse wave contour analysis.

4.1 Introduction

An accruing body of evidence suggests that oxidative stress, of which reduced NO bioactivity is a hallmark, is significantly involved in the pathogenesis of vascular disease including hypertension, atherosclerosis, type 2 diabetes mellitus, heart failure and hypercholesterolemia (Landmesser 2001, Hamilton 2004).

A previously stated in detail within section 1.6 the *NOS3* single nucleotide polymorphism (SNP) (894 G→T) that encodes a Glu298→Asp amino acid substitution in the eNOS protein has been implicated with cardiovascular disorders in which NO bioactivity is impaired including coronary artery disease (Hingorani 1999, Hibi 1998, Colombo 2002), hypertension (Jáchymová 2001, Miyamoto 1998), stroke (Elbaz 2000) and end stage

renal disease (Noiri 2002). It is also associated with endothelial dysfunction (Leeson 2002).

A key characteristic of increased vascular oxidative stress is a decrease in the compliance of large and small blood vessels. This alteration in vascular stiffness can be measured non-invasively using pulse wave analysis for example. We used diastolic pulse wave contour analysis utilising the modified Windkessel model as al non invasive intermediate phenotype for the assessment of arterial function in patients with coronary artery disease. Consistent characteristic changes in the pulse pressure wave shape have been associated with ageing and disease states predisposing to vascular events in which NO bioactivity is impaired. Small artery compliance has been associated with coronary artery disease in post menopausal women (Cohn 1995) and also with impaired NO bioactivity (McVeigh 2001).

We therefore hypothesised that this functional SNP within an oxidative stress gene, which potentially modulates part of its effect on cardiovascular function through altering arterial stiffness possibly due to altered eNOS function, would affect the non invasive cardiovascular phenotype small artery compliance.

4.2 Methods

4.2.1 Subjects

103 volunteers were recruited from patients attending the Western Infirmary Glasgow for coronary artery bypass grafting for coronary artery disease. As detailed in section 2.2 each volunteer attended the BHF Glasgow Cardiovascular research Unit at the University of Glasgow the week prior to surgery fasted and abstinent from tobacco, alcohol, tea and coffee from the 10 pm previous night. Each study was performed by myself between 8.00 am and 11 am. Ethical permission for this study was obtained from the Ethics Committee of the West Glasgow Hospitals University NHS trust and each subject gave informed consent before a detailed clinical history and examination was performed including an ECG to ensure sinus rhythm.

4.2.2 Clinical procedures and laboratory analysis.

Morphological measurements were performed upon the arrival of the subject and then following 60 minutes supine rest in a quiet, temperature controlled room the clinical protocol outlined in section 2.3 was undertaken. Following pulse wave contour analysis a portion of blood was obtained for quantification of IL-6, Adiponectin, sICAM-1 and highly sensitive CRP and analysis of the *NOS3* G894T genotype. Small and large artery compliance values were obtained using a calibrated, proprietary tonometer (model CR 2000 Hypertension Diagnostics Inc) and used according to manufacturers specifications.

Augmentation Index was measured using the SphygmoCor system (PWV Medical). A full description of these techniques is detailed in section 2.3.1 and 2.3.2. IL-6, CRP, Adiponectin and sICAM1 were measured using the techniques detailed in 2.5. From the portion of blood taken DNA was extracted using the Wizard Genomic DNA Purification kit (Promega) and genotyping of the *NOS3* G894T SNP was performed by members of the BHF Glasgow Cardiovascular Research Centre who were unaware of the patients' phenotypes. The G894T polymorphism was genotyped by sequencing performed using SeqScape (Applied Biosystems) software and two individuals checked all genotypes. Concordance between the two individuals was 100%. A full description is detailed in section 2.4.

4.2.3 Statistical Evaluation.

All data are presented as mean ± SEM. Power calculations were performed before sample acquisition and 100 subjects were projected to provide 80% power for primary analysis based on two sample *t*-tests to compare mean C2 values between the two homozygous groups at each locus. The Chi squared statistic was used to ensure that the observed allele frequencies did not differ from that expected under Hardy-Weinberg equilibrium. One-way ANOVA was used to examine the effect of genotype of the *NOS3* SNP on vascular parameters as with this SNP there has been no documented dominant allele (Hibi 1998). Thereafter multiple regression analysis was preformed to identify the variables that independently predicted the relationships. Statistical significance was defined as $p<0.05$.

4.3 Results.

4.3.1 Clinical Characteristics

Baseline characteristics of the study population are those displayed in table 3.1. 78 patients were male and 27 female. The mean age of the patients was 61.84, mean blood pressure 131/71 and cholesterol 4.29 mmol/l. In terms of smoking status 37 patients had never smoked, 26 patients were ex smokers as defined by more than 6 months of abstinence and 40 patients continued to smoke cigarettes. The mean large artery compliance (C1) was 14.74 ml/mmHg x 10, mean small artery compliance (C2) 4.63 ml/mmHg x 100 and Augmentation Index (AIx) 28.89%. 75 patients were on Aspirin, 10 on Clopidogrel alone and 12 on both Aspirin and Clopidogrel. 76 were taking Beta Blockers, 55 on calcium channel blockers, 89 on HMG Co A Reductase Inhibitors, 43 on a form of Nitroglycerin, 51 on Angiotensin Converting Enzyme Inhibitors and 5 on Angiotensin II type 1 receptor antagonists.

4.3.2 The distribution of the genotypes and frequency of the alleles.

GG	GT	TT
43/103	47/103	13/103
42%	45%	13%

Table 4.1 The distribution of genotypes and frequency of the alleles of the *NOS3* gene.

The distribution of the genotypes and the frequency of the T alleles did not differ significantly from that expected under Hardy-Weinberg equilibrium (Chi = 0.007, p=0.65, q= 0.35).

4.3.3 The NOS3 G894T genotype and cardiovascular phenotypes.

The effect of the G894T SNP of the *NOS3* gene on cardiovascular phenotypes is shown in Table 4.2. There was no association observed between this polymorphism and blood pressure or large artery compliance (Table 4.2, Figures 4.1, 4.2, 4.3 and 4.4). ANOVA revealed a statistically significant association for TT homozygosity and small artery compliance (p=0.01, Table 4.2, Figure 4.5). The highest small artery compliance was seen in the patients homozygous for the G allele (5.51 ± 0.51 ml/mmHg x 100), an intermediate value observed in heterozygotes (4.21 ± 0.33 ml/mmHg x 100) and the lowest value demonstrated in patients homozygous for the T allele (3.18 ± 0.38ml/mmHg x 100). No association was evident in this cohort between *NOS3* G894T genotype and augmentation index (Table 4.2 and Figure 4.6)

Table 4.2-. Associations between the *NOS3* G894T genotype and cardiovascular phenotypes in patients with coronary artery disease.

Variable	Genotype	(n)	ANOVA
	GG	GT	TT	p value
	(43)	(47)	(13)	
Age (years)	58.98 ± 1.4	63.17 ± 1.2	63.85 ± 2.5	0.05
BMI (kg/m^2)	29.85 ± 0.89	29.56 ± 0.80	29.55 ± 0.85	0.97
SBP (mmHg)	129.9 ± 2.9	132.6 ± 2.3	127 ± 5.2	0.55
DBP (mmHg)	71.1 ± 1.6	71.11 ± 1.3	69.4 ± 2.8	0.85
PP (mmHg)	58.9 ± 2.0	61.5 ± 2.0	57.5 ± 2.9	0.51
HR (beats/min)	61.9 ± 1.6	62.5 ± 1.9	58.33 ± 2.3	0.53
C1 (ml/mmHg x 10)	15.87 ± 0.89	13.98 ± 0.64	14.47 ± 1.1	0.20
C2 (ml/mmHg x 100)	5.51 ± 0.51	4.21 ± 0.33	3.18 ± 0.38	0.01
Augmentation Index (%)	29.48 ± 2.01	28.99 ± 1.74	32.67 ± 2.43	0.61
CHOL (mmol/l)	4.26 ± 0.11	4.321 ± 0.14	4.43 ± 0.23	0.81
logTRIG (mmol/l)	0.24 ± 0.03	0.21 ± 0.04	0.21 ± 0.01	0.80
LDL (mmol/l)	2.30 ± 0.09	2.35 ± 0.12	2.55 ± 0.21	0.57
HDL (mmol/l)	1.06 ± 0.06	1.07 ± 0.06	1.08 ± 0.10	0.99
Log CRP (mg/l)	0.34 ± 0.07	0.42 ± 0.08	0.14 ± 0.17	0.21
Log Adiponectin (ng/ml)	3.49 ± 0.06	3.57 ± 0.05	3.66 ± 0.11	0.32
Log ICAM (ng/ml)	2.56 ± 0.02	2.55 ± 0.02	2.53 ± 0.05	0.84
Log IL-6 (pg/ml)	0.52 ± 0.06	0.33 ± 0.09	0.29 ± 0.18	0.19

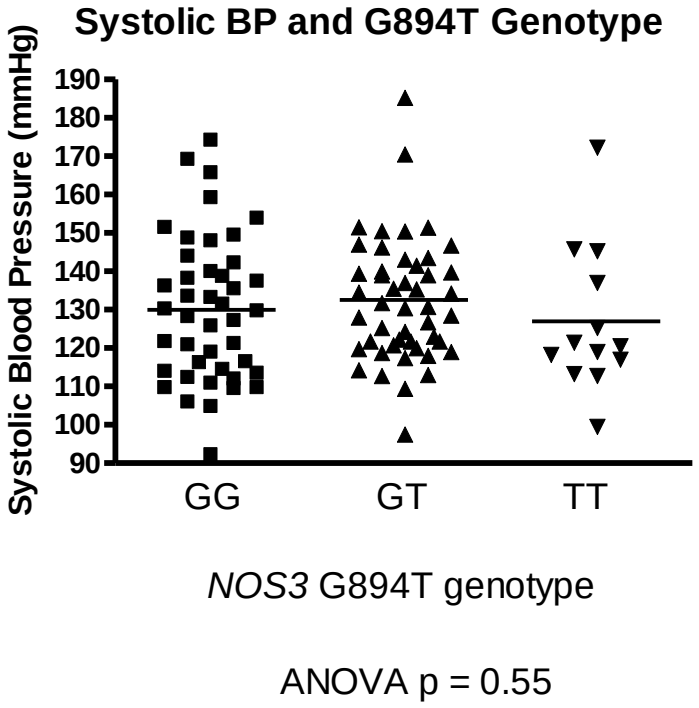


Figure 4.1:- The G894T SNP of the *NOS3* gene and systolic blood pressure in patients with coronary artery disease.

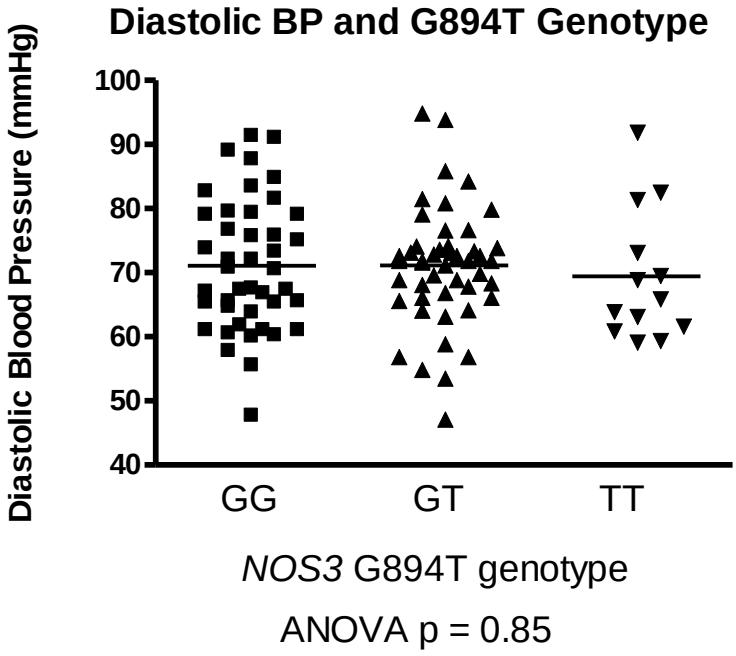


Figure 4.2:- The G894T SNP of the *NOS3* gene and diastolic blood pressure in patients with coronary artery disease.

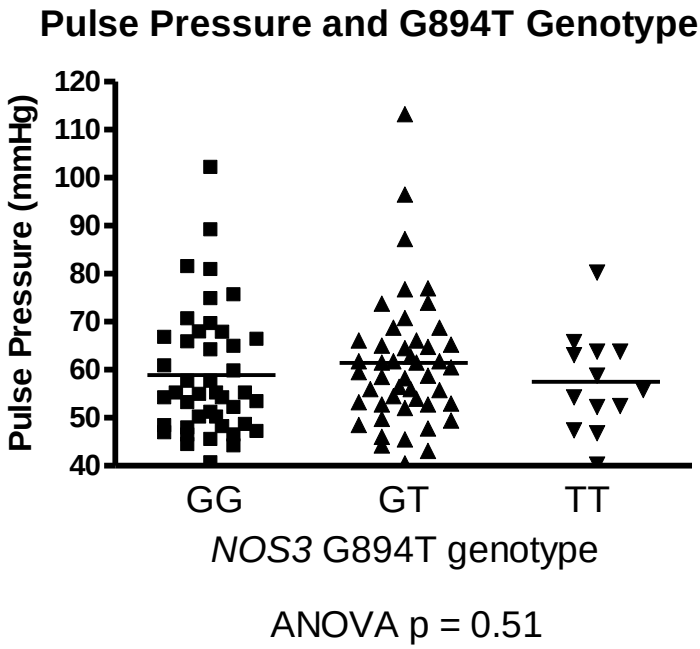


Figure 4.3:- The G894T SNP of the *NOS3* gene and pulse pressure in patients with coronary artery disease.

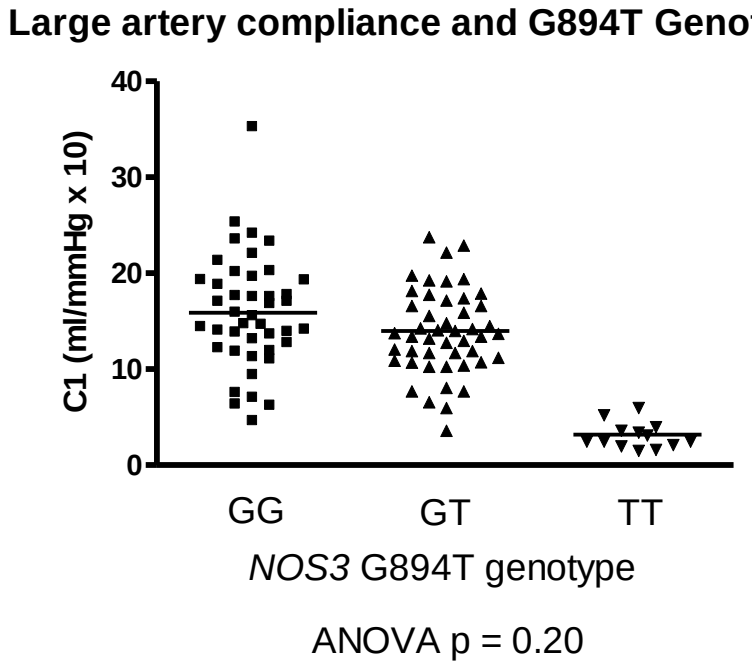


Figure 4.4:- The G894T SNP of the *NOS3* gene and large artery compliance in patients with coronary artery disease.

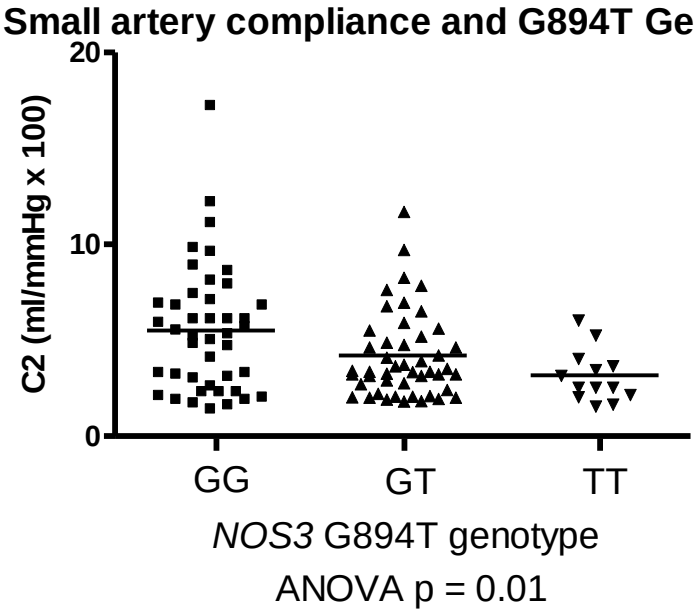


Figure 4.5:- The G894T SNP of the *NOS3* gene and small artery compliance in patients with coronary artery disease.

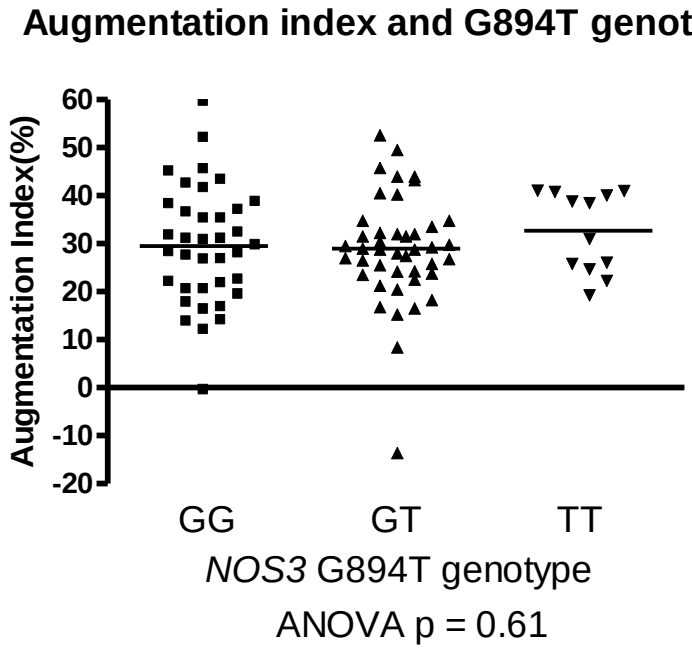


Figure 4.6:- The G894T SNP of the *NOS3* gene and augmentation index in patients with coronary artery disease.

4.3.4 Multiple Regression Analysis.

Multiple regression analysis, examining the possible contribution of age, BMI, SBP, DBP, total and LDL cholesterol showed that only small artery compliance was significant when *NOS3* G894T genotype was assigned as the dependent variable (p=0.01).

Predictor	Regression Coefficient	Standard Error	*T* value	*p* value
Age (years)	0.01	0.01	1.55	0.13
BMI (kg/m^2)	0.01	0.01	0.68	0.50
SBP (mmHg)	-0.01	0.01	1.63	0.11
DBP (mmHg)	0.0001	0.01	0.02	0.99
C1 (ml/mmHg x10)	-0.02	0.02	1.47	0.15
C2 (ml/mmHg x 100)	-0.07	0.03	2.64	0.01
Chol mmol/l	0.04	0.14	0.33	0.74
LDL mmol/l	0.05	0.15	0.36	0.72

Table 4.3: Multiple regression analysis of vascular phenotypes with *NOS3* G894T genotype as the dependent variable.

R^2= 16.11%, adjusted R^2= 16.11%, p=0.03 for the entire study group.

6.4 Discussion

Despite a growing body of evidence that implicates the *NOS3* G894T polymorphism in both human physiology and pathology there is still no complete consensus as to its role or indeed its functional mechanism. This SNP has been associated with altered baseline production of nitric oxide (Veldman 2002), blood pressure response to endurance training (Rankinen 2000), heamodynamic response to stress (Malhotra 2004), maternal vascular adaptation to healthy pregnancy (Savvidou 2001) and flow mediated dilatation and carotid intima media thickness in young healthy subjects (Paradossi 2004). There are many studies in different populations associating the T allele with susceptibility to ischaemic heart disease (Hingorani 1999, Hibi 1998, Colombo 2002, Gardemann 2002, Casas 2004). Some controversy remains with negative studies also reported (Rossi 2003, Schmoelzer 2003). The G894T SNP, though, has been associated with cerebrovascular disease (Elbaz 2000), survival in patients with congestive cardiac failure (McNamara 2003), coronary in stent restenosis (Gomma 2002) and as previously stated, end stage renal disease (Noiri 2002).

There is argument as to whether this SNP is functional. Glutamate and aspartate are both conservative substitutions and, indeed, analysis of the crystal structure indicates that residue 298 is situated externally away from the active site so that the aspartate substitution should have negligible effect on enzyme activity (Fischmann 1999). Though the initial experiment by Tesauro and colleagues (Tesauro 2000) suggesting that the mutant rather than the wild type enzyme was more amenable to proteolytic cleavage was

discounted as an artefact caused by the acidic pH used (Fairchild 2001), this group did suggest, using Chou-Fasman secondary structure predictions, that potentially significant structural changes would occur, even with the seemingly small conservative replacement (Tesauro 2000). Golser *et al* (Golser 2003) did not elicit any difference between the 2 variants with respect to enzyme kinetic parameters, bound cofactors, uncoupled NAD(P)H oxidase activity and binding activities for calcium calmodulin and tetrahydrobiopterin. Moreover, it has recently been suggested via a series of experiments examining the complex intracellular regulation of eNOS that the aspartate variant is unlikely to directly modulate eNOS activity (McDonald 2004). However, Noiri *et al* (Noiri 2002) have demonstrated, utilising stably transfected Chinese hamster ovary cells for comparing nitric oxide activity, a statistically significant difference in nitric oxide production between the 298 glutamate and 298 aspartate variants. This is in keeping with our finding that the number of T alleles was associated with small artery compliance, which has been found to be altered in parallel with pharmacological manipulation of nitric oxide bioavailability (McVeigh 2001).

Measurement of small artery compliance using the modified Windkessel model of diastolic pulse contour analysis has been suggested as a useful marker for the non invasive detection of vascular abnormalities in disease states where nitric oxide bioactivity is known to be impaired (McVeigh 1991, McVeigh 1993, Cohn 1995). Recent prospective study suggested possible prognostic (survival) significance for the Asp298 variant of the *NOS3* gene in patients with heart failure (McNamara 2003). One can extrapolate from this and our current data that the non-invasive measurements of arterial

compliance (especially C2) might be used as a marker of severity of cardiovascular disease and ultimately PWA *per se* as a non invasive phenotype which would delineate those most at benefit of aggressive intervention.

Chapter 5 The C242T single nucleotide polymorphism of the CYBA gene and blood pressure and arterial compliance in patients with coronary artery disease.

5.0 Summary

This chapter describes the effect of the C242T single nucleotide polymorphism upon blood pressure and arterial compliance in patients with coronary artery disease. In short we found that the 242T allele was associated with elevated systolic and pulse pressure and large artery compliance but not diastolic blood pressure nor small artery compliance in patients with coronary artery disease.

5.1 Introduction

Oxidative stress is increasingly implicated, as described in sections 1.3 and 1.4, in vascular disease. Moreover that induced by O_2^- is especially important as many other reactive oxygen species are subsequently derived from it (Taniyama 2003). The membrane bound NADPH enzyme system is putatively the major source of vascular O_2^-. Expression of the p22phox has been found to be more intense in atherosclerotic human coronary arteries than non atherosclerotic human arteries, indicating that the p22phox might participate in the pathophysiology and pathogenesis of atherosclerotic coronary artery disease (Azumi 1999). The gene coding for p22phox, a critical component of the NADH/NAD(P)H oxidase enzyme system, a major source of vascular O_2^-, is *CYBA*.

Among the allelic polymorphisms reported in *CYBA* is C242T, which has been demonstrated to affect NADPH oxidase activity (Wyche 2004, Guzik 2000). There have been several, contradictory, associations of the C242T SNP with coronary atherosclerosis some associating the T allele as being associated with coronary artery disease and others indeed suggesting that the T allele may be protective. 'Positive' associating studies have been reported by Cia (Cai 1999), Cahilly (Cahilly 2000) and Nasti (Nasti 2006) within European and American populations whereas 'negative' non associating studies have emanated from several groups most recently Finland (Fan 2006, Inoue 1998, Li 1999, Saha 1999, Zafari 2002). This SNP has also been associated with cerebrovascular disease (Ito 2000), carotid atherosclerosis (Hayaishi-Okano 2003) and susceptibility to diabetic nephropathy (Hodgkinson 2003). No relation has been found however with peripheral vascular disease (Renner 2000), pre eclampsia (Raijmakers 2002) or with endothelial function in patients with hypercholesterolaemia (Schneider 2003).

Consequently given the importance of O_2^- in the generation of vascular disease and the association of genetic variation in the p22phox gene with vascular disease we sought to ascertain if there was a relationship between vascular compliance measured using systolic and diastolic pulse contour analysis and blood pressure and variation of the C242T SNP of *CYBA* in patients with angiographically proven coronary artery disease.

5.2 Methods

5.2.1 Subjects

103 volunteers were recruited from patients attending the Western Infirmary for coronary artery bypass grafting for symptomatic, obstructive coronary artery disease. As detailed in section 2.2 each volunteer attended the BHF Glasgow Cardiovascular research Unit at the University of Glasgow the week prior to surgery fasted and abstinent from tobacco, alcohol, tea and coffee from the previous night. Ethical permission for this study was obtained from the Ethics Committee of the West Glasgow Hospitals University NHS trust and each subject gave informed consent before a detailed clinical history and examination was performed including an ECG to ensure sinus rhythm.

5.2.2 Clinical Procedures and laboratory analysis.

Morphological measurements were performed upon the arrival of the subject and then following 60 minutes supine rest in a quiet, temperature controlled room the clinical protocol outlined in section 2.3 was undertaken. Subsequently a portion of blood was taken for quantification of IL-6, Adiponectin, sICAM-1 and highly sensitive CRP and analysis of the C242T *CYBA* genotype. Small and large artery compliance values were obtained using a calibrated, proprietary tonometer (model CR 2000 Hypertension Diagnostics Inc) and used according to manufacturers specifications. Augmentation

Index was measured using the SphygmoCor system (PWV Medical). A full description of these techniques is detailed in section 2.3.1 and 2.3.2. IL-6, CRP, Adiponectin and sICAM1 were measured using the techniques detailed in 2.5. From the portion of blood taken DNA was extracted using the Wizard Genomic DNA Purification kit (Promega) and genotyping of the *CYBA* C242T SNPs was performed by members of the BHF Glasgow Cardiovascular Research Centre who were unaware of the patients' phenotypes. The C to T substitution, at position 242 in the *CYBA* was typed by *Rsa*I digestion of specific polymerase chain reaction products amplified from genomic DNA as described in section 2.4. Analysis was preformed on a 1.5% agarose gel.

5.2.3 Statistical Evaluation

All data are presented as mean ± SEM. Power calculations were performed before sample acquisition and 100 subjects, based on pilot data, were projected to provide 80% power for primary analysis based on two sample *t*-tests to compare mean C1 or C2 values between the two homozygous groups at each locus. The Chi squared statistic was used to ensure that the observed allele frequencies did not differ from that expected under Hardy-Weinberg equilibrium

A two tailed Student's *t*-test was used to analyse differences in phenotype between subjects with presence or absence of the T allele for the *CYBA* C242T SNP, as this has previously been reported as being the dominant allele (Cai 1999, Cahilly 2000 and Nasti 2006). Thereafter multiple regression analysis was preformed to identify the variables

that independently predicted the relationships. Statistical significance was defined as $p<0.05$.

5.3 Results

5.3.1 Clinical Characteristics.

The baseline characteristics of the study population are those displayed in table 3.1. 78 patients were male and 27 female. The mean age of the patients was 61.84, mean blood pressure 130.94/70.88and cholesterol 4.29 mmol/l. In terms of smoking status 37 patients had never smoked, 26 patients were ex smokers as defined by more than 6 months of abstinence and 40 patients continued to smoke cigarettes. The mean large artery compliance (C1) was 14.74 ml/mmHg x 10, mean small artery compliance (C2) 4.63 ml/mmHg x 100 and Augmentation Index (AIx) 28.89%. 75 patients were on Aspirin, 10 on Clopidogrel alone and 12 on both Aspirin and Clopidogrel. 76 were taking Beta Blockers, 55 on calcium channel blockers, 89 on HMG Co A Reductase Inhibitors, 43 on a form of Nitroglycerin, 51 on Angiotensin Converting Enzyme Inhibitors and 5 on Angiotensin II type 1 receptor antagonists.

5.3.2 The distribution of the genotypes and frequency of the alleles.

CC	CT	TT
44/103	51/103	8/103
43%	50%	7%

Table 5.1 The distribution of genotypes and frequency of the alleles of the p22phox *CYBA* gene.

The distribution of the genotypes and the frequency of the T alleles did not differ significantly from that expected under Hardy-Weinberg equilibrium (Chi = 1.69, p=0.67, q= 0.33).

5.3.3 The CYBA C242T genotype and cardiovascular phenotypes.

We sought to examine the influence of the C242T SNP of *CYBA* upon vascular compliance and blood pressure using the dominant allele model. The analysis of all measured cardiovascular phenotypes and *CYBA C*242T genotype are illustrated in Table 5.2. As displayed in table 5.2 and Figure 5.1 the presence of the 242T allele was associated with significantly higher systolic blood pressure. Patients homozygous for the C allele had lower systolic blood pressure (125.7 ± 2.3 mmHg) than heterozygotes and patients homozygous for the T allele(134.6 ± 2.4 mmHg) (p=0.010). There was no statistically significant effect upon diastolic blood pressure (Table 5.2 and Figure 5.2).

There was however a significant association observed between the 242T allele and pulse pressure (Table 5.2 and Figure 5.3). Patients that were homozygous for the C allele had a PP of 56 ± 1.6 mmHg where as patients heterozygous or homozygous for the T allele had a PP of 62.7 ± 1.8 mmHg (p =0.01).

In terms of arterial stiffness the 242T allele was associated with lower large artery compliance (Table 5.3 and Figure 5.4). CC homozygotes had higher large artery compliance (17.07 ± 0.82 ml/mmHg x10), than heterozygotes and TT homozygotes (13.16 ± 0.53 ml/mmHg x10) (p = < 0.001). No difference was observed between the C242T SNP and small artery compliance nor Augmentation Index in our study population (Table 5.3 and Figures 5.5 and 5.6)

Table 5.2 -. Associations between the *CYBA* C242T genotype and cardiovascular phenotypes in patients with coronary artery disease.

Variable	Genotype (n)				p value
	CC (44)	CT (51)	TT (8)	CT + TT (59)	CC vs CT+TT
Age (years)	61.91 ± 1.1	61.6 ± 1.4	58.63 ± 1.3	61.2 ± 1.3	0.68
BMI (kg/m^2)	28.89 ± 0.69	29.82 ± 0.65	33.2 ± 3.8	30.27 ± 0.76	0.18
SBP (mmHg)	125.7 ± 2.3	134.4 ± 2.6	135.6 ± 6.3	134.6 ± 2.4	0.01
DBP (mmHg)	69.6 ± 1.7	71.93 ± 1.2	71.16 ± 2.0	71.82 ± 1.1	0.28
PP (mmHg)	56 ± 1.6	62.5 ± 2.0	64.5 ± 5.3	62.7 ± 1.8	0.01
HR (beats/min)	62.2 ± 1.8	61.7 ± 1.7	58.83 ± 3.0	61.3 ± 1.5	0.70
C1 (ml/mmHg x 10)	17.07 ± 0.82	13.39 ± 0.57	11.71 ± 1.5	13.16 ± 0.53	0.00
C2 (ml/mmHg x 100)	4.66 ± 0.35	4.74 ± 0.45	3.73 ± 0.69	4.60 ± 0.40	0.91
Augmentation Index (%)	30.57 ± 1.4	28.3 ± 2.0	32.78 ± 3.0	29.0 ± 1.8	0.49
CHOL (mmol/l)	4.40 ± 0.12	4.24 ± 0.11	4.28 ± 0.42	4.25 ± 0.11	0.40
logTRIG (mmol/l)	0.18 ± 0.03	0.26 ± 0.04	0.25 ± 0.09	0.26 ± 0.04	0.12
LDL (mmol/l)	2.50 ± 0.10	2.21 ± 0.10	2.45 ± 0.36	2.24 ± 0.10	0.07
HDL (mmol/l)	1.13 ± 0.06	1.04 ± 0.05	0.90 ± 0.10	1.02 ± 0.05	0.14
Log CRP (mg/l)	0.36 ± 0.08	0.31 ± 0.07	0.57 ± 0.17	0.34 ± 0.07	0.84
Log Adiponectin (ng/ml)	3.45 ± 0.10	3.59 ± 0.05	3.32 ± 0.14	3.55 ± 0.05	0.34
Log ICAM (ng/ml)	2.57 ± 0.02	2.54 ± 0.02	2.56 ± 0.03	2.54 ± 0.02	0.32
Log IL-6 (pg/ml)	0.41 ± 0.09	0.42 ± 0.07	0.27 ± 0.27	0.40 ± 0.07	0.96

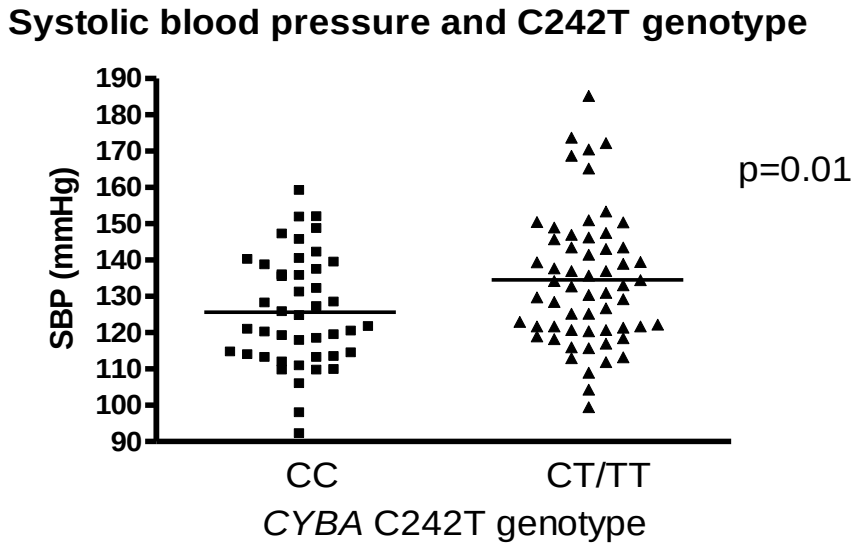


Figure 5.1:- The C242T SNP of the CYBA gene and SBP in patients with coronary artery disease.

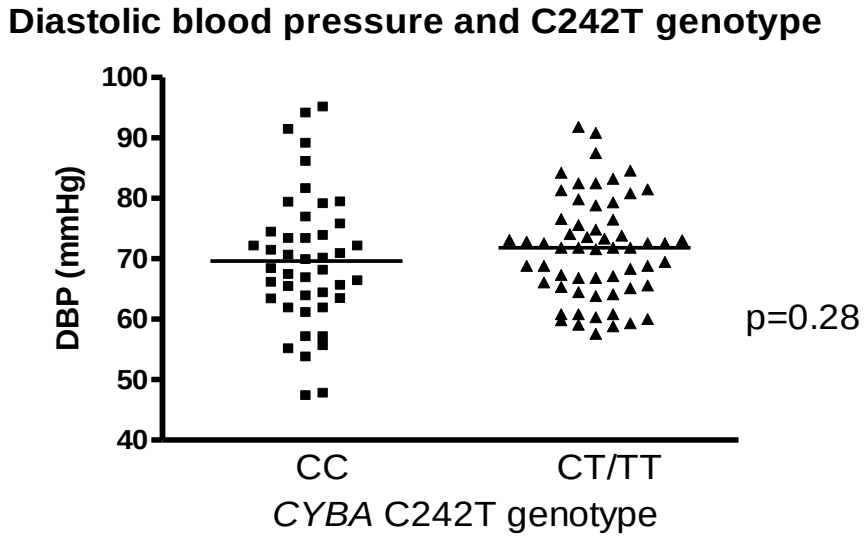


Figure 5.2:- The C242T SNP of the CYBA gene and DBP in patients with coronary artery disease.

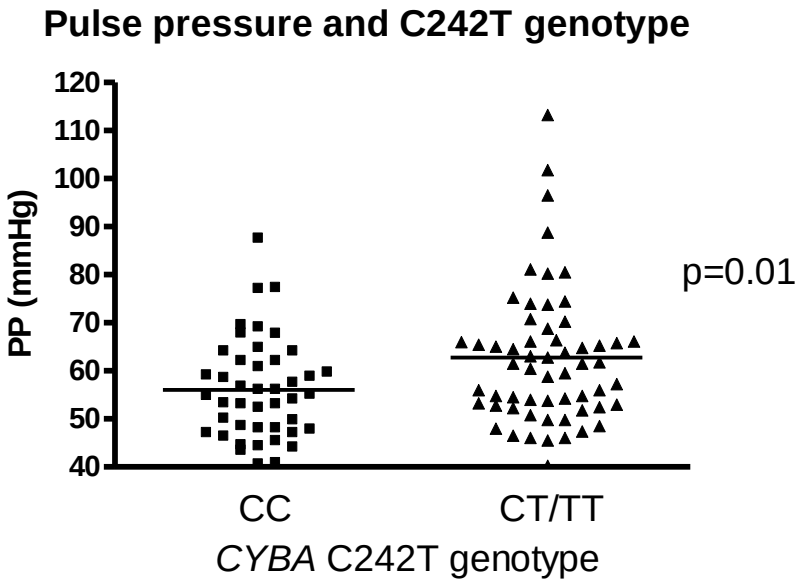


Figure 5.3:- The C242T SNP of the *CYBA* gene and PP in patients with coronary artery disease.

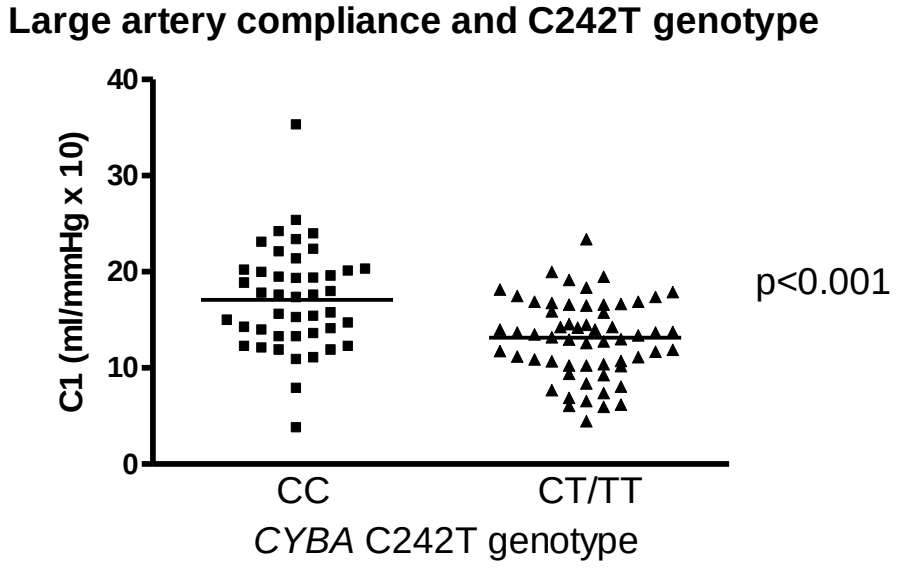


Figure 5.4:- The C242T SNP of the *CYBA* gene and C1 in patients with coronary artery disease.

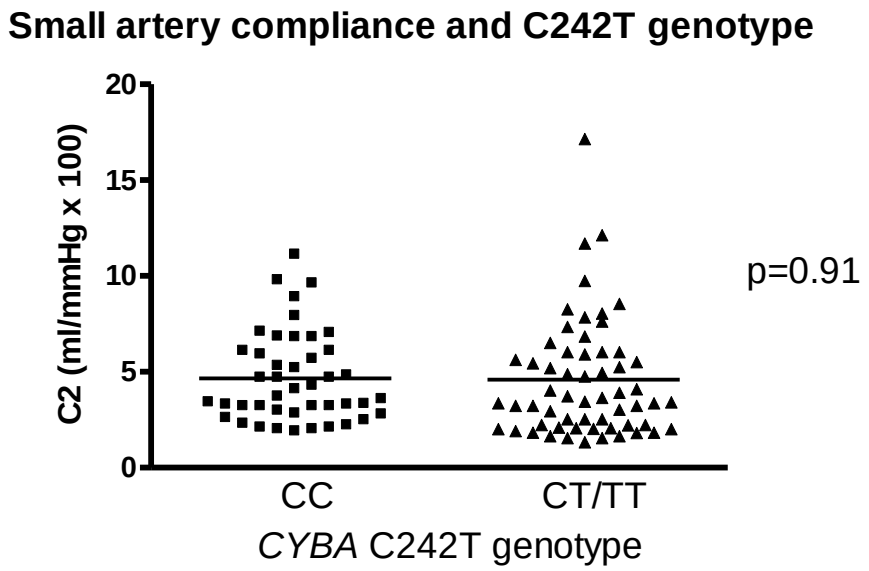


Figure 5.5:- The C242T SNP of the *CYBA* gene and C2 in patients with coronary artery disease.

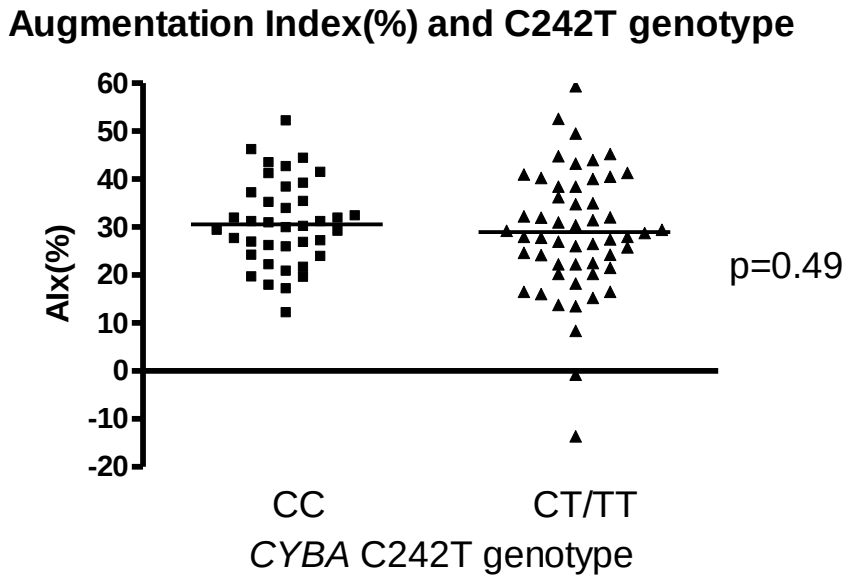


Figure 5.6:- The C242T SNP of the *CYBA* gene and AIx in patients with coronary artery disease.

5.3.4 Multiple Regression Analysis.

Multiple regression analysis, examining the possible contribution of age, BMI, SBP, DBP and total cholesterol showed that only large artery compliance was significant when *CYBA* C242T genotype was assigned as the dependent variable (p=0.001).

Predictor	Regression Coefficient	Standard Error	*t* value	*p* value
Age (years)	-0.005	0.006	0.771	0.443
BMI (kg/m^2)	0.010	0.009	1.138	0.258
SBP (mmHg)	0.003	0.004	0.647	0.519
DBP (mmHg)	-0.004	0.007	0.523	0.602
C1 (ml/mmHg x10)	-0.040	0.012	3.316	0.001
C2 (ml/mmHg x 100)	0.021	0.019	1.107	0.271
Chol mmol/l	-0.040	0.057	0.705	0.483

Table 5.3: Multiple regression analysis of vascular phenotypes with *CYBA* C242T genotype as the dependent variable.

R^2= 19%, adjusted R^2= 13.3%, p=0.004 for the entire study group.

5.4 Discussion

The p22phox subunit of NAD(P)H oxidase has been associated with vascular disease. Azumi and co workers (Azumi 1999) demonstrated increased p22phox expression that paralleled increasing disease severity in atherosclerotic coronary artery endothelial cells and vascular smooth muscle cells. Guzik *et al* (Guzik 2002) showed that the p22phox protein subunit was significantly increased in human diabetic veins and arteries.

Debate exists as to the role of the common C242T polymorphism of the p22phox gene in the pathogenesis of vascular disease. This SNP results in a substitution of histidine-72 with tyrosine that has been speculated to modulate NAD(P)H oxidase enzyme activity by affecting its heme binding site thus influencing the amount of superoxide present and consequently nitric oxide bioactivity (Guzik 2000). Some groups have found that this single nucleotide polymorphism has no effect upon risk of ischaemic heart disease (Li 1999, Gardemann 1999, Saha 1999). Others have also suggested that the T allele may even be protective in terms of less superoxide generation within human blood vessels and less development of carotid atherosclerosis in patients with type 2 diabetes mellitus (Hayaishi-Okano 2002). Schächinger et al (Schächinger 2001) suggested that the CC but not the TT homozygosity was associated with blunted coronary endothelial vasodilatation.

On the contrary, and in keeping with the findings of our study, the T allele of the p22phox gene has been associated with premature coronary artery disease in an Australian population (Cai 1999) and with progression of angiographically determined atherosclerosis in an American population in a prospective study (Cahilly 2000). These observations were more recently corroborated in an Italian population by Nasti and colleagues who noted that in 494 Caucasian Italians undergoing diagnostic coronary angiography to ascertain the cause of chest pain that the frequency of the mutant T allele was significantly higher in the 276 patients with angiographically documented coronary artery disease (Nasti 2006). Furthermore this relationship with the T allele was even stronger within patients with early onset coronary artery disease (aged < 55 years) (Nasti 2006). Within a Japanese population the 242T allele has been associated with ischaemic cerebrovascular disease (Ito 2000). Additionally the T allele has been shown to be a marker of susceptibility to microvascular disease (nephropathy) in patients with type 1 diabetes mellitus (Hodgkinson 2003).

This is the first report of an association between the 242T allele of *CYBA* and the large artery stiffness as measured non-invasively. Further more we have shown an association between the T allele and higher systolic and pulse pressure. A recent previous report by Moreno and coworkers found, conversely, that the prevalence of the CC genotype and the C allele were significantly higher in hypertensives than in normotensives (Moreno 2006). The disparity between these results is possibly explained by the severity of vascular disease displayed in our cohort evidenced by prior diagnostic angiography necessitating bypass grafting. The strength of our observation finding an association with non invasive

arterial stiffness is in keeping with recent evidence that suggests that differences in central aortic pressures and hence large artery stiffness may be a determinant of differing clinical outcomes, as documented in the Conduit Artery Function Evaluation sub study of the Anglo-Scandinavian Outcomes Trial (ASCOT) investigators (Williams 2006). A prospective study combining pulse contour analysis and p22phox genotype would enable a definitive observation regarding the importance of this genotype in cardiovascular survival and the strength of the observed associations between the T allele and systolic and pulse pressure and large artery stiffness. Though the study was performed blind to genotype the weakness of this study is in both the small sample size and small numbers of homozygotes and the degree of overlap between each of the groups analysed as displayed within the scatter points and hence the results should be interpreted with a degree of caution.

Chapter 6 Combined analysis of NOS3 G894T and CYBA C242T genotypes upon arterial stiffness.

6.0 Summary

This chapter describes the collated analysis of the *NOS3* G894T and *CYBA C242T* genotypes upon arterial stiffness. When the favourable and unfavourable genotypes were compared an additive effect was observed such that patients homozygous for the 'deleterious' alleles had lower large and arterial compliance values when contrasted with the 'favourable' alleles.

6.1 Introduction

The involvement of the two SNPs examined in chapters' 4 and 5 in vascular disease combined with the observations that eNOS protein expression and NO release are reduced in human atherosclerosis (Oemar 1998) and augmented p22phox expression in atherosclerotic human coronary arteries (Azumi 1999) with the severity of atherosclerosis correlating with NAD(P)H oxidase subunit mRNA expression (Sorescu 2002) leads to the hypothesis that these two genes may interact. Given that both SNPs have been shown to have the potential to be functional, the C242T *CYBA* polymorphism via the alteration of the heme binding site (Guzik 2002) and the G894T *NOS3* via altered cleavage at the cleavage altering NO generation (Tesauro 1999) we hypothesized that the combination of

the deleterious homozygote alleles of both SNPs may result in reduced arterial compliance and hence increased arterial stiffness when compared to the favourable alleles.

6.2 Methods

6.2.1. Subjects, clinical procedures and statistical evaluation.

The subjects utilised in the analysis of a possible gene gene interaction were those detailed in chapters 4 and 5 and documented in detail in section 2.2. Additionally the genotyping of both genes was performed utilising polymerase chain reaction and restriction digestion for the *CYBA* C242T SNP and sequencing for the *NOS3* G894T SNP. A comparison using the Student's *t*-test was carried out between patients homozygous for the *NOS3*T allele and possessing the *CYBA* 242T allele and patients homozygous for the *NOS3* G allele and for the *CYBA* C allele. Thereafter multiple regression analysis was performed to identify the variables that independently predicted the relationships.

6.3 Results

The combined analysis of *NOS3* G894T and *CYBA C242T* genotypes are detailed in table 6.1. There was no difference evident in age, BMI, BP or cholesterol parameters between

the two groups. In order to contrast the arterial stiffness between the favourable versus the non-favourable genotypes patients homozygous for the *NOS3* G allele and homozygous for the *CYBA* C allele were compared with those homozygous for the *NOS3*T allele and possessing the *CYBA* 242T allele. Hence the numbers analysed are small (18 *CYBA* CC & *NOS3* GG and 8 *CYBA* CT/TT & *NOS3* TT) as only patients with the 'opposite' genotype are included in the analysis. The former displayed higher large and small artery compliance than the latter group. Patients with the CC/GG genotype had a mean C1 of 19.68 ± 1.3 ml/mmHg x10, with patients with the CT+TT/TT genotype a mean C1 13.90 ± 1.6 ml/mmHg x10 (p=0.01, Table 6.1, Figure 6.1). Likewise patients with the CC/GG genotype had a mean C2 of 5.48 ± 0.63 ml/mmHg x100, with patients with the CT+TT/TT genotype a mean C2 of 3.24 ± 0.52 ml/mmHg x100 (p=0.01, Table 6.1, Figure 6.2). Using multifactor dimensionality reduction to detect epistasis there was no evidence of epistasis between the two genotypes analysed using the constructive induction algorithm (Ritchie 2001).

Table 6.1. Gene gene interaction. Associations between the *CYBA* C242T and *NOS3* G894T SNPs and cardiovascular phenotypes.

Variable	Genotype(n)		p value
	***CYBA* CC**	**CYBA CT/TT**	**CC+GG vs**
	***NOS3* GG**	**NOS3 TT**	**CT/TT + TT**
	(18)	**(8)**	
Age(years)	59.44 ± 1.5	60.13 ± 3.3	0.85
BMI (Kg/m2)	28.79 ± 0.92	29.50 ± 1.00	0.61
SBP (mmHg)	122.6 ± 4.00	126.8 ± 8.00	0.64
DBP (mmHg)	69.7 ± 2.5	69.6 ± 4.2	0.98
PP (mmHg)	52.81 ± 2.1	57.2 ± 4.4	0.39
HR (beats/min)	60.7 ± 2.9	55.79 ± 2.5	0.21
C1 (ml/mmHg x10)	19.68 ± 1.3	13.90 ± 1.6	0.01
C2 (ml/mmHg x100)	5.48 ± 0.63	3.24 ± 0.52	0.01
CHOL (mmol/l)	4.13 ± 0.15	4.51 ± 0.35	0.34
logTRIG (mmol/l)	0.17 ± 0.04	0.262 ± 0.06	0.20
LDL (mmol/l)	2.28 ± 0.14	2.58 ± 0.93	0.33
HDL (mmol/l)	1.12 ± 0.09	1.04 ± 0.13	0.13

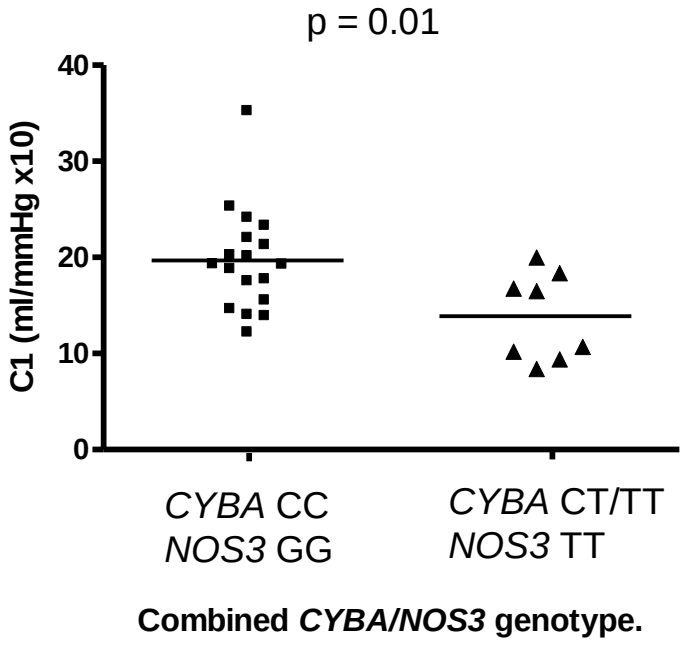


Figure 6.1 Combined analysis of the *CYBA* C242T and *NOS3* G894T genotypes and large artery compliance.

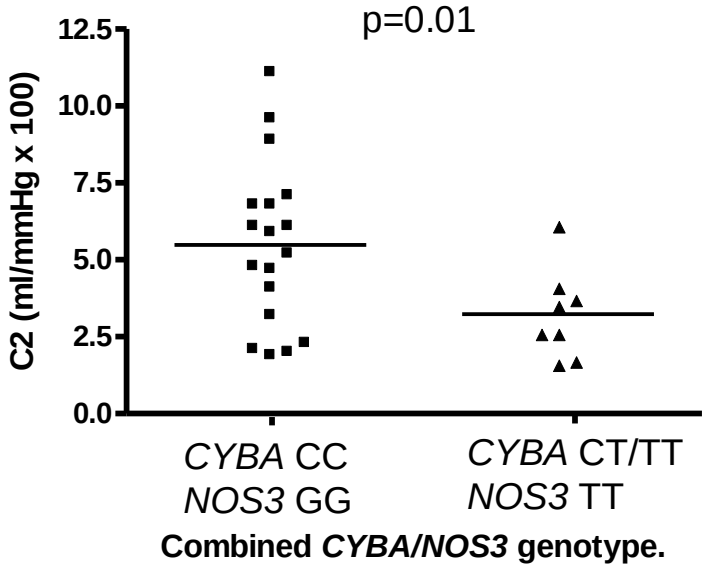


Figure 6.2 Combined analysis of the *CYBA* C242T and *NOS3* G894T genotypes and small artery compliance.

6.3.4 Multiple Regression Analysis.

Multiple regression analysis, examining the possible contribution of age, BMI, SBP, DBP, total, ldl cholesterol and triglyceride concentration showed that only the large(p=0.02) and small(p=0.05) artery compliance values contributed significantly when genotype was assigned as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t* value	*p* value
Age (years)	0.014	0.01	1.04	0.32
BMI (kg/m^2)	0.0004	0.02	0.02	0.99
SBP (mmHg)	0.002	0.01	0.16	0.87
DBP (mmHg)	-0.02	0.02	1.18	0.26
C1 (ml/mmHg x10)	-0.04	0.02	2.65	0.02
C2 (ml/mmHg x 100)	-0.07	0.03	2.14	0.05
Chol mmol/l	-0.06	0.25	0.23	0.82
LDL mmol/l	0.34	0.26	1.33	0.20
Log Trig mmol/l	0.19	0.51	0.36	0.72

Table 6.2: Multiple regression analysis of vascular phenotypes with combined *NOS3* G894T - *CYBA* C242T genotype as the dependent variable.

R^2= 61.9%, adjusted R^2= 69.2%, p=0.03 for the entire study group.

6.4 Discussion

This chapter outlines the potentially additive effects of two common allelic variants within oxidative stress genes that potentially modulate the action of the gene products and are both implicated in the pathogenesis of vascular disease.

Other groups have also attempted to look at the association of cardiovascular phenotypes with combinations of allelic variants. A large meta analysis was undertaken by Zintzaras *et al* (Zintzaras 2006) looking at the meta analysis of all the available studies relating the G894T, eNOS VNTR 4 b/a, -786T/C and G23T polymorphisms and their associations with hypertension. They preformed cumulative and recursive cumulative meta analyses which supported an association between hypertension and the eNOS VNTR 4 b/a polymorphism such that under a recessive model the allele b provided evidence of protection, especially when the analysis was confined to whites. Notably they found no detectable influence of the G894T, -786T/C nor G23T polymorphisms (Zintzaras 2006). A sib-pair study analysis and haplotype study was carried out by Persu and co workers who examined for linkage and association of the three commonly reported di allelelic polymorphisms (G894T, -786T/C, eNOS VNTR 4 b/a) and the intron 13 CA-repeat of *NOS3* with blood pressure as a continuous trait (Persu 2005). The *NOS3* haplotypes which associated with ambulatory blood pressure recordings all harboured the 894T allele (Persu 2005).

Other studies looking to ascertain the influence of combinations of allelic variants have been Casas *et al* (Casas 2004) have previously reported that individuals homozygous for the *NOS3* 894T and the *NOS3* intron-4a allele are at increased risk of ischemic heart disease (Casas 2004). Further more a gene-gene interaction has also been documented between another *NOS3* SNP (-786T/C) and the angiotensin converting enzyme gene insertion/deletion polymorphism in patients with early coronary artery disease (Álvarez).

Sampians *et al* (Sampians 2007) investigated the relationship with the three commonly reported di allelelic polymorphisms (G894T, -786T/C, eNOS VNTR 4 b/a) and endothelial dysfunction in a population of 128 patients with an acute myocardial infarction aged less than 40. They found that a NOS3 -786TT/894 GT haplotype was associated with increased flow mediated dilatation than the other haplotypes.

The observed interaction between the SNPs studied in this study and arterial compliance is the first to concentrate on a non invasive cardiovascular phenotype. It is the first study additionally to attempt to combine the effect of the *CYBA* C242T SNP with the *NOS3* G894T SNP. Chapter 4 describes that the 894T allele of the *NOS3* gene is associated with attenuated small artery compliance and chapter 5 that the 242T allele of the *CYBA* gene is associated with high large artery stiffness. When the 2 alleles coincide the negative effect could be further potentiated. The limitations are naturally the small number of patients that were involved in the analysis as well as that the subjects are drawn from a single geographic are and all have established vascular disease. The fact that both genes are involved in ROS generation and implicated, as described, in the pathophysiology of

atherosclerotic vascular disease lends further weight to the potential usefulness of PWA as an intermediate phenotype for cardiovascular clinical functional genomics.

Chapter 7 Chronic low grade inflammation and insulin resistance and arterial compliance in healthy volunteers.

7.0 Summary

This clinical study was designed to ascertain whether, in healthy individuals free of vascular disease, markers of chronic low grade inflammation and insulin resistance were associated with arterial compliance. Fifty three healthy volunteers attended the clinical research unit to have a non invasive vascular profile performed and serum taken for biochemical analysis. In multiple regression analysis there was an association between vascular compliance and some, but not all markers of low grade chronic inflammation and insulin resistance.

7.1 Introduction

The advent of both non invasive vascular and biochemical surrogate markers for cardiovascular disease has provided intermediate phenotypes that may guide the clinician in treatment and risk stratification before established vascular disease is present (Davies 2003, Oliver 2003, McVeigh 2003, Cohn 2004). Cardiovascular disease is complicit with alterations in the structure, properties, and function of wall and endothelial components of blood vessels (Gibbons 1994). This study examines biochemical compounds which are either markers of, or pathophysiologically involved in, the genesis of vascular disease and

compares them to the non-invasively obtained large and small artery compliance values and augmentation index. Plasma levels of CRP are strong independent predictors of atherosclerotic events in apparently healthy individuals (Ridker 1997, Ridker 1998). Its association with aortic and brachial PWV and augmentation index has been examined previously (Yasmin 2004, Kampus 2004). IL-6 plasma levels within the highest quartile of the 'normal' range have been associated with future cardiovascular events (Harris 1999, Ridker 1999). Lower levels of Adiponectin are associated with insulin resistance, type 2 diabetes mellitus, hypertension and ischaemic heart disease (Matsuzawa 2004, Mallamaci 2002, Kumada 2003). There has also been an association of levels of Adiponectin with endothelial function (Lawlor 2005). Increasing levels of ICAM1 are associated with dyslipidaemia, hypertension and ischaemic heart disease (Hackman 1996, Chae 2001, Ridker 1998). Changes in the pulse pressure wave shape have been described in all these entities both in terms of AIx and small and large compliance values (Wilkinson 2000, Wilkinson 2002, Cohn 1995, McVeigh 1999). As with each marker studied statistical strength does not imply causality as confounding factors or reverse causality offer alternative explanations for the association (Schunkert 2008). An association thus between arterial stiffness and these biochemical markers would perhaps strengthen their relative associations with vascular disease and putatively add to their potential role in risk stratification.

7.2 Methods

7.2.1 Subjects

Fifty three healthy normotensive volunteers were recruited via advertisements within Glasgow University and local newspapers. This clinical study was approved by the Ethics Committee of the West Glasgow University NHS trust. No subjects were taking any medication and all abstained from alcohol, nicotine, tobacco, food and strenuous activity overnight before the study day. All subjects attended fasted in the morning between 7.00 and 9.00 am depending on availability. Physical health was confirmed by screening with history, a full physical examination, ECG and supine blood pressure measurement in triplicate (Dinamap Critikon, Johnson and Johnson Professional Products Ltd.). Ninety volunteers were screened and those with raised blood pressure, an antecedent history of vascular disease or an abnormal ECG excluded. Of the 53 patients recruited the mean age was 52.74 years (range 37-72) years. Mean systolic blood pressure was 125 ±13 mmHg and diastolic blood pressure 72 ± 8 mmHg. The mean BMI was 26.14 ± 3.34 kg/m^2 and total cholesterol 4.75 ± 0.78 mmol/l. 4 subjects were smokers and 12 were ex smokers with more than 6 months since their last cigarette. Of the ex smokers 8 had more than a 10 pack year history. Table 3.3 details the baseline characteristics of the healthy volunteers.

7.2.2 Clinical Procedures

Following screening morphometric parameters were taken as detailed in section 2.3. Thereafter 60 minutes supine rest was taken before vascular phenotyping and then blood obtained for quantification of IL-6, Adiponectin, sICAM-1 and highly sensitive CRP. Small and large artery compliance values were obtained using a calibrated, proprietary tonometer (model CR 2000 Hypertension Diagnostics Inc) and used according to manufacturers specifications. Similarly Augmentation Index was measured using the The SphygmoCor system (PWV Medical). A full description of these techniques is detailed in section 2.3.1 and 2.3.2. IL-6, CRP, Adiponectin and sICAM1 were measured using the techniques detailed in 2.5.

7.2.3 Statistical Evaluation.

The package used for analysis was Minitab for Windows (Minitab Inc). P value of <0.05 was considered significant.

Using the Kolmogorov Smirnov test for normality with the Dallal and Wilkinson approximation to Lilliefors' method the data sets for sICAM-1 and adiponectin were normally distributed whereas those for IL-6 and CRP were not. For data that did not follow the Gaussian distribution, for statistical analysis, the data set was log transformed to create a normal distribution.

Simple regression was used to create a model with only one predictor utilizing the least squares estimation. Where the data was normally distributed the Pearson correlation was used (sICAM-1 and Adiponectin). Where the data did not fit a Gaussian distribution Spearman Rank correlation was used, i.e. for CRP and IL-6. Thereafter multiple regression was used to describe the statistical relationship between a response and 2 or more predictors. Again this used the method of least squares, which determines the equation for the straight line that minimizes the sum of the vertical distances between the data points and the line. Augmentation Index has been shown to vary with heart rate (Wilkinson 2002b) hence AIx was corrected for a mean heart rate of 75.

7.3 Results

In the fifty three volunteers without vascular disease the procedures were completed without complication and were well tolerated.

7.3.1 Arterial stiffness.

The mean Large artery compliance value (C1) in this group was 15.20 ± 4.14 ml/mmHg x 10. The mean small artery compliance (C2) was 6.74 ml/mmHg x 100. This is comparable to previous work undertaken using this technique (McVeigh2001). Similarly

the mean augmentation index measurement of 23.85 ± 11.26 % was akin to measurements obtained by other groups (Kampus 2004).

7.3.2 Interleukin 6.

IL-6 was measured with a commercial assay kit (Quantikine human IL-6, R and D System).The mean IL 6 level in this group was 3.49 ± 6.2 pg/ml. While in the normal range for this assay (1-10 pg/ml) our results are slightly higher than those from Chae *et al* from the United States who observed correlations across quartiles of systolic blood pressure of less than 3pg/ml although exact means are not detailed in their publication (Chae 2001).

Table 7.1 Linear Correlation of IL6 Using Spearman Rank Correlation

Parameter	Spearman r	95% CI	p value
Age(years)	0.343	0.072 to 0.567	0.011
Height(m)	-0.076	-0.347 to 0.206	0.588
Weight(kg)	-0.003	-0.280 to 0.276	0.986
BMI	0.035	-0.245 to 0.310	0.802
Smoking Status	0.011	-0.268 to 0.288	0.939
SBP(mmHg)	0.356	0.0860 to 0.576	0.009
DBP(mmHg)	0.258	-0.021 to 0.500	0.062
PP(mmHg)	0.366	0.0979 to 0.584	0.007
HR	0.177	-0.107 to 0.433	0.206
C1(ml/mmHgx10)	-0.325	-0.557 to -0.045	0.020
C2(ml/mmHgx100)	-0.527	-0.705 to -0.286	< 0.0001
AIx(%)	0.361	0.093 to 0.581	0.008
AIx(%)/HR75	0.429	0.172 to 0.632	0.001
CHOL(mmol/l)	-0.201	-0.453 to 0.082	0.150
Log TRIG(mmol/l)	-0.036	-0.311 to 0.245	0.799
LDLChol(mmol/l)	-0.201	-0.454 to 0.081	0.148
HDL(mmol/l)	-0.134	-0.397 to 0.149	0.338

The spearman rank associations are detailed in table 7.2. We found in our group a significant correlation with age (p=0.011) as has been found previously (Bermudez 2002). Spearman rank correlation with only IL 6 levels as the predictor also showed that IL 6 was correlated positively with AIx (p=0.008). When AIx was adjusted for heart rate the relationship persisted (p=0.001). Higher levels of IL6 within the normal range were therefore associated with increased arterial stiffness. Akin to this was the finding that higher levels of IL 6 were associated with lower levels of arterial compliance, i.e. stiffer arteries with a significant correlation for both C1 (p=0.02) and C2 (p=<0.001). Associations were also seen with both SBP (p=0.009) and PP (p=0.007) but not DBP.

7.3.3 Multiple regression analysis: IL 6 in healthy volunteers.

Multiple regression analysis with C1 as the dependent variable showed significant associations for height, BMI, smoking and systolic blood pressure but not IL-6 (table 7.3). The 'good of the fit' for this multiple regression analysis was a R^2 of 61.5%, and highly significant with p value <0.001. Incorporating pulse pressure into any of the analysis generated, as would be expected, redundant information. Table 7.4 shows that when C2 was taken as the dependent variable only log normalized IL 6 remained significant (p=0.05). Therefore even in healthy individuals where vascular disease is not apparent small artery compliance is associated with mediators of low grade chronic inflammation. With augmentation index corrected for heart rate of 75 age and systolic blood pressure were significant but not IL 6 (table 7.5)

Table 7.2 Results for the multiple regression analysis of IL 6 with Large Artery Compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	0.07	0.05	1.35	0.18
Gender	-0.70	1.15	-0.60	0.55
Height (m)	0.22	0.06	3.71	0.001
BMI (kg/m^2)	0.22	0.12	1.88	0.07
Smoking	-1.92	0.60	-3.18	0.003
SBP	-0.18	0.03	-5.73	0.000
Log IL6	-1.15	0.87	-1.31	0.20

R^2= 66.9%, adjusted R^2= 61.5%, p=0.000 for the entire study group (n=53).

Table 7.3 Results for the multiple regression analysis of IL 6 with Small Artery Compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	-0.03	0.04	-0.58	0.57
Gender	-0.79	1.05	-0.76	0.45
Height (m)	0.04	0.05	0.83	0.41
BMI (kg/m^2)	0.14	0.10	0.39	0.17
Smoking	-0.23	0.53	-0.43	0.67
SBP	-0.07	0.05	-1.47	0.15
DBP	-0.08	0.07	-1.04	0.31
Log IL6	-1.59	0.77	-2.05	0.05

R^2= 51.0%, adjusted R^2= 41.7%, p=0.000 for the entire study group (n=53).

Table 7.4 Results for the multiple regression analysis of IL 6 with Augmentation Index as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t* value	*p* value
Age (years)	0.34	0.17	2.07	0.04
Gender	3.48	3.92	0.89	0.38
Height (m)	-0.34	0.19	-1.76	0.09
BMI (kg/m^2)	-0.19	0.40	-0.48	0.64
Smoking	1.66	2.05	0.81	0.42
SBP	0.30	0.11	2.81	0.01
Log IL6	3.35	3.00	1.12	0.27

R^2= 49.5%, adjusted R^2= 41.7%, p=0.000 for the entire study group (n=53).

7.3.4 CRP in healthy volunteers

CRP was measured using a sensitive double-antibody sandwich ELISA with rabbit anti-human CRP and peroxidase-conjugated rabbit anti-human CRP. The assay was linear up to 5 mg/l and logarithmic thereafter. The inter- and intra-assay coefficients of variation were <10% across the range of measured results. Intra-assay coefficients of variation were <7% for all analytes. Mean CRP levels in this group were 2.11 ± 7.06 mg/L. The mean levels are lower than those in a recent similar study where mean level of 4.0 ± 5.6 were found (Yasmin 2004).

Linear correlations of hsCRP are displayed in table 7.6. As the data did not follow Gaussian distribution Spearman Rank correlations were used and thereafter for further analysis the data was log transformed. As with previous work we found that highly sensitive CRP correlated with BMI and hence weight. Moreover in accordance with Abramson *et al* we found that CRP correlated with pulse pressure (Abramson 2002). There was a trend, but not statistically significant, between small artery compliance and hs CRP (p=0.097). Additionally CRP correlated with HDL (p=0.005).

Table 7.5 Linear Correlation: hsCRP Using Spearman Rank Correlation

Parameter	Spearman r	95% CI	p value
Age(years)	0.199	-0.084 to 0.452	0.154
Height(m)	-0.018	-0.295 to 0.261	0.897
Weight(kg)	0.397	0.133 to 0.608	0.003
BMI	0.521	0.284 to 0.698	< 0.0001
Smoking Status	0.159	-0.124 to 0.419	0.254
SBP(mmHg)	0.242	-0.038 to 0.487	0.081
DBP(mmHg)	0.117	-0.166 to 0.383	0.403
PP(mmHg)	0.292	0.015 to 0.527	0.034
HR	0.018	-0.262 to 0.294	0.900
C1(ml/mmHgx10)	-0.165	-0.429 to 0.124	0.246
C2(ml/mmHgx100)	-0.235	-0.486 to 0.052	0.097
AIx(%)	0.181	-0.103 to 0.437	0.196
Aix(%)/HR75	0.184	-0.099 to 0.439	0.188
CHOL(mmol/l)	0.066	-0.216 to 0.337	0.641
Log TRIG(mmol/l)	0.202	-0.080 to 0.455	0.146
LDLChol(mmol/l)	0.159	-0.125 to 0.418	0.256
HDL(mmol/l)	-0.378	-0.594 to -0.112	0.005

7.3.5 Multiple Regression Analysis: CRP

In multiple regression analysis with C1 as the dependent variable (table 7.7, adjusted R^2= 48.2, p=0.000) correlated with gender (p=<0.01), BMI (p=0.05) and SBP (p=0.004). Though a tendency existed with log normalized CRP it did not reach clinical significance. Where the dependent variable was C2 (table 7.8, adjusted R^2=39.8%, p=0.000) a significant correlation existed with gender (p=0.03), BMI (p=0.02), and log CRP (p=0.02). Table 7.9 shows multiple regression analysis with AIx corrected for heart rate as the dependent variable (adjusted R^2= 44%, p=0.000). Age (p=0.007), Gender (0.000) and DBP (p=0.01) but not log CRP (0.43) were significant correlates. The lack of association between CRP and Aix mirrors that of Yasmin *et al* (Yasmin 2004) but is at odds with the work of Kampus and co workers (Kampus 2003). Given that the same technology was utilized in each study technical differences are unlikely to explain the different findings. That C2 and AIx are not analogous is further strengthened by the disparity in the associations described.

Table 7.6 Multiple Regression Analysis of log CRP in healthy volunteers with large artery compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	-0.03	0.05	-0.51	0.61
Gender	-3.95	0.974	-4.17	0.00
BMI (kg/m^2)	0.31	0.15	2.02	0.05
Smoking	-1.29	0.70	-1.86	0.07
SBP	-0.18	0.06	-3.02	0.004
DBP	0.04	0.10	0.45	0.65
Chol mmol/l	0.13	0.55	0.23	0.82
HDL mmol/l	0.62	1.66	0.37	0.71
Log CRP	-2.38	1.27	-1.87	0.07

R^2= 57.6%, adjusted R^2= 48.2%, p=0.000 for the entire study group (n=53).

Table 7.7 Multiple Regression Analysis of log CRP in healthy volunteers with small artery compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	-0.06	0.04	-1.44	0.16
Gender	-1.67	0.73	-2.29	0.03
BMI (kg/m^2)	0.28	0.12	2.34	0.02
Smoking	0.003	0.53	0.01	0.99
SBP	-0.07	0.05	-1.43	0.16
DBP	-0.08	0.08	-1.07	0.29
Cholesterol	0.12	0.42	0.27	0.79
HDL mmol/l	1.30	1.27	1.02	0.31
Log CRP	-1.93	0.97	-1.98	0.05

R^2= 50.7%, adjusted R^2= 39.8%, p=0.000 for the entire study group (n=53).

Table 7.8 Multiple Regression Analysis of log CRP in healthy volunteers with Augmentation Index as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	0.43	0.15	2.82	0.007
Gender	10.54	2.69	3.93	0.000
BMI (kg/m^2)	-0.28	0.44	-0.64	0.53
Smoking	0.50	1.99	0.25	0.80
SBP mmHg	-0.09	0.17	-0.54	0.60
DBP mmHg	0.72	0.28	2.56	0.01
Chol mmol/l	-1.49	1.59	-0.94	0.35
HDL mmol/l	-5.55	4.78	-1.16	0.25
Log CRP	2.95	3.67	0.80	0.43

R^2= 53.7%, adjusted R^2= 44.0%, p=0.000 for the entire study group (n=53).

7.3.6 ICAM in healthy volunteers

sICAM-1 was measured, as described in section 2.5 by ELISA. The mean level was 298.25 ± 84.82 which is comparable with levels found elsewhere in the literature (Chae 2001). Simple linear regression, as detailed in table 7.10 highlighted positive correlations with age (p=0.01), weight (p=0.04), BMI (p=0.02), smoking status (p=0.004), HDL (p=0.01). A positive correlation was seen for augmentation index when corrected for heart rate (p=0.006) and a negative relationship was seen between ICAM and small artery compliance (p=0.05). No relationship was seen with large artery compliance however (p= 0.112).

Table 7.9 ICAM and Vascular Parameters: Simple Linear Regression

Parameter	Pearson r	95% CI	r^2	p value
Age(years)	0.335	0.071 to 0.555	0.112	0.014
Height(m)	0.090	-0.185 to 0.352	0.008	0.521
Weight(kg)	0.290	0.021 to 0.520	0.084	0.035
BMI	0.328	0.064 to 0.550	0.108	0.016
Smoking Status	0.385	0.128 to 0.594	0.148	0.004
SBP(mmHg)	0.228	-0.045 to 0.469	0.052	0.101
DBP(mmHg)	0.182	-0.0926 to 0.431	0.033	0.191
PP(mmHg)	0.194	-0.0811 to 0.441	0.038	0.165
HR	0.192	-0.0830 to 0.439	0.037	0.169
C1(ml/mmHgx10)	-0.225	-0.472 to 0.054	0.051	0.112
C2(ml/mmHgx100)	-0.278	-0.515 to -0.003	0.077	0.048
AIx(%)	0.279	0.010 to 0.511	0.078	0.043
AIx(%)/HR75	0.376	0.117 to 0.586	0.141	0.006
CHOL(mmol/l)	-0.120	-0.378 to 0.155	0.015	0.391
Log TRIG(mmol/l)	-0.027	-0.295 to 0.245	0.001	0.849
LDLChol(mmol/l)	0.023	-0.249 to 0.292	0.001	0.870
HDL(mmol/l)	-0.352	-0.568 to -0.090	0.124	0.010

r^2 = Coefficient of determination

7.3.7 Multiple regression analysis: ICAM

Multiple regression analysis with large artery compliance as the dependent variable confirmed the lack of association between large artery compliance (p=0.07) and ICAM in our study population (table 7.11, adjusted R^2=31.6%, p=0.001). Only weight was significant in this analysis (p=0.00). The significant associations seen within simple linear analysis with small artery compliance and augmentation index were corroborated with multiple regression analysis. With small artery compliance as the dependent parameter only ICAM remained a significant correlate (p=0.05) (table 7.12 adjusted R^2 20.9%, p=0.01). No other significant correlates were observed. With augmentation index, multiple regression analysis (table 7.13 adjusted R^2 38.9%, p=0.000) correlates were seen no only with ICAM (p=0.01) but also with age (p=0.01) and weight (p=0.001). The finding that the intracellular adhesion molecule ICAM, a cellular mediator of inflammation with a role in atherogenesis is associated with arterial stiffness is a novel finding. It didn't matter whether either SPCA or DPCA was used. These results would certainly be in keeping with those of Ridker (Ridker 1998) who found that plasma concentrations of ICAM was associated with risks of future myocardial infarction in apparently healthy men.

Table 7.10 Multiple Regression Analysis of ICAM in healthy volunteers with Large Artery Compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	-0.02	0.06	-0.30	0.76
Weight (kg)	0.25	0.06	4.35	0.00
BMI (kg/m^2)	-0.37	0.23	-1.60	0.12
Smoking	-0.89	0.85	-1.05	0.30
ICAM (ng/ml)	-0.01	0.01	-1.87	0.07
HDL (mmol/l)	1.18	1.90	0.62	0.54

R^2= 39.8%, adjusted R^2= 31.6%, p=0.001 for the entire study group (n=53)

Table 7.11 Multiple Regression Analysis of ICAM in healthy volunteers with Small Artery Compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	-0.09	0.05	-1.87	0.07
Weight (kg)	0.08	0.04	1.87	0.07
BMI (kg/m^2)	0.06	0.18	0.31	0.76
Smoking	0.45	0.65	0.70	0.49
ICAM (ng/ml)	-0.01	0.01	-1.99	0.05
HDL (mmol/l)	1.47	1.45	1.01	0.32

R^2= 30.4% adjusted R^2= 20.9% p=0.01 for the entire study group (n=53)

Table 7.12 Multiple Regression Analysis of ICAM in healthy volunteers with Augmentation Index(%) as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*t*value	*p* value
Age (years)	0.42	0.15	2.79	0.01
Weight (kg)	-0.50	0.14	-3.57	0.001
BMI (kg/m^2)	0.74	0.58	1.29	0.20
Smoking	-1.09	2.19	-0.50	0.62
ICAM (ng/ml)	0.05	0.02	2.71	0.01
HDL (mmol/l)	-3.81	4.97	-0.77	0.45

R^2= 45.9% adjusted R^2= 38.9 % p=0.000 for the entire study group (n=53)

7.3.8 Adiponectin in healthy volunteers

Adiponectin measurements were quantified using commercially available ELISA (R&D Systems, Minneapolis, MN, USA). The mean levels were 6234 ± 4824.2 ng/ml – comparable with the work of others (Lawlor 2005). The data were normally distributed so simple linear regression was performed. As table 7.14 show adiponectin was positively correlated with sex (p=0.002), SBP (p=0.04), DBP (p=0.04), HDL (p=0.05) as well as augmentation index corrected for heart rate. Inverse correlations were observed with height (p=0.02), weight (p=0.0001), BMI (p=0.02) and large artery compliance (p=0.02).

Table 7.13 Adiponectin and Vascular Parameters: Simple Linear Regression

Parameter	Pearson r	95% CI	r^2	p value
Age(years)	0.138	-0.138 to 0.393	0.019	0.325
Height(m)	-0.329	-0.550 to -0.064	0.108	0.016
Weight(kg)	-0.440	-0.635 to -0.193	0.194	0.001
BMI	-0.314	-0.539 to -0.048	0.099	0.022
Smoking Status	-0.051	-0.317 to 0.223	0.003	0.717
SBP(mmHg)	0.279	0.009 to 0.511	0.078	0.043
DBP(mmHg)	0.283	0.014 to 0.514	0.080	0.040
PP(mmHg)	0.176	-0.099 to 0.426	0.031	0.208
HR	0.085	-0.190 to 0.347	0.007	0.547
C1(ml/mmHgx10)	-0.325	-0.551 to -0.054	0.106	0.020
C2(ml/mmHgx100)	-0.269	-0.507 to 0.007	0.073	0.056
AIx(%)	0.427	0.178 to 0.626	0.183	0.001
AIx(%)/HR75	0.434	0.186 to 0.631	0.189	0.001
CHOL(mmol/l)	-0.128	-0.385 to 0.147	0.017	0.360
Log TRIG(mmol/l)	-0.352	-0.569 to -0.091	0.124	0.010
LDLChol(mmol/l)	-0.131	-0.388 to 0.144	0.017	0.349
HDL(mmol/l)	0.275	0.005 to 0.508	0.076	0.046

r^2 = Coefficient of determination

7.3.9 Multiple Regression Analysis. Adiponectin in Healthy Volunteers.

Once more multiple regression analysis was preformed for large artery compliance (table 7.15, adjusted R^2 55.9%, p = 0.000), small artery compliance (table 7.16, adjusted R^2 37.3%, p = 0.001) and augmentation index corrected for heart rate (table 7.17, adjusted R^2 46.4%%, p = 0.000). No association was documented between either of the non invasive pulse contour measurements although correlations were seen with large artery compliance and height, SBP and log normalized triglyceride levels; small artery compliance and weight; and augmentation index corrected for heart rate and age and DBP. Although pulse contour analysis generated arterial stiffness measurements are not synonymous with endothelial function both are dependent up on structure and function of the arterial wall and hence potentially affected by atherogenesis. The lack of association seen in relation to DPCA and SPCA is therefore in keeping with the findings of Singhal and colleagues who found that whereas plasma adiponectin levels were associated with Insulin resistance they were not with endothelial function in young, healthy adolescents (Singhal 2005)

Table 7.14 Multiple Regression Analysis of Adiponectin in healthy volunteers with Large Artery Compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*T* value	*p* value
Age	0.02	0.05	0.43	0.67
Gender	-0.87	1.41	-0.62	0.54
Height	0.15	0.08	2.00	0.05
Weight	0.07	0.05	1.54	0.13
SBP	-0.22	0.06	-3.92	0.00
DBP	0.07	0.09	0.80	0.43
Log Trig	4.76	2.22	2.14	0.04
HDL mmol/l	2.03	1.56	1.30	0.20
Adiponectin(ng/ml)	0.0001374	0.0001086	1.27	0.21

R^2= 63.8% adjusted R^2= 55.9 % p=0.000 for the entire study group (n=53)

Table 7.15 Multiple Regression Analysis of Adiponectin in healthy volunteers with Small Artery Compliance as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*T* value	*p* value
Age	-0.05	0.05	-1.16	0.25
Gender	-1.18	1.20	-0.98	0.33
Height	-0.02	0.06	-0.31	0.76
Weight	0.08	0.04	2.04	0.05
SBP	-0.09	0.05	-1.92	0.06
DBP	-0.07	0.08	-0.86	0.40
Log Trig	-0.09	1.88	-0.05	0.96
HDL mmol/l	1.37	1.32	1.04	0.30
Adiponectin(ng/ml)	0.00008	0.00009	0.88	0.38

R^2= 48.6% adjusted R^2= 37.3 % p=0.001 for the entire study group (n=53)

Table 7.16 Multiple Regression Analysis of Adiponectin in healthy volunteers with Augmentation Index as the dependent variable.

Predictor	Regression Coefficient	Standard Error	*T* value	*p* value
Age	0.33	0.16	2.11	0.04
Gender	3.98	4.33	0.92	0.36
Height	-0.27	0.22	-1.24	0.22
Weight	-0.04	0.15	-0.29	0.77
SBP	-0.03	0.17	-0.18	0.86
DBP	0.58	0.28	2.06	0.05
Log Trig	-3.02	6.77	-0.45	0.66
HDL mmol/l	-7.20	4.77	-1.51	0.14
Adiponectin(ng/ml)	0.0003	0.0003	1.03	0.31

R^2= 55.7% adjusted R^2= 46.4 % p=0.000 for the entire study group (n=53)

7.4 Discussion

In brief we have described an association between parameters of low grade inflammation and vascular compliance in healthy volunteers. Similar analysis was performed in the cohort of patients with coronary artery disease but no statistical association was found in patients with the established phenotype. By multiple regression analysis there were significant associations between small artery compliance and IL-6, high sensitivity CRP and sICAM-1. There was also an association between augmentation index and ICAM. The importance of these results rests upon firstly what a change in arterial compliance may represent and secondly whether there is a link between this and biochemical markers of low grade inflammation. In short C2 and augmentation index are both sensitive to decreased NO bioactivity, a scenario common to atherosclerotic risk factors and low grade chronic inflammation.

Alterations in arterial compliance mirror the functional and structural changes that accompany the progression of atherosclerosis, a process that these molecules are either risk markers for or indeed themselves culprit vehicles. The relationships that are shown further lend utility to pulse contour analysis as a useful tool for non invasive vascular assessment both physiologically and in risk assessment.

The small artery compliance value does not represent a specific arterial bed or anatomical localization but reflects function (endothelial NO dependent) and structural changes (McVeigh 2001, Cohn 2004). While both are age dependent large artery compliance

reflects structural alterations in the conduit arteries that are accelerated by hypertension and atherosclerosis. This parameter has not been found to be sensitive to pharmacological manipulation of NO (McVeigh 2001). Augmentation index has also been found to be sensitive to inhibition of NO synthase with L-NMMA (Wilkinson 2002) but that C2 is not analogous to Aix is sufficient to explain why the correlations between C2 and CRP, IL6 and ICAM were significant but only an association existed only between ICAM and AIx. In studies comparing the two AIx and C2 were related in part but not completely related to small artery compliance ($r = -0.487$, Rietzschel 2001, $r= -0.36$, Segers 2001).

Inflammation constitutes an important role within the development of atherosclerosis (Ross 1996, Libby 2002). The series of ensuing events involves recruitment of leukocytes to the arterial wall promoting development of atherosclerotic lesions. The vascular endothelium contributes to the inflammatory response by, following activation by proinflammatory cytokines or 'classical cardiovascular risk factors, expressing leukocyte adhesion molecules which themselves promote adhesion of monocytes and T lymphocytes to the endothelial surface (Libby 2002, Widlansky 2003). The phenotypic changes in the endothelium induced by cardiovascular disease risk factors, cytokines and inflammatory markers including CRP, IL-6 and sICAM-1 in turn promotes a decrease in the production and/or biological activity of NO (Widlansky 2003,Verma 2002). Decreased NO activity promotes leukocyte adhesion, thrombosis, vasoconstriction and cellular proliferation; the principal components of atherogenesis (Vita 2002). CRP decreases expression of eNOS and NO synthesis in part by reducing the half life of eNOS (Verma 2002) and has been demonstrated to decrease eNOS expression and bioactivity in

human aortic endothelial cells (Venugopal 2002). These observed effects may also account form the relationship between IL-6 and small artery compliance as IL 6 is a potent stimulus for hepatic CRP production (Pearson 2003). Other vascular phenotypes which associate with NO bioactivity have been shown to correlate with low grade inflammation. Vita *et al* (Vita 2004) examined the cross sectional relationship between vasodilator function in the forearm (both in terms of brachial artery flow-mediated dilatation and reactive hyperaemia) and CRP, IL-6 and sICAM-1 in the Framingham Offspring Study. They found that there was an incremental contribution of CRP, IL-6 and sICAM-1 to reactive hyperaemia above and beyond traditional risk factors (Vita 2004). Other groups have suggested that there is a correlation between CRP and brachial artery flow mediated dilatation (Tan 2002, Brevetti 2003) and CRP and coronary circulation endothelial dysfunction via the cold pressor test (Tomai 2001). Chapter 4 described how, in a group of patients with CAD, small artery compliance was lower in patients homozygous for the 894T allele of the *NOS3* gene. The T allele has also been found to not only increase the risk for premature MI by Antoniades and coworkers (Antoniades 2005) but also to be associated with a modified response of the vascular endothelium during the acute phase of the MI by affecting the release of IL-6. IL- 6 triggers an acute phase reaction in the liver, regulating the release of acute phase reactants such as CRP, and up regulating the expression of adhesion molecules in endothelial cells and depressing NO production by inhibiting eNOS (Cardaropoli 2003). A genome wide linkage scan for arterial stiffness was undertaken within 204 families from the Framingham Offspring study with tonometry data by Mitchell and colleagues (Mitchell 2005). They found 4 regions of suggestive linkage for carotid femoral PWV including a

peak on chromosome 7 at 29 cM which includes IL-6. Not only does this implicate arterial stiffness as heritable but also that IL-6 may account for a proportion of the heritability.

The lack of association between adiponectin and any of the pulse contour analysis variables given recent investigations are not surprising. Though the association between adiponectin and insulin resistance, diabetes mellitus and hypertension (Stefan 2002, Hotta 2000, Iwashima 2004) has been clearly established the association with vascular phenotypes remains more obtuse. Adiponectin did not predict endothelial function in 294 adolescents (Singhal 2005) and most recently high molecular weight Adiponectin was not associated with incident coronary heart disease within the British Women's Heart and Health Study (Sattar 2008).

Chapter 8 General Discussion, Conclusions and Future Work.

The cardiovascular disease epidemic continues to advance and increase both geographically to developing countries and numerically such that the projected increase in the proportion of all deaths attributable to cardiovascular causes increasing from approximately 25% in 1990 to more than 40% by 2020 (Reddy 2004). The 'post genome era' permits the ability to study gene function and gene gene relationships which has been critical to the investigation of complex cardiovascular traits. The ultimate clinical dividend of this approach will include mechanistic classification of the common cardiac phenotypes, diagnostic markers and improved clinical therapy in terms of prevention and intervention on the basis of a putative risk assessment based upon an individual's cardiovascular risk haplotype (Dominiczak 2005). While others have utilised case-control association studies, i.e. the occurrence of a particular allele of a polymorphism in a group of subjects more frequently than expected by chance, as a process of the evaluation of two candidate genes in cardiovascular disease this project focused upon a non invasive cardiovascular phenotype, pulse wave analysis.

The pathogenesis of vascular disease including hypertension, atherosclerosis, type 2 diabetes mellitus, heart failure and hypercholesterolemia is, at least in part driven by oxidative stress (Landmesser 2001, Hamilton 2004). The relation of oxidative stress to atherosclerotic vascular disease is one where reactive oxygen species modulate the accumulation of sub endothelial LDL particles (Sorescu 2002). The principle reactive oxygen species are $\bullet O2^-$ and NO of which the associated genes *CYBA* and *NOS3* have

been implicated in vascular disease where NO bioactivity is impaired (Hingorani 2000) which is associated with a decrease in the compliance of large and small blood vessels.

The objective of this thesis was to investigate the effect of single nucleotide polymorphisms of oxidative stress genes and low grade inflammation upon pulse wave contour analysis which has emerged as a useful non invasive, intermediate vascular phenotype. In this way the continuum from gene to protein to function has been paralleled by the examination of genotype, oxidative stress markers and arterial stiffness.

8.1 The reproducibility of diastolic pulse wave contour analysis and its relation to systolic pulse contour analysis.

Using Bland Altman plots the calculated intra-observer bias for C1 was -0.1(SD of bias was 0.36, 95% CI -0.8 to 0.6) and for C2 the observed bias was -0.04 (SD of bias was 0.20, 95% CI -0.44 to 0.36). Moreover over 95% of the variability fell within 2 standard deviations. Furthermore there was a significant correlation between both AIx and C1and AIx and C2 in healthy volunteers and though there was no association between AIx and C1 in patients with coronary artery disease AIx did correlate with C2 in this population.

Non invasive measures of assess the arterial pulse, while not a new arterial phenotype (Sharpey 1866), is becoming increasingly popular as measures of central aortic pulse pressure is recognized as providing additional information beyond that given by conventionally measured brachial pulse pressure (Williams 2006). PWV remains pre-

eminent as the current gold standard (Laurent 2006) especially as it has been shown to correlate with coronary arterial plaque load (McLeod 2004) and has been shown to be an independent predictor of clinical outcome, albeit in high risk patient groups (Blacher 1999, Blacher 1999, Blacher 2003, Guerin 2001). Systolic and diastolic pulse pressure pulse contour analysis also has its advocates. The former also has outcome data (Kingwell 2002, London 2001) and the recent Anglo Cardiff Collaborative Trial in 4001 healthy, normotensive individuals aged 18-90 has not only provided 'normal reference values' but also suggested that while PWV may be a better assessment of arterial stiffness in older individuals AIx might be a more sensitive marker of arterial stiffness and risk in younger individuals who potentially have more to gain from a biological marker driven treatment regimen (McEniery 2005). Moreover endothelial function, characterized by impaired NO bioactivity and oxidative stress has, within healthy individuals, been shown to be inversely associated with aortic PWV and AIx (McEniery 2006). The correlations observed in this thesis between C2 and AIx suggests that this may also be the case with the Windkessel derived arterial compliance measurement. Consistent characteristic changes in the pulse pressure wave shape have been associated with ageing and disease states predisposing to vascular events and application of the four element 3rd order modified Windkessel model has been shown to generate useful information pertaining to hypertension and diabetes (C1) (McVeigh 1991, McVeigh 1993) and increasing age, coronary artery disease in post menopausal women and diabetes (C2) (McVeigh 1999, Cohn 1995, McVeigh 1993). Additionally the consistent and predictable changes found with pharmacologically impaired NO bioactivity suggest that C2 may be sensitive to conditions underpinned by oxidative stress (McVeigh 2001).

8.2 The effect of the G894T SNP of the NOS3 gene upon arterial stiffness in patients with coronary artery disease.

There was no association observed between this polymorphism and blood pressure or large artery compliance however ANOVA revealed a statistically significant association for TT homozygosity and small artery compliance (p=0.01). The highest small artery compliance was seen in the patients homozygous for the G allele (5.51 ± 0.51 ml/mmHg x 100), an intermediate value observed in heterozygotes (4.21 ± 0.33 ml/mmHg x 100) and the lowest value demonstrated in patients homozygous for the T allele (3.18 ± 0.38ml/mmHg x 100). Multiple regression analysis, examining the possible contribution of age, BMI, SBP, DBP, total and LDL cholesterol showed that only small artery compliance was significant when *NOS3* G894T genotype was assigned as the dependent variable (p=0.01).

Arterial stiffness measures and mean and pulsatile components of blood pressure are heritable and appear to have genetic determinants that are postulated to be linked to separate genetic loci in humans (Mitchell 2005). Durier *et al* investigating the physiological genomics of human arteries in an elegant study by examining gene expression of human aortic specimens and correlating the results with PWV found patterns of expression correlating with arterial stiffness including genes which may mediate, in part NO dependent vasodilatation (Durier 2003). The observations therefore that a common allelic variation is associated with an arterial stiffness parameter

associated with impaired NO bioactivity is consistent with these previous studies. This gene variant has been shown to be associated with survival in patients with heart failure (McNamara 2003) and the ultimate determinant of potential clinical utility of our finding would be a survival or treatment effect that would modulate clinical decision making in terms of risk stratification and treatment.

8.3 The C242T single nucleotide polymorphism of the CYBA gene and blood pressure and arterial compliance in patients with coronary artery disease.

We sought to examine the influence of the C242T SNP of *CYBA* upon vascular compliance and blood pressure using the dominant allele model. The presence of the 242T allele was associated with significantly higher systolic blood pressure. Patients homozygous for the C allele had lower systolic blood pressure (125.7 ± 2.3 mmHg) than heterozygotes and patients homozygous for the T allele(134.6 ± 2.4 mmHg) (p=0.010). There was no statistically significant effect upon diastolic blood pressure but there was however a significant association observed between the 242T allele and pulse pressure. Patients that were homozygous for the CC allele had a PP of 56 ± 1.6 mmHg where as patients heterozygous or homozygous for the T allele had a PP of 62.7 ± 1.8 mmHg (p =0.01).

In terms of arterial stiffness the 242T allele was associated with lower large artery compliance. CC homozygotes had higher large artery compliance (17.07 ± 0.82

ml/mmHg x10), than heterozygotes and TT homozygotes (13.16 ± 0.53 ml/mmHg x10) (p = < 0.001). No difference was observed between the C242T SNP and small artery compliance nor Augmentation Index in our study population. Multiple regression analysis, examining the possible contribution of age, BMI, SBP, DBP and total cholesterol showed that only large artery compliance was significant when *CYBA* C242T genotype was assigned as the dependent variable (p=0.001).

The p22phox subunit is a common component of all types of NADPH oxidases and is the reputed redox centre of the enzyme system. The allelic variant C242T of the enzyme system has been demonstrated to affect enzyme activity (Guzik 2000, Wyche 2004) but as has been detailed there have been case control candidate gene association studies which have provided incongruous results as to which allelic variant is deleterious or advantageous (Cai 1999, Cahilly 2000, Nasti 2006, Inoue 1998, Saha 1999, Fan 2006). The strength of this thesis is thus in seeking to establish whether a gene function relationship is present. The finding that large artery stiffness, associated with hypertension (McVeigh 1991), is associated with the 242T allele is in keeping with the recent findings of Moreno *et al* who found a relationship between the C242T polymorphism in a case control study (Moreno 2006). They found however, that the CC genotype was associated with higher blood pressure levels and also NADPH oxidase activity and p22phox expression quantified from phagocytic cells by chemiluminescence and by northern and western blots (Moreno 2006). A larger study combining protein expression with arterial phenotype is therefore required before the relationship of this polymorphism and vascular disease is clearly delineated.

8.4 Combined analysis of NOS3 G894T and CYBA C242T genotypes upon arterial stiffness.

In order to contrast the arterial stiffness between the favourable versus the non-favourable genotypes patients homozygous for the *NOS3* G allele and homozygous for the *CYBA* C allele were compared with those homozygous for the *NOS3*T allele and possessing the *CYBA* 242T allele. The former displayed higher large and small artery compliance than the latter group. Patients with the CC/GG genotype had a mean C1 of 19.68 ± 1.3 ml/mmHg x10, with patients with the CT+TT/TT genotype a mean C1 13.90 ± 1.6 ml/mmHg x10 (p=0.01). Likewise patients with the CC/GG genotype had a mean C2 of 5.48 ± 0.63 ml/mmHg x100, with patients with the CT+TT/TT genotype a mean C2 of 3.24 ± 0.52 ml/mmHg x100 (p=0.01). There was no difference evident in age, BMI, BP or cholesterol parameters between the two groups. Multiple regression analysis, examining the possible contribution of age, BMI, SBP, DBP, total, ldl cholesterol and triglyceride concentration showed that only the large(p=0.02) and small(p=0.05) artery compliance values contributed significantly when genotype was assigned as the dependent variable.

The interplay of these two enzyme systems may be hypothesized to modulate the oxidative milieu within vascular cells and the observed gene gene interaction adds weight to this theory. Most previous studies examining combinations of polymorphisms have concentrated on one gene such as the meta analysis by Zintzaras and co workers

(Zintzaras 2006). The combination of two genes that are associated with diminished NO bioactivity may therefore provide useful information. NO is a potent anti atherogenic molecule and endothelial dysfunction has been established as determining all cause mortality in coronary heart disease (Schächinger 2000). The interplay of two genes which, implicated in the genesis of oxidative stress and associated therefore with impaired NO bioactivity and consequently potentially endothelial dysfunction, and an observed gene gene interaction is entirely in keeping with prior studies examining reactive oxygen species vascular biology.

8.5 Chronic low grade inflammation and insulin resistance and arterial compliance in healthy volunteers.

Within healthy volunteers multiple regression analysis showed that small artery compliance was significantly associated with IL 6 ($p=0.05$), CRP ($p=0.02$) and ICAM ($p=0.01$). Augmentation index showed only an association with ICAM ($p=0.01$). There was no significant correlation between Adiponectin levels and either of the arterial stiffness parameters studied.

Inflammation plays a critical role in all stages of atherosclerosis from the nascent lesion to the acute coronary syndrome (Libby 2002). There has been considerable interest and speculation about the interrelation between vascular inflammation and atherosclerosis (Ross 1999). The link with endothelial function and consequently arterial stiffness has been explored and CRP, IL-6 and sICAM-1 have previously been shown to associate

with endothelial function (Vita 2004). The observation that Windkessel derived pulse contour analysis is also related to these markers of low grade inflammation is hence consistent with these observations. Additionally CRP has also been reported to be associated with coronary microvascular endothelial function (Teragawa 2004). This thesis describes that in patients with CAD, small artery compliance is lower in patients homozygous for the 894T allele of the *NOS3* gene. The T allele has also been shown to modulate the response of the vascular endothelium during the acute phase of the MI by affecting the release of IL-6 (Antoniades 2005). IL- 6 triggers an acute phase reaction in the liver, regulating the release of acute phase reactants such as CRP, and up regulating the expression of adhesion molecules in endothelial cells and depressing NO production by inhibiting eNOS (Cardaropoli 2003). Furthermore Mitchell and colleagues utilized tonometry within the Framingham Offspring study and discovered 4 regions of suggestive linkage for carotid femoral PWV including a peak on chromosome 7 which includes IL-6 (Mitchell 2005) This suggests that arterial stiffness may not only be heritable but also that IL-6 may account for a proportion of the heritability and also links these two non invasive phenotypes. CRP itself has been demonstrated to decrease eNOS expression, enzymatic activity and bioactivity within human aortic endothelial cells further supporting its role in atherogenesis. The observations in this thesis associating arterial stiffness with low grade chronic inflammation are hence in keeping with current knowledge and extends the robustness of diastolic pulse contour analysis as a vascular phenotype. The lack of association with adiponectin is congruent with that of Iwashima and co workers (Iwashima 2004).

8.6 Conclusions

a) Diastolic pulse wave contour analysis is a reproducible assessment of arterial stiffness with the potential to represent a high fidelity non invasive vascular phenotype.

b) Small artery compliance is correlated with Augmentation Index and although the measurements are not analogous they both represent useful means of acquiring quantitative data concerning arterial stiffness.

c) The 242T allele of the p22phox gene, *CYBA*, is associated with decreased large but not small artery compliance and increased systolic and pulse pressure.

d) Homozygosity for a common *NOS3* polymorphism (894 G→T) was associated with decreased small artery compliance but not with large artery compliance or blood pressure.

e) The markers of chronic inflammation Interleukin 6, ICAM and hsCRP but not Adiponectin, a marker of Insulin resistance, predict small artery compliance in healthy individuals apparently free of vascular disease.

8.7 Future work

There are several important considerations which relate to future investigations regarding the relation of genotype and phenotype in cardiovascular disease. Firstly the sample size

required must be sufficient to detect small genetic effects and gene-gene and gene-environment interactions. Secondly standardized high-fidelity phenotyping methods are required and in terms of the applicability of pulse wave analysis the current gold standard remains carotid femoral pulse wave velocity (Laurent 2006). Thirdly the use of proteomic markers to link the gene function relationship would add weight to conclusions.

In terms of diastolic pulse waveform analysis the next step in assessing the clinical utility of this technology is to ascertain whether it represents an independent predictor of clinical outcome much in the way that has been established with pulse wave velocity (Blacher 1999, Blacher 1999, Blacher 2003, Guerin 2001) and Augmentation Index (Kingwell 2002, London 2001). Only then would small and large artery compliance values form comparable clinical parameters alongside other measurements of arterial stiffness or markers of low grade chronic inflammation such as CRP which although popular have also not always generated encouraging results (Danesh 2004).

This thesis describes what is in essence a small gene function candidate gene study and the clinical relevance of the C242T *CYBA* and G894T *NOS3* SNPs will only be fully clarified when an appropriately powered study allowing inclusion of other potentially involved loci to form a full haplotype analysis. As stated there have been discrepant case control association studies which such a study would provide a definitive answer to. The completion of the Human Genome Project in 2003 has conferred upon researchers the ability to find the genetic contributions to human disease phenotypes for genetic variations that contribute to their onset. Such a genome wide association study would

hence allow definitive associations with vascular phenotypes and genetic variation including pulse wave analysis and biochemical markers associated with vascular disease.

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