Endothelial Dysfunction:  
Mechanisms and Reversal in  
Insulin Resistance and Diabetes Mellitus

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Dedication

To Donna

for being there
ABSTRACT

Injury to the vascular wall resulting in endothelial dysfunction is recognised as a key early event that initiates and promotes progression of atherosclerosis. Studies have shown that endothelial dysfunction, characterised by impaired endothelium-dependent flow mediated dilatation (FMD), is present from early in life in association with cardiovascular risk factors. This thesis investigates the mechanisms of impaired FMD and describes its relation to risk factors and inflammatory markers in healthy subjects with insulin resistance and type 1 diabetes mellitus.

In healthy volunteers, the role of nitric oxide in conduit artery dilatation, in response to flow stimuli with differing dynamic characteristics, was examined. It has been demonstrated that NO is an important mediator of transient flow stimuli, whilst dilatation to sustained flow occurs by a NO independent mechanism. Furthermore, in subjects with hypercholesterolaemia impaired flow-mediated dilatation was confined to the NO pathway.

In a large population based cohort, the determinants of vascular dysfunction have been studied with particular reference to the roles of insulin resistance, glucose intolerance and the metabolic syndrome. These studies demonstrate an abnormality of vascular function in association with the metabolic syndrome that is not mediated by insulin resistance per se. In contrast, associations between classical risk factors, inflammation and impaired endothelial function and arterial elasticity are demonstrated.

In experimental models reversal of endothelial dysfunction retards the progression of atherosclerosis. Whilst such an approach remains unproven in humans, this thesis describes studies designed to test the effects of specific interventions, on endothelial function in young patients with insulin-dependent diabetes. No benefit has been demonstrated following ACE inhibition or administration of L-arginine, the precursor of NO but significant improvement in FMD was seen after cholesterol reduction.
Conclusion

The studies presented in this thesis enhance the understanding of conduit artery physiology and the mechanisms of endothelial dysfunction in patients with risk factors for atherosclerosis. Understanding these mechanisms will be important in the development of strategies targeted at retarding atherosclerosis early in its natural history. Using non-invasive techniques, the benefit of such interventions, on arterial physiology, can be demonstrated from an early age.
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PUBLICATIONS ARISING FROM THIS WORK


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Other publications not arising directly from this work


CHAPTER 1

INTRODUCTION
1.1 OVERVIEW

The endothelium, which lines all blood vessels, is now recognised to have a diverse range of physiological functions pivotal in regulating vascular homeostasis. Injury to the endothelium, and its transformation into a dysfunctional phenotype, may be an important event which underlies many of the known vascular disorders. Of these atherosclerosis has the largest impact, and is responsible for a major proportion of all morbidity and mortality in Western societies. This thesis focuses on the potential role for endothelial dysfunction in atherosclerosis, particularly in young individuals with predisposing risk factors. In this chapter, the epidemiology of atherosclerosis, its impact on cardiovascular morbidity and mortality and its relations to proven and proposed risk factors is discussed. The pathophysiology of atherosclerosis and its progression to anatomical disease is briefly reviewed. The diverse functional activities of healthy endothelium and the many potential methods for studying early atherosclerosis and endothelial function are then considered.

1.2 EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE

In the United Kingdom coronary vascular disease alone accounts for 24 per cent of total mortality and with carotid vascular disease atherosclerosis is responsible for over 500 000 deaths per year (National Audit Office, 1996). World-wide the World Health Organisation estimate that 12 million people die each year from cardiovascular diseases (World Health Organisation, 1993; World Health Organisation, 1995). The cost of treating coronary vascular disease in the United Kingdom has been estimated at over £500 million per year (National Audit Office, 1996). Furthermore, cardiovascular diseases result in substantial disability, absence from work and loss of productivity and represent a major economic burden.

There are marked differences in the incidence of clinical complications and mortality from cardiovascular diseases between the sexes and within different age groups. Below the age of 65 years cardiovascular death is three times as common in men as in women, whilst beyond 75 years mortality in women may be even greater than in
men (Sans et al., 1997). This is important as over 50 per cent of deaths occur in patients over 75 years of age. Despite this sharp increase in mortality in the elderly, coronary vascular disease is still the commonest cause of death before the age of 65 years in men and is second only to breast cancer in women (National Audit Office, 1996).

Marked geographical differences in cardiovascular mortality, at a national and international level, are also apparent (Sans et al., 1997) (Figure 1.2.1). The highest death rates are seen in eastern European countries with 1000-1500 deaths per 100,000 men aged 45-75 years. At the opposite end of the scale in France the equivalent death rate is only 330 deaths per 100,000. In the United Kingdom the overall death rates from cardiovascular diseases in men and women aged 45-75 years are 800 and 400 per 100,000 respectively. However within the United Kingdom, regional differences are also apparent with mortality in Scotland and Northern Ireland being 20 to 30 per cent higher than in England (Rayner, 1994; Sans et al., 1997).

1.2.1 Trends

In post war Europe and North America cardiovascular disease became the leading cause of death and disability. In 1950, one out of every three men developed cardiovascular disease before reaching 60 (Thom and Kannel, 1981). However, over the past 20 to 30 years, a marked decline in coronary heart disease mortality of between 20 to 40 per cent has been noted in developed countries and particularly in younger age groups (Beaglehole, 1990; McGovern et al., 1996). Cardiovascular morbidity as determined by the prevalence of angina pectoris and incidence of myocardial infarction however, has not declined to the same extent (McGovern et al., 1996) and the impressive reduction in mortality is likely to largely reflect the major advances that have been made in the treatment of life threatening events with specialised prehospital care, the use of aspirin, thrombolysis, angiotensin converting enzyme inhibitors and revascularisation. Of great concern, however, is the increasing incidence of cardiovascular disease in developing countries (World Health Organisation, 1993; World Health Organisation, 1995). This is particularly apparent in eastern Europe, where in many countries cardiovascular mortality has risen by as
much as 80% and rates are now amongst the highest in the world (Sans et al., 1997) (Figure 1.2.2). In Africa, Western Asia and Southeast Asia, a similar increase in cardiovascular mortality has also been reported (Reddy and Yusuf, 1998). With a projected increase in global population, in parallel with an increase in risk factors and decline in infectious diseases the total economic burden of cardiovascular disease is likely to increase (Murray and Lopez, 1997).

1.3 RISK FACTORS FOR ATHEROSCLEROSIS

Until the middle of this century atherosclerosis was often regarded as a degenerative disease and an inevitable consequence of ageing. However, the wide variations that were evident in the prevalence of cardiovascular diseases between individuals and the apparent ability for mortality to both increase and decrease within populations led several observers to suggest that environmental (and therefore potentially reversible) factors might be important in the pathogenesis of atherosclerosis (Snapper, 1941; Strom and Jensen, 1951). A number of early cross-sectional studies reported that patients with manifest cardiovascular disease were predominantly male and had higher levels of cholesterol and blood pressure suggesting a possible causal relationship (Gertler et al., 1950; Master et al., 1939; Steiner and Domanski, 1943). In 1953, Ancel Keys proposed that “clinical coronary disease usually represents the cumulative effect of a factor, or factors operating over a period of years”. The term ‘risk factor’ was first coined by Doyle in 1963 and defined as a measurable trait or characteristic that predicts the probability of an individual developing clinically manifest disease. The Framingham study was initiated in 1948 to prospectively examine the relationship between proposed risk factors and the later incidence of coronary disease. Initial findings, reported after 4 and 6 years follow-up, demonstrated significant associations between the incidence of new coronary disease and maleness, cholesterol levels, hypertension and smoking (Dawber et al., 1957; Dawber et al., 1959). Since then a large number of further epidemiological studies in Europe and North America have confirmed and refined the Framingham findings and facilitated the development of primary prevention of atherosclerosis as a viable concept.
Figure 1.2.1 Age-standardized mortality from cardiovascular disease in European regions in 1990-91. All ages in men. Wide differences in mortality are noted in different regions with a bimodal distribution (see key). Adapted from Sans et al., 1997.

Figure 1.2.2 Time trends in mortality from cardiovascular diseases in selected European regions 1970-92. Men ages 45-74 years. Whilst in many countries mortality is steadily declining, in eastern Europe an increase in cardiovascular disease is apparent. Adapted from Sans et al., 1997.
1.3.1 Risk factor: causal agent or epiphenomena

Recently, a wide range of additional parameters which are independently associated with the incidence of atherosclerotic disease have been proposed as risk factors (e.g., homocysteine levels, birthweight, fibrinogen levels). However, it should be noted that the statistical association seen in these studies does not prove causality, which is necessary if effective preventative strategies are to be devised. Furthermore, it is also necessary to differentiate between factors which might predispose to an acute event (e.g., increased thrombogenicity) and factors which are intimately involved in the pathogenesis of atherosclerosis itself. Hill (1964) suggested a number of criteria by which a causal relationship between risk factor and disease might be inferred:

1. Strength of the association
2. Dose dependent association
3. Temporal precedence
4. Consistency within and between different populations
5. Independence of other known or suspected risk factors
6. Plausibility: a plausible scientific rationale for the association can be made
7. Specificity: the risk factor predicts the risk of specific diseases, e.g., most disease occurs in the elderly and therefore age is a very non-specific predictor for atherosclerosis.
8. Reversibility: the demonstration that the disease is preventable by modification of specific risk factors is the most convincing evidence of a causal relationship.

1.3.2 Hyperlipidaemia

Cholesterol was first postulated to be related to atherosclerosis when it was found to be a major component of advanced atherosclerotic plaques. A large body of evidence now exists linking serum cholesterol levels to risk of cardiovascular disease and particularly coronary artery disease. Data from large epidemiological studies have consistently indicated a dose dependent ‘J’ shaped relationship between cholesterol levels and cardiovascular risk (Dawber et al., 1959; Keys et al., 1984; Menotti et al., 1994). This relationship is progressive, operates even at low levels of cholesterol and
is apparent in populations with both high and low levels of prevalent vascular disease (Keys et al., 1984; Menotti et al., 1994). Differences in mortality rates between different populations may be largely explained by variation in serum cholesterol levels (Simons, 1986). Increasing levels of cholesterol, as seen in first and second generation Japanese men who have migrated to western countries, are associated with a parallel increase in cardiovascular risk (Kagan et al., 1974).

Anitschkow and Chalatow, first demonstrated in 1913 that cholesterol fed rabbits developed a pathological disease indistinguishable from human atherosclerosis, a finding, which has subsequently been confirmed in other species including rodents and primates. In foetal aortic tissue, excised from spontaneously aborted foetuses of mothers with hypercholesterolemia, widespread early atherosclerotic lesions are apparent during the first and second trimester when foetal cholesterol levels are closely linked to the maternal serum cholesterol level (Colwell and Lopes-Vivella, 1988). On histological examination of these lesions, cholesterol accumulation was noted to occur prior to other pathological events. That these lesions regress with the adoption of a neonatal pattern of low cholesterol indicates the dynamic and mutable nature of early atherosclerosis.

The importance of cholesterol as a risk factor for cardiovascular disease from an early age is also supported by the findings of autopsy and longitudinal studies. Cholesterol levels have been linked to the presence and extent of atherosclerotic lesions in teenagers and young adults killed in road traffic accidents (Reed et al., 1989; Wissler, 1995). These early lesions demonstrate a temporal and spatial relationship with atherosclerotic plaques known to cause clinical vascular events in later life (Stary, 1989). In a large longitudinal study, cholesterol levels measured in young adults were found to predict the incidence of clinical cardiovascular events in middle age (Klag et al., 1993).

A causal link between cholesterol and cardiovascular disease has been firmly established by the results of interventional trials of cholesterol reduction. Early studies using fibrates and weak hypolipidaemic agents, whilst demonstrating significant benefit on cardiovascular mortality were not associated with a decrease in
overall deaths (Frick et al., 1987). In the POSCH study a surgical approach was used to lower cholesterol levels and significant benefit in this relatively small group was seen after 6 years follow up (Buchwald et al., 1990). Recently with the development of 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitors, marked and rapid reductions in serum cholesterol levels have been safely achieved. In the Scandinavian Simvastatin Survival Study (Scandinavian Simvastatin Survival Group, 1994) and the West of Scotland Prevention Study (Shepherd et al., 1995), reductions in cardiovascular mortality of 20-30% have been demonstrated in patients with hypercholesterolemia, with and without established coronary artery disease, after as little as two years treatment. The reduction in clinical events seen in these trials is quantitatively close to that that would be predicted from the relationships derived in epidemiological studies. Further studies have demonstrated that the benefit from cholesterol reduction extends to cerebral and peripheral vascular morbidity and mortality and may also be derived at normal levels of cholesterol (The LIPID Study Group, 1998; Downs et al., 1998; MacMahon et al., 1998; Sacks et al., 1996). In angiographic trials, lowering cholesterol levels is associated with reduced progression of atherosclerotic plaque (Brown et al., 1990; Jukema et al., 1995).

1.3.2.1 Lipoproteins and cardiovascular risk

Cholesterol is insoluble in the aqueous phase and circulates bound to apolipoproteins, which can be differentiated on the basis of their density and separated into 4 major subgroups: high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein and intermediate-density lipoprotein (IDL). Whilst, initial studies reported that total cholesterol levels predict risk of coronary artery disease, later trials examined the relationship between the cholesterol content of the various lipoprotein moieties, their relative balance and cardiovascular risk. These studies have consistently demonstrated a positive correlation between LDL cholesterol concentration, which accounts for approximately two-thirds of the total serum cholesterol, and the later incidence of clinical atherosclerotic disease (Dawber et al., 1959; Kannel et al., 1971; Simons, 1986). In contrast HDL cholesterol levels were independently associated with decreased risk (Gordon et al., 1977; Miller and Miller, 1975). A pathogenic role for LDL cholesterol in atherosclerosis is supported by the very high prevalence of atherosclerotic disease
seen in patients with familial hypercholesterolaemia, a genetic trait characterised by
gross elevations of LDL-cholesterol secondary to complete or partial absence of the
LDL receptor, which is responsible for its clearance in the liver (Goldstein and
Brown, 1975).

High-density lipoprotein is thought to be important in reverse cholesterol transport,
the process whereby cholesterol is removed from the tissues and returned to the liver.
Elevated HDL-cholesterol levels are associated with a decreased incidence of
coronary events and the ratio of HDL to total or LDL cholesterol has been proposed
as the best predictor of cardiovascular risk (Kannel et al., 1971). In the Helsinki
Heart Study, treatment with gemfibrozil resulted in significant elevation in HDL
cholesterol levels and a 34% reduction in the incidence of new coronary heart disease
(Frick et al., 1987). However, fibrates also reduce non-LDL cholesterol levels,
triglycerides and shift the pattern of LDL subtypes towards larger less dense moieties
and a specific antiatherogenic role for HDL remains unproven.

1.3.2.2 The atherogenic lipoprotein profile

Whilst familial hypercholesterolemia and very high levels of LDL cholesterol are
strong predictors of cardiovascular risk, these are rare and the majority of clinical
events occur in patients with only moderate elevations in LDL cholesterol. Many of
these patients however will have predominantly small dense LDL particles, which
are rich in cholesterol and more susceptible to oxidation, low HDL cholesterol levels,
moderately raised triglycerides and increased postprandial lipaemia. This complex
metabolic stew has been termed the ‘atherogenic lipoprotein profile’ (ALP) (Gofman
et al., 1966; Krauss, 1994) and is thought to represent the interaction between a
number of heritable and environmental factors. In two recent longitudinal studies, the
presence of an ALP was associated with a threefold increase in cardiovascular risk,
which was independent of other established risk factors (Gardner et al., 1996;
Stampfer et al., 1996). In intervention studies, angiographic regression of
atherosclerosis, following HMG-CoA-reductase inhibitor therapy, occurs
predominantly in subjects whose concentration of small dense LDL particles
decreases (Watts et al., 1993). Furthermore, in the Helsinki Heart Study the majority
of benefit on morbidity and mortality following gemfibrozil therapy, was seen in
subjects with characteristics likely to indicate an ALP (Manninen et al., 1992). Gemfibrozil like other fibrates, has little effect on LDL cholesterol content but alters the profile to more larger more buoyant moieties, decreases the levels of triglyceride rich particles and elevates HDL cholesterol content.

1.3.2.3 Lipoprotein(a)

Lipoprotein(a) (Lp(a)) is an immunologically distinct form of LDL that has been associated with an increased risk of cardiovascular disease independently of LDL cholesterol levels (Berg et al., 1974; Schaefer et al., 1994). The nature of this association remains unclear as Lp(a) levels are not modified by standard hypolipidaemic agents. Recently homology between apolipoprotein(a) and plasminogen has been reported and inhibition of thrombolysis might be a mechanism by which increased risk occurs (Loscalzo et al., 1990). The risk from Lp(a) is also reported to be higher in subjects with elevated cholesterol levels and other risk factors (Schaefer et al., 1994).

1.3.2.4 Triglycerides

Whilst triglyceride levels consistently correlate with cardiovascular risk in epidemiological studies (Austin, 1989) the association is usually confounded by other risk factors particularly an inverse relationship with HDL cholesterol levels (Assmann and Schulte, 1992), and positive relationships with obesity, blood pressure and insulin resistance (Reaven, 1988). A recent meta-analysis reported an association of triglycerides with cardiovascular risk over and above HDL cholesterol levels (Austin et al., 1998). Hypertriglyceridaemia may be particularly important in the context of low HDL levels and as part of an ALP (Sprecher et al., 1993).

1.3.3 Smoking

The epidemiological evidence linking cigarette smoking to coronary artery disease has been supported by multiple studies over a period of 30 years. Community based cohort studies such as the Framingham study have shown that cigarette smokers are at a markedly elevated risk of developing cardiovascular events (Freund et al., 1993; Kannel, 1978; Pooling Project Research Group, 1978). These studies have also
shown that former cigarette smokers have a decline in the excess incidence of cardiovascular disease over a period of several years to approach that of non-smoking populations (Kannel, 1978). In autopsy studies, exposure to tobacco smoke has been associated with the presence of atherosclerosis from a young age (McGill et al., 1997; Stary, 1989; Tracy et al., 1995). However, it remains less clear whether, the degree to which, the increased risk associated with cigarette smoking is due to direct initiation of atherogenesis or effects of smoking on established atherosclerotic plaque. Studies in smokers have demonstrated increased coagulability (Kannel et al., 1987b), inflammatory activation (Blann et al., 1997b), lipid peroxidation (Morrow et al., 1995), endothelial dysfunction (Pepine et al., 1998) and other abnormalities that might predispose to cardiac events. Cessation of smoking will rapidly remove many of these effects, and is associated with an immediate reduction in risk of acute events, possibly by inactivation of unstable lesions and reduced thrombogenicity. The combined evidence from the epidemiological, experimental and pathological studies supports a unifying hypothesis of chronic effects of cigarette smoking resulting in vascular damage that may promote the development of atherosclerosis as well as acute effects, in the setting of established atherosclerosis, that increase the risk of cardiovascular events.

1.3.3.1 Passive smoking

Compounds present in environmental tobacco smoke, such as polycyclic aromatic hydrocarbons, are toxic at low concentrations. Epidemiological studies, conducted in a variety of locations, have suggested that environmental tobacco smoke might be associated with as much as a 30% increase in risk of death from ischaemic heart disease or myocardial infarction among non-smokers (Glantz and Parmley, 1991; He et al., 1994; La Vecchia et al., 1993). A number of these studies also demonstrate a significant dose dependent effect, with greater exposure to environmental tobacco smoke associated with greater risk of death from heart disease. In clinical studies, passive smoking is associated with early vascular damage (Celermajer et al., 1996) and adversely affects platelet function (Davis et al., 1989) in a manner that is similar to that seen in smokers. Furthermore, chronic smoking leads to physiological adaptations that may partially offset the damaging effects of tobacco smoke and thus, non-smokers may be more sensitive to the effects of environmental tobacco smoke.
than smokers (Glantz and Parmley, 1991). It has been estimated that passive smoking may responsible for as many as 53,000 deaths annually from passive smoking in the United States, making passive smoking the third leading preventable cause of death (AHA, 1999).

### 1.3.4 Hypertension

In a meta-analysis of nine major prospective epidemiological studies both diastolic and systolic blood pressure were related in a dose-dependent and continuous manner with incidence of stroke coronary and vascular events (MacMahon et al., 1990). In the Multiple Risk Factor Intervention Trial (1976) only 25% of men had a systolic blood pressure less than 120 mmHg and a diastolic blood pressure less than 80 mmHg. The attributable risk from blood pressure above these ideal levels was 32% and 42% for coronary vascular deaths for diastolic and systolic blood pressure respectively. Interestingly however, the greatest attributable risk, which depends on not only the relative risk but also the prevalence, was highest for a diastolic blood pressure of 85-89 mmHg, and a systolic blood pressure of 140-144 mmHg (Stamler, 1987). In clinical practice both of these levels are often considered within an acceptable range.

A wide range of environmental, pathological and genetic factors are important in the control of blood pressure. Hypertension is often associated with obesity, abnormal lipid profiles and insulin resistance (Brunner et al., 1997; Ferrannini et al., 1991). As such, establishing whether blood pressure causes cardiovascular disease or occurs as consequence of vascular injury is likely to be complicated by confounding factors. Clinical studies however, have indicated that anti-hypertensive treatment results in major reduction in the incidence of stroke (38%) and coronary vascular events (17%) (Yusuf et al., 1993). That benefit is seen with a wide range of antihypertensive treatments with different mechanisms of action is strong evidence for a direct role of blood pressure in the pathogenesis of cardiovascular disease. Interestingly, reduction in blood pressure has a greater benefit on the incidence of strokes rather than coronary events. This is in contrast to the effects of cholesterol reduction, which has its major benefit on coronary heart disease and highlights the differences that exist in the pathophysiology of vascular disease in different vascular beds.
1.3.5 Diabetes mellitus

Compared to non-diabetic populations, both type 1 insulin-dependent and type 2 non-insulin-dependent diabetes mellitus are associated with a marked increase in cardiovascular risk (Kannel and McGee, 1979; Krolewski et al., 1987; Pyorala et al., 1987). As many as 70% of diabetic patients die as a result of vascular complications often at an early age (Figure 1.3.1). The increased risk is particularly apparent in women whose risk is similar to that of men (Pyorala et al., 1987). Autopsy and angiographic studies have demonstrated more widespread and advanced atherosclerosis in diabetic compared to non-diabetic patients (Seiler et al., 1993; Strong et al., 1995).

Figure 1.3.1 Diabetes and cardiovascular mortality. A marked increase in mortality is apparent in diabetic populations from the age of 35 years, compared to the death rate in the Framingham study. By age 50 years up to 35% of diabetic subjects have died. Adapted from Krowelski et al., 1987.
The mechanisms, which underlie the increased risk of cardiovascular disease in diabetes, are complex and have not been fully elucidated. Hyperglycaemia increases osmotic pressure and shear damage to the vascular wall. However, the benefit derived from tight glycaemic control on the incidence of macrovascular complications in diabetes mellitus has been disappointing (The Diabetes Control and Complications Trial (DCCT) Research Group, 1995). Increased oxidative stress and reduced levels of endogenous antioxidants have been demonstrated in diabetes and may be important proatherogenic factors (Cominacini et al., 1994; Giugliano et al., 1996). In addition to the direct effects of hyperglycaemia, diabetes mellitus is often associated with dyslipidaemia as manifest by an atherogenic lipoprotein profile, hypertension and renal disease (Hughes et al., 1998). The risk of clinical cardiovascular disease increases dramatically, in both type 1 and type 2 diabetes once renal disease becomes manifest.

1.3.5.1 *Glucose intolerance, insulin resistance and the metabolic syndrome*

In epidemiological studies plasma glucose concentrations lower than that conventionally used to define diabetes mellitus or even impaired glucose tolerance are associated with an increased risk of coronary artery disease (Fuller et al., 1980; Singer et al., 1992). Moderate hyperglycaemia is thought to represent decreased insulin sensitivity and is associated with compensatory hyperinsulinaemia, central obesity, raised systolic blood pressure and an atherogenic lipoprotein profile (Grundy, 1998; Reaven, 1988). This clustering of risk factors has been termed the metabolic syndrome and appears to effect up to 10% of healthy working individuals (Brunner et al., 1997). The metabolic syndrome is associated with an increased risk of cardiovascular disease in men (Lempiainen, et al., 1999), though its effect in women is not yet clear. However, the impact of the metabolic syndrome on the initiation of vascular damage and the progression of atherosclerosis over and above the effects of the individual risk factors and the relative role for the different factors associated with the metabolic syndrome is not clear and the subject of debate (Ferrannini et al., 1991; Pinkney et al., 1997). Hyperinsulinaemia might itself be atherogenic and insulin levels have been associated with cardiovascular morbidity and mortality (Fontbonne et al., 1991; Perry et al., 1996).
1.3.6 Family history

In the Framingham Study and the Munster Heart Program a positive family history of coronary heart disease was identified as an independent predictor of cardiovascular risk (Assmann et al., 1997; Myers et al., 1990). The aggregation of risk factors is well recognised and, in the majority of cases, the mechanisms that underlie this relationship are likely to be heterogeneous and represent the interaction between both genetic and environmental factors. However, a number of specific genetic abnormalities are known to predispose to cardiovascular disease, whilst many more have been proposed but as yet their role remains less well defined (Hwang et al., 1997). Many of these are likely to be recessive traits with impact on atherogenesis only in homozygotes (e.g. polymorphisms of endothelial nitric oxide synthase (Hingorani et al., 1999)).

Familial hypercholesterolemia is the most widely recognised hereditary risk factor for atherosclerosis (Goldstein and Brown, 1975). The defect is now recognised to be due to mutations of the gene encoding for the LDL receptor, which prevents the uptake of LDL cholesterol by cells (Brown and Goldstein, 1976). In the United Kingdom heterozygous familial hypercholesterolemia affects one person in 500. Affected individuals have isolated elevations of LDL cholesterol and usually present with xanthomatosus manifestations in childhood or clinical coronary disease in middle age.

Familial combined hyperlipidaemia is a common abnormality with a range of phenotypic manifestations. In the majority of patients, elevated levels of both triglycerides and LDL cholesterol are apparent often with low levels of HDL cholesterol. Occasionally isolated elevation in triglycerides or LDL cholesterol levels are seen. Austin and colleagues (1990) have recently established a Mendelian dominant inheritance for the dyslipidaemic profile of familial combined hyperlipidaemia. The majority of other familial dyslipidaemias are rare and have little overall impact on the incidence of cardiovascular disease. Lipoprotein(a) levels are thought to be largely genetically determined and may be high in up to 20% of the population (Scanu and Gless, 1990).
1.3.7 Other Risk Factors

Epidemiological studies have also identified a wide range of additional factors which are associated with the incidence of cardiovascular morbidity and mortality in later life. For many of these a causative role has not been established and putative mechanisms remain the topic of controversy and debate.

1.3.7.1 Birthweight and prenatal factors

Socio-economic factors have long been thought to be important in the pathogenesis of atherosclerosis. In 1989 Barker (Barker et al., 1989) has reported a continuous inverse relationship between birthweight and weight at one year, an index of nutrition in prenatal and early life, and the later incidence of cardiovascular disease. This has subsequently been confirmed in other large cohorts in both developed and underdeveloped countries (Rich-Edwards et al., 1997; Stein et al., 1996). These associations might reflect the aggregation of genetic, economic and environmental risk factors, whereby poor conditions in early life, related to maternal ill-health, poor housing, inappropriate diet and smoking, result in foetal malnutrition and reduced birthweight, continue to operate in later life and influence the pathogenesis of atherosclerosis (Forsdahl, 1977). However, no similar association exists between birthweight and all cause mortality or death from lung cancer in later life. This has lead to the proposition of an alternative hypothesis whereby inadequate nutrition at crucial stages in foetal development and early postnatal life result in permanent alterations in the physical and metabolic characteristics of the subject such predisposing them to cardiovascular disease many decades later (Barker, 1994; Lucas and Morley, 1994). Interestingly, increased blood pressure, glucose intolerance and dyslipidaemia have also been associated with low birthweight and represent a potential mechanism by which such programming might influence atherogenesis in adult life (Barker and Osmond, 1988; Ravelli et al., 1998).

1.3.7.2 Infection and Inflammation

Histopathological studies have now demonstrated that atherogenesis represents an inflammatory response to vascular injury. As such a possible role for chronic infections its aetiology has been proposed. Higher levels of cardiovascular disease have been reported in patients with chronic helicobacter pylori infection (Markus and
Mendall, 1998). Immunological studies have also demonstrated the presence of Chlamydia pneumoniae and cytomegalovirus (Chiu et al., 1997) within atherosclerotic plaques suggesting a direct pathogenic role (Campbell et al., 1998; Wong et al., 1999). Recent studies have demonstrated higher levels of inflammatory mediators in patients with risk factors for atherosclerosis, including hypercholesterolaemia and insulin resistance (Festa et al., 2000; Kannel et al., 1987a; Mendall et al., 1996) that are predictive of later cardiovascular morbidity and mortality (Ridker et al., 2000a; Ridker et al., 2000b). The role of inflammation in atherogenesis and its potential for modification is a highly topical area of investigation.

1.3.7.3 Psychosocial factors

Distinct socio-economic differences in the incidence of cardiovascular disease are apparent men in social class V having twice the mortality of men in social class I (Marmot and McDowell, 1986). These might be accounted for by co-associations with other risk factors as outlined above. However in studies in primates, increased psychosocial stress enhances the progression of atherosclerosis (Williams et al., 1993) and, in humans, low work place control has also been identified as a possible risk factor for cardiovascular morbidity and mortality (Bosma et al., 1998).
1.4 ATHEROSCLEROSIS AND ATHEROGENESIS: PATHOLOGY AND MECHANISMS

1.4.1 Atherosclerosis: a historical perspective

Although atherosclerosis as a pathological entity has only been recognised for about 100 years (Marchand, 1904) it is an old disease, having been identified in aortic samples taken from the mummified remains of King Merneptah (1224 –1214 BC), thought to be the Pharaoh of the Exodus (Shattock, 1909). The name is derived from the Greek for porridge (athero) and for hardening (sclerosis). Despite its antiquity the clinical consequences of atherosclerosis on the coronary, cerebral and peripheral circulations are the leading cause of death before the age of 65 years in Western societies and atherosclerosis remains a major public health issue.

1.4.2 The normal artery

Healthy arteries are composed of 3 layers (Ross and Glomset, 1976) (Figure 1.4.1). The intima is the innermost layer. It is lined by the endothelium and consists of a relatively thin layer of connective tissue containing occasional vascular smooth muscle cells and resident macrophages. The lesions of atherosclerosis are entirely confined to the intima. The media is the muscular wall of the artery and is separated from the intima by the internal elastic lamina. The number and configuration of the smooth muscle cells is not uniform. In muscular arteries the cells are arranged in spiralling layers, whilst in elastic arteries the smooth muscle is arranged in multiple lamellae each bounded by an elastic lamina. The adventitia is the outermost layer and consists mostly of connective tissue, fibroblasts and smooth muscle cells. It is highly vascular containing nutrient vasa vasorum, and is the only layer of the artery that is directly innervated.

1.4.2.1 Adaptive intimal thickening

Adaptive intimal thickening (Figure 1.4.2) is present at birth often at arterial bifurcations, sites which are predisposed to develop atherosclerosis in later life (Velican and Velican, 1975). The intima at these sites contains increased connective tissue and smooth muscle cells, responses, which are thought to represent adaptive
changes to low shear stress (Zarins et al., 1987). Although not a pathological lesion the spatial relationship with atherosclerotic lesions in later life has led some workers to suggest that adaptive intimal thickening might be involved in the pathogenesis of atherosclerosis, possibly by increasing the retention time for lipids and macrophages within the intima (Williams and Tabas, 1995). Indeed areas of adaptive intimal thickening, in humans, have higher levels of lipid content and numbers of macrophages than in normal sections of the arterial wall (Schwenke and Carew, 1989a; Stary et al., 1992).

1.4.3 The lesions of atherosclerosis

1.4.3.1 Fatty streaks

Fatty streaks are considered to be the earliest pathological lesions of atherosclerosis (Stary et al., 1994). They are visible to the naked eye (Figure 1.4.3a) and consist of lipid laden macrophages and foam cells within the intima (Figure 1.4.3b). Fatty streaks are often present from early life, being seen in more than 40% of infants aged one month to one year (Schwartz et al., 1967). In infants, fatty streaks are localised to the aortic valve ring and the ostia of intercostal vessels. In young adults, the ascending aorta, arch and ostia of major vessels are involved and coronary fatty streaks are usually apparent by the second decade. Whilst many fatty streaks regress spontaneously, pathological studies suggest that, at predilicted sites and in the presence of risk factors, some early lesions may develop through a series of intermediate lesions into raised fibrolipid plaques (Faggiotto et al., 1984; Stary, 1989). Based on morphological characteristics and the temporal and spatial relationship of these lesions, (Stary and colleagues (1994) classified the lesions of atherosclerosis into a number of grades (I-VI, Figure 1.4.4) and proposed a pathological sequence of events.

1.4.3.2 Fibrolipid plaques

Fibrolipid plaques are the archetypal lesions of atherosclerosis, which begin to appear during the second and third decade of life (Stary et al., 1995) (Figure 1.4.4). Complications such as plaque rupture or encroachment on the lumen are the basis of the vast majority of clinical events. Whilst fatty streaks are apparent with equal propensity in populations with low and high incidences of cardiovascular disease, the
extent of intimal involvement by the fibrolipid plaque predicts the risk of clinical vascular events (Stary, 1989). The raised fibrolipid plaque has two characteristic features: a fibrous cap below the endothelium overlying a lipid rich pool containing, mainly, necrotic debris derived from foam cells. However, a wide spectrum of morphological variants have been described ranging from a densely fibrous lesion with a thick cap and small lipid pool to lesions with a large lipid pool separated from the lumen only by a thin cap depleted of connective tissue (Stary et al., 1994) (Figures 1.4.5 and 1.4.6). The pathophysiological consequences of these lesions are likely to differ. Plaques with a large lipid pool and a thin cap are more prone to rupture and result in a major thrombo-occlusive event. In many advanced plaques, thinning of the media and an inflammatory infiltrate within the adventitia are apparent. As a result, despite the presence of a large lesion, the arterial lumen may be normal in size. Alternatively, the plaque may protrude into the lumen and limit blood flow resulting in tissue ischaemia.

1.4.4 Molecular and cellular events in atherogenesis

Immunohistochemical techniques have facilitated the identification of the origin of cells within atherosclerotic lesions. In fatty streaks, foam cells are largely derived from circulating monocytes, which adhere to the endothelium and enter the intima by diapedesis. Once in the intima, monocytes differentiate into macrophages and phagacytose lipoproteins. Lipid laden macrophages may fuse and are recognised microscopically as foam cells. An important aspect of the formation of foam cells is the oxidative modification of lipoproteins (Steinberg et al., 1989). Native LDL is normally taken up by the LDL receptor first described by Goldstein and Brown (Goldstein and Brown, 1975). The uptake of LDL by this mechanism is under feedback control, which limits the cholesterol content within cells. However, following oxidative modification of lipoproteins, they are rapidly taken up by macrophages via the 'scavenger' pathway, which is not rate limited (Parthasarathy et al., 1989). Oxidative modification of LDL is likely to occur if the LDL particle has a long residence time in the intima, when its own anti-oxidants may be exhausted and macrophages are potent sources of oxidative stress and enzymes that promote lipid esterification.
Figure 1.4.1 Cross section through a normal artery wall. The lumen is large without any narrowing by atheromatous plaque. There is a thin media which underlies the endothelium. The muscular wall is of normal proportion.

Figure 1.4.2 Adaptive intimal thickening. This normal physiological response to low shear stress is present at birth often at arterial bifurcations. Although these sites are predilected to atherosclerosis in later life, no clear pathological link has been identified.
Figure 1.4.3a Macroscopic appearance of coronary artery fatty streaks, the earliest pathological lesion of atherosclerosis.

Figure 1.4.3b Histological appearance of a fatty streak. Lipid (purple stain) can be clearly seen within the intima surrounded by macrophages.
<table>
<thead>
<tr>
<th>Earliest onset</th>
<th>Composition or main growth mechanism</th>
<th>Sequences in the development of individual lesion types</th>
<th>Effect on wall thickness and arterial lumen</th>
<th>Clinical correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>from first decade</td>
<td>macrophages increased; isolated macrophage foam cells</td>
<td>complete reversal to normal possible</td>
<td>no or minimal increase in wall thickness; no reduction in lumen</td>
<td>clinically silent</td>
</tr>
<tr>
<td>from third decade</td>
<td>more intra-cellular lipid: in macrophages and in smooth muscle cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>from fourth decade</td>
<td>type II changes and isolated pools of extracellular lipid</td>
<td>increased wall thickness but often only minimal lumen reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>from fifth decade</td>
<td>type II changes and core of extracellular lipid</td>
<td>additional increases in wall thickness; progressive reduction of lumen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>type IV changes and smooth muscle and collagen increase</td>
<td>thrombotic deposits increase thickness; lumen often completely blocked</td>
<td></td>
<td>clinically overt or silent</td>
</tr>
<tr>
<td></td>
<td>type IV or type V changes and thrombotic deposits, hematoma, fissure</td>
<td>episodes of thrombosis recurring at close intervals increase type V size rapidly</td>
<td></td>
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<tr>
<td></td>
<td>mineralization predominates</td>
<td>formation of a thrombotic deposit increases type V size</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>collagen predominates; lipid absent or minimal</td>
<td>progression? stabilization? regression?</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>residual of thrombosis? regression?</td>
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</tbody>
</table>

Figure 1.4.4 Classification of the lesions of atherosclerosis. Adapted from Stary et al., 1994.
Figure 1.4.5 The coronary artery shown on the left has narrowing of the lumen due to build up of an eccentric lipid rich coronary atherosclerotic plaque. The lipid pool is separated from the lumen by a fibrous cap containing connective tissue and smooth muscle cells. The plaque on the right is "complex" in that there is, widespread calcification which appears bluish on this H&E stain and a severe degree of luminal narrowing.

Figure 1.4.6 Cross sections of coronary arteries stained for collagen. The lumen of the artery on the left is significantly narrowed by this densely fibrous plaque. This might be important in limiting blood flow though the plaque is likely to be relatively stable. By contrast on the right although the luminal diameter is well preserved due to thinning and expansion of the adventitia the plaque has only a thin fibrous cap with a large lipid pool. This plaque is unlikely to cause any limitation in blood flow but is at high risk of rupture.
The other major cell type identified in atherosclerotic plaques, by immunohistochemistry, is that of vascular smooth muscle cells (Stary et al., 1995). In response to growth factors, smooth muscle cells migrate through fenestrations in the internal elastic lamina into the intima, where they proliferate and transform from a contractile cell into a synthetic phenotype (Ross, 1993). These cells are largely responsible for secreting the large amounts of connective tissue seen in fibrolipid plaques. These processes are mediated by growth factors (for example, platelet-derived growth factor, transforming growth factor β) secreted by the endothelium, platelets and synthetic smooth muscle cells, thus setting up a vicious circle promoting the progression of large fibrous plaques. In advanced lesions reduction in the number of smooth muscle cells and thus connective tissue may be an important factor in destabilising the plaque rendering it susceptible to rupture (Stary et al., 1995).

1.4.4.1 Initiation of atherogenesis

The concept that atherosclerosis represents an inflammatory response to injury was first proposed by Virchow in 1858. In 1973, Ross and Glomset formulated a ‘response to injury’ hypothesis which incorporated many of Virchows ideas and have since updated the hypothesis taking into account new insights into the mechanisms of atherogenesis (Ross, 1993). The basic tenant of the hypothesis is that early atherosclerosis represents an inflammatory response to noxious substances which injure the vascular wall. In advanced atherosclerotic plaques, a fibroproliferative or ‘healing’ process predominates although the inflammatory response may continue to have an important role in mediating plaque stability.

Retention of lipoproteins within the vascular wall is thought to be a key factor that initiates these pathophysiological processes (Anitschkow and Chalatow, 1913; Williams and Tabas, 1995). In cholesterol fed rabbits, Schwenke and Carew (1989a) have demonstrated that LDL accumulation occurs at sites predilicted to develop atherosclerosis before the appearance of inflammatory cells. This process seems to be dependent on the retention of lipids within the intimal matrix rather than the rate of entry of lipoproteins (Schwenke and Carew, 1989b). Once within the intima, LDL particles may become trapped and form crosslinks with proteoglycans within the
extracellular matrix (Camejo et al., 1993; Nivelstein-Post et al., 1994). As a result, lipoprotein concentrations within the intima may exceed plasma levels (Smith, 1974). The reason for the focal distribution of lipid accumulation remains unclear but may reflect differences in the thickness of the intima, function of the overlying endothelium and the molecular configuration of proteoglycan molecules at lesion prone sites (Cardosa and Mourão, 1994).

1.4.4.2 Oxidative modification of lipoproteins

Trapped lipoproteins sequestered within the intima are isolated from plasma antioxidants and undergo oxidative modification, an event that may be obligatory in atherogenesis (Steinberg et al., 1989; Yla-Herttuala et al., 1989). This process results in the production of oxidised lipid moieties, which have potent biological effects, and modification of the apolipoprotein molecule rendering it unrecognisable by the LDL receptor. In experimental studies oxidatively modified lipids inhibit endothelial-dependent dilatation (Deckert et al., 1997) and stimulate the expression of leucocyte adhesion molecules on the endothelial surface (Erl et al., 1998; Kume et al., 1993; Quinn et al., 1988), thus facilitating the binding of monocytes and their egress into the intima (Drake et al., 1991). Napoli et al., (1997) have recently demonstrated that oxidised epitopes of LDL are present in the aortic intima of hypercholesterolaemic foetuses prior to the infiltration of macrophages and it seems likely that the products of minimally oxidised lipoproteins represent the initial inflammatory stimulus. Furthermore, minimally oxidised LDL stimulates endothelial cells to synthesise potent monocyte activators (monocyte chemoattractant protein 1 and monocyte colony stimulating factor). Once within the intima, monocytes differentiate into macrophages and avidly take up the modified LDL by the scavenger pathway, which is not under feedback control, coalescing to form foam cells that characterise arterial fatty streaks. An important aspect of this pathophysiological process is that macrophages have the potential to promote further oxidation of LDL and synthesise growth factors, thus setting up a vicious cycle that acts to amplify the inflammatory response. High-density lipoproteins may have important antiatherogenic effects by removing cholesterol from the artery but also by inhibiting LDL oxidation (Mackness et al., 1991b).
Understanding the mechanisms of atherogenesis and the interaction of the vascular wall with environmental, acquired and genetic risk factors will be crucial in the development of strategies targeted at retarding its progression. It is likely that the vascular endothelium both bears the brunt of the initial insult and mediates many of the cellular responses, which ultimately result in the formation of atherosclerotic plaques (Ross, 1993). Risk factors might promote these atherogenic processes by; enhancing lipoprotein retention within the intima; increasing oxidative stress, which will promote lipoprotein oxidation and directly injury endothelial cells; or directly altering endothelial homeostasis thus rendering it dysfunctional.
1.5 THE ENDOTHELIUM

Until recently vascular endothelium, which lines all blood vessels, was considered as a relatively inactive layer of cells whose sole function was to act as a semipermeable and nonthrombogenic interface between circulating blood and parenchymal tissues. However, it is now recognised that the endothelium is a multifunctional homeostatic organ with a combined weight greater than that of the liver and a diverse range of endocrine and paracrine functions, which regulate local metabolic requirements and maintain vascular integrity (Henderson, 1991; Vane et al., 1990). It presents a non-thrombogenic interface to the flowing blood, actively regulates the transport of macromolecules, controls vasomotor tone through the synthesis and release of vasodilators and vasoconstrictors and regulates growth and inflammatory responses. Healthy endothelium normally operates in an inhibitory mode; resisting thrombostasis and platelet and leucocyte adhesion and in many vessels the endothelium maintains the smooth muscle in a state of relaxation. Endothelial cells in vivo align in the direction of blood flow, thus minimising shear stress gradients (Barbee et al., 1994). The ability of the endothelium to detect and respond to local biochemical and biophysical stimuli is an important factor in regulating homeostatic functions within different organs and vascular beds throughout the body. As such phenotypic differences may exist within different vascular beds whereby the endothelium is functionally specialised for that particular organs needs (Gerritsen, 1987).

In view of the diverse nature of normal functions of the endothelium, it is not therefore, surprising that injury to the endothelium or the onset of endothelial dysfunction, as represented by loss of its inhibitory role might be important in the pathophysiology of vascular disease.
1.5.1 Functions of normal endothelium

1.5.1.1 Regulation of vascular tone

The ability of blood vessels to relax and constrict in response to agonists and physical stimuli has been recognised for many years. However, it is only relatively recently that the central role of the endothelium in regulating vascular tone, through the synthesis of both relaxing (e.g., nitric oxide, prostacyclin) and vasoconstrictor substances (e.g., endothelin angiotensin II), has been appreciated (Mombouli and Vanhoutte, 1995). The interaction between these factors provides a control mechanism that regulates regional blood flow and systemic blood pressure and limits the potentially damaging effects of shear stress on the vascular wall.

1.5.1.1.1 Endothelium derived relaxing factor (EDRF)

In 1980, Furchgott and Zawadzki first reported their observation that an intact endothelium was a prerequisite for rabbit aortic rings to relax in response to acetylcholine and postulated the existence of an endothelium-derived relaxing factor (EDRF). The action of EDRF could not be blocked by inhibitors of cyclooxygenase suggesting that it was not a prostanoid, similar to prostacyclin, which had been identified earlier. EDRF had a short half-life, was inactivated by haemoglobin and caused smooth muscle to relax by stimulating guanylate cyclase to increase cyclic GMP levels. This action had many similarities to the actions of nitrosovasodilators and in 1987 Ignarro proposed that EDRF was the free radical nitric oxide (NO) or a molecule elaborating NO. Subsequently, Palmer and colleagues (1987) demonstrated that EDRF and NO had identical biological and pharmacological properties in bioassay studies. Nitric oxide is a small highly soluble lipophilic molecule, which rapidly diffuses through biological membranes without the need for a specialised transport mechanism. The binding of NO to the haem group of guanylate cyclase results in a rapid increase in cyclic guanosine monophosphate, which results in smooth muscle cell relaxation by decreasing intracellular calcium levels (Ignarro et al., 1981).

Nitric oxide is synthesised enzymatically from the semi-essential amino acid L-arginine by the enzyme NO synthase (Palmer et al., 1988). The reaction is
stereospecific and L-arginine is converted to NO and L-citrulline (Figure 1.5.1). Three isoforms of NO synthase have been characterised and mapped to three distinct genes (Forstermann et al., 1994). An endothelial isoform and neuronal isoform are constitutively expressed whereas inflammatory cytokines and endotoxin activate an inducible isoform. The constitutive isoforms of NO synthase are calcium dependent and synthesise NO in response to receptor mediated agonists whilst the inducible isoform is calcium independent and can synthesise large amounts of NO which may be directly cytotoxic. The activities of both constitutive and inducible isoforms of NO synthase are dependent on the availability of sufficient tetrahydrobiopterin as a cofactor (Knowles and Moncada, 1994). Activation of NO synthase in the presence of suboptimal concentrations of tetrahydrobiopterin may paradoxically result in the generation of superoxide rather than NO (Wever et al., 1997). This may reflect the close amino acid sequence homology between NO synthase and cytochrome P450.

A number of methylated forms of L-arginine have been identified, which competitively inhibit NO synthesis. These occur naturally and are normally found in very low concentrations in human plasma. However in some disease states (e.g. renal failure, hypercholesterolaemia) levels of endogenous inhibitors might be increased and contribute to reduced NO synthesis (Boger et al., 1998; Vallance et al., 1992). Pharmacological use of these specific inhibitors has also facilitated the study of the physiological and pathophysiological roles of NO in both animals and the human circulation. In healthy humans, small amounts of NO are synthesised continuously and this basal NO synthesis is a major determinant of systemic vascular resistance and blood pressure (Vallance et al., 1989). In contrast to small resistance arterioles, basal NO synthesis in large conduit arteries is low (Mullen et al., 1998a). The major physiological stimulus to NO synthesis in these vessels is likely to be the physical forces of shear stress (Rubanyi et al., 1986), pressure and endothelial tension induced by pulsatile blood flow (Lamontagne et al., 1992). NO dependent flow mediated vasodilatation has been demonstrated in the coronary and peripheral circulation of humans (Joannides et al., 1995; Mullen et al., 1998a; Nabel et al., 1990). Vasodilatation in response to the receptor mediated agonists acetylcholine, bradykinin and substance P can also be significantly attenuated by NO synthase inhibition both in vitro and in vivo (Chowienczyk et al., 1993; Quyyumi et al., 1997).
However, these pharmacologically mediated responses are unlikely to represent important physiological pathways for NO synthesis.

Figure 1.5.1 The L-arginine nitric oxide pathway

1.5.1.1.2 Endothelium-derived hyperpolarization factor (EDHF)

The presence of additional non-prostanoid endothelium-dependent vasodilators distinct from NO has been postulated from experiments, in animal models, where agonist mediated vasodilatation is not attenuated by either inhibitors of NO synthesis or cyclooxygenase inhibitors (Corriu et al., 1998). These agonists in addition to stimulating release of NO may also result in hyperpolarization of the smooth muscle, and the findings are consistent with the release of substance(s) which have the capacity to hyperpolarize smooth muscle cells (Nagao and Vanhoutte, 1992). Thus, the presence of a diffusible endothelium-derived hyperpolarization factor (EDHF) has been postulated (Feletou and Vanhoutte, 1996; Mombouli et al., 1996) though its biochemical identity has not been determined and, as no specific inhibitors have been developed, its biological relevance in humans is not known.
1.5.1.1.3 Prostacyclin

Prostacyclin, like NO, is a potent vasodilator substance derived from vascular endothelium, which also inhibits platelet aggregation (MacIntyre et al., 1978; Moncada et al., 1976). It is a prostanoid derived from arachidonic acid and cyclooxygenase inhibitors such as aspirin and indomethacin can inhibit its synthesis. Prostacyclin acts via the stimulation of adenylate cyclase to increase intracellular levels of cyclic adenosine monophosphate in platelets and smooth muscle cells. Unlike NO, prostacyclin does not seem to have an important role in regulating systemic vascular tone, however in specialised vascular beds such as the pulmonary circulation and in some disease states decreased prostacyclin production may be linked to the pathogenesis of disease (Jones et al., 1987; Willis et al., 1986).

1.5.1.1.4 Endothelin

The endothelins are a family of 21 amino acid polypeptides which are potent vasoconstrictors (Yanagisawa et al., 1989). Three isoforms have been identified to date; endothelin-1 is the major isoform and is almost entirely endothelial in origin. It is synthesised from the precursor molecule big-endothelin, by the metalloprotease enzyme ‘endothelin converting enzyme’, which in turn is cleaved from preproendothelin. Endothelin-1 is not stored within endothelial cells and is synthesised on a de novo basis in response to a variety of stimuli. These include shear stress, hypoxia, angiotensin II, bradykinin, adrenaline, insulin, glucocorticoids, thromboxane A2 and inflammatory cytokines. In humans the biological effects of endothelin-1 are mediated through the activation of two specific membrane receptors, ET_\text{A} and ET_\text{B}. Activation of ET_\text{A} receptors, which are found only on vascular smooth muscle cells, results in a calcium dependent vasoconstriction and promotes growth. In contrast to the short-lived effects of NO and EDHF, the endothelin-1 mediated vasoconstriction is profound and long-lasting. In addition ET_\text{A} activation promotes smooth muscle cell growth and proliferation. ET_\text{B} receptors are found on both the endothelium and smooth muscle cells. Activation of endothelial ET_\text{B} receptors stimulates the release of NO and prostacyclin resulting in vasodilatation (Takase et al., 1995).
Although endothelin is found in many primitive species, its role in human physiology has not been established. In healthy subjects plasma endothelin levels are generally low. However, infusion of endothelin receptor antagonists decreases peripheral arteriolar tone and blood pressure suggesting that endogenous endothelin-1 has a role in the maintenance of vascular tone (Haynes and Webb, 1994; Haynes et al., 1996). In a variety of clinical situations, including patients with heart failure (Stewart et al., 1992), ischaemic heart disease (Wieczorek et al., 1994; Zeiher et al., 1995) and otherwise healthy subjects with high-altitude pulmonary oedema (Goerre et al., 1995), markedly increased levels of circulating endothelin-1 have been demonstrated and might contribute to pathophysiology of these diseases. The development of orally active endothelin antagonists represents a potentially valuable therapeutic approach.

1.5.1.5 Angiotensin II

Like endothelin-1 angiotensin II is a potent vasoconstrictor substance but also is promitogenic and stimulates vascular superoxide production (Heagerty, 1991; Rajagopalan et al., 1996). Angiotensin II is derived from the precursor angiotensin I by the action of the kininase angiotensin converting enzyme. Whilst the majority of circulating angiotensin I is not of endothelial origin, tissue bound angiotensin converting enzyme is likely to be an important local source of angiotensin II (Campbell, 1987). Furthermore angiotensin-converting enzyme expression may be increased in atherosclerotic plaques (Diet et al., 1996).

1.5.1.2 Maintenance of vascular permeability

The maintenance of a selective barrier between the blood and tissues represents the most fundamental function of vascular endothelium (van Hinsberg et al., 1997). Small soluble molecules may diffuse through the endothelium along concentration gradients, whilst the distribution of fluid is determined by a balance of osmotic and hydrostatic forces. It is now recognised that endothelial cells also actively transport a wide variety of macromolecules, including lipoproteins, both to and from the intima by a process of endocytosis. In addition, in the microvasculature intracellular fenestrations might be an important route for large molecules to enter the tissues.
A range of different factors regulate endothelial permeability and considerable heterogeneity is apparent between different vascular beds (Gerritsen, 1987). In the liver, exocrine pancreas and other endocrine organs and the renal glomeruli, high permeability facilitates the rapid transfer of substances to and from the blood. In large conduit arteries, increased permeability to macromolecules is apparent at sites predilicted to develop atherosclerosis, where mean shear stress is low and endothelial cells are poorly aligned (Schwenke and Carew, 1989b). This might be an important factor in the accumulation of lipids at these sites. The composition of the basement membrane and extracellular matrix, structural aspects of endothelial glycocalyx, the tightness of intercellular junctions and shear stress all seem to be important factors in regulating endothelial permeability. In addition, nitric oxide, circulating mediators such as histamine, cytokines, vascular endothelial growth factor and adrenaline may result in hyperpermeability and oedema (Cardona-Selemente and Born, 1995; Murohara et al., 1998).

1.5.1.3 Anti-thrombogenic role of endothelium

The maintenance of an intact endothelial monolayer is crucial to prevent intravascular thrombosis. The endothelium is a major source of both pro- and anti-thrombogenic factors. Normally tonic production of NO and prostacyclin inhibit platelet activation and aggregation (Murohara et al., 1995; Radomski et al., 1987) whilst fibrinolysis is regulated by the release of tissue-plasminogen activator and plasminogen activator inhibitor type 1 (Newby et al., 1998). Furthermore, the endothelium is the major source of the large glycoprotein von Willebrand factor which is important in the formation of crosslinks between platelets and endothelial cells (Mannucci, 1998). Thus, whilst healthy endothelium maintains a non-thrombogenic interface, in disease states dysfunctional endothelium is likely to be an important factor that promotes thrombogenesis.

1.5.1.4 Regulation of inflammatory responses

The endothelium plays a key role in regulating the entry of inflammatory cells into the tissues. In healthy vessels, leucocyte adhesion is inhibited by the production of NO (Tsao et al., 1996). However, in response to proinflammatory stimuli, endothelial cells synthesise and express specific adhesion molecules on their luminal
surface (Albelda et al., 1994). These include P and E selectin, vascular cellular adhesion molecule-1 and intracellular adhesion molecule-1, which recognise ligands on circulating platelets and leucocytes and facilitate their binding and transmigration between adjacent endothelial cells (Adams and Shaw, 1994). The importance of adhesion molecules in regulating inflammatory responses is illustrated by the rare deficiencies of adhesion molecules. Although patients with the hypergammaglobulinaemia syndrome have functionally normal leucocytes they have adhesion molecule deficiencies and are at risk from profound sepsis. In atherosclerosis, adhesion molecule deficient mice are protected from the development of fatty streaks the earliest inflammatory lesion (Nageh et al., 1997).

1.5.1.5 Regulation of vascular growth

The endothelium synthesises a number of growth factors including platelet derived growth factor and the growth inhibitor transforming growth factor β (Hart and Clowes, 1997). In addition endothelium derived NO, endothelin and angiotensin II may all influence the growth and proliferation of cells within the vascular wall (Daemen et al., 1991; Garg and Hassid, 1989). The endothelium is therefore, likely to be a major determinant of growth acting through the interaction between these pro- and anti-mitogenic factors.

1.5.2 Consequences of endothelial dysfunction

Because of its strategic position the endothelium has a fundamental role in orchestrating the interaction between blood and parenchymal tissue. The brief overview above illustrates the functional diversity of the endothelium in normal physiology but also its dynamic and mutable nature and its potential to regulate many of the pathological processes associated with vascular disease. Alterations in endothelial function include; increased endothelial cell apoptosis and turnover; increased vascular permeability; the expression of adhesion molecules on the abluminal surface, which facilitates the binding of monocytes and their egress into the intima; release of growth factors including platelet derived growth factor, which promotes smooth muscle migration and proliferation; abnormal vasomotion,
mediated by increased release of vasoconstrictors and decreased synthesis of the endothelial derived relaxing factor, nitric oxide.

The phenotypic modulation of the endothelium to this dysfunctional state represents the basis for the ‘response to injury’ hypothesis of atherosclerosis as described by Ross and Glomset (Ross, 1993). Potentially noxious substances which might result in endothelial dysfunction include; oxidised lipids, oxygen derived free radicals, substances within cigarette smoke, hyperglycaemia, glycosylated proteins, growth factors, inflammatory mediators, high or low shear stress, hyperhomocysteinaemia, reduced endogenous antioxidant vitamins and reduced cofactors necessary for the synthesis of nitric oxide. Once established however, dysfunctional endothelium will promote vasoconstriction, thrombogenesis, and the inflammatory infiltrate, which characterises the earliest lesions of atherosclerosis. Through its effects on smooth muscle cell growth and proliferation, continued endothelial dysfunction might also be important in determining the progression of fatty streaks to the more advanced fibroproliferative plaques. In advanced disease, impaired endothelial-dependent dilatation is likely to result in abnormalities of coronary blood flow, which might underlie episodes of transient myocardial ischaemia, and endothelial dysfunction might be implicated in the events which lead to plaque instability and rupture. Thus the transformation of healthy endothelium into a dysfunctional phenotype might represent a fundamental step in the initiation of atherosclerosis and in the later manifestation of clinical cardiovascular disease.

1.5.2.1 Nitric oxide: an endogenous antiatherogenic molecule?

A key aspect of endothelial dysfunction that promotes many of the pathophysiological processes in atherosclerosis might be reduced synthesis or bioavailability of endothelium-derived NO. Nitric oxide in addition to its role as a potent vasodilator (Furchgott and Zawadski, 1980), regulates endothelial permeability (Murohara et al., 1998), inhibits leucocyte endothelial cell interaction (Lefer, 1997), platelet aggregation (Radomski et al., 1987), and smooth muscle cell migration and growth (Sarkar et al., 1996). Nitric oxide might also be important in regulating the secretion of fibrinolytic factors (Newby et al., 1998). These properties have lead many researchers to postulate that NO is an endogenous antiatherogenic
molecule (Cooke and Tsao, 1994). In cholesterol fed animals, chronic inhibition of NO synthesis results in enhanced progression of atherosclerosis (Cayatte et al., 1994). In humans the role of NO as an antiatherogenic molecule remains unproven though impaired NO mediated vasodilatation has been demonstrated in a wide range of subjects with risk factors for atherosclerosis and in patients with established cardiovascular disease (Celermajer et al., 1992).
1.6 MEASURING ATHEROSCLEROSIS IN HUMANS

Detection and quantification of atherosclerosis are important in both clinical and research settings. In patients with clinically manifest cardiovascular disease, treatment is often based on the precise delineation of the severity and extent of atherosclerotic disease. In younger patients detection of atherosclerosis at an early stage in its natural history might be helpful in assessing the risk of cardiovascular morbidity and mortality in later life and guiding the institution of appropriate interventional strategies. The success of such an approach will depend on the availability of suitable surrogate endpoints with which, to measure atherosclerosis and determine the benefit of treatment. These techniques will need to be sensitive and specific and be able to detect and quantify the severity of disease at an early stage. As the majority of subjects are likely to be asymptomatic a non-invasive approach will be required. Current methods for assessing atherosclerosis can be broadly divided into three groups and these will be reviewed.

1.6.1 Assessment of adequacy of tissue perfusion

The extent and severity of atherosclerosis may be judged indirectly by assessing its consequences on end organ function or the presence of ischemia, at rest or under conditions of stress. Abnormalities of normal physiology or evidence of reversible ischemia are suggestive of significant occlusive vascular disease. These techniques which include electrocardiography, exercise testing, cardiac radionuclide perfusion scans and stress echocardiography are widely used in the assessment of patients with suspected or established clinical coronary heart disease. However, by definition, they are only sensitive to the presence of significant flow limiting stenoses and are therefore likely to become positive only late in the disease process and have limited application in the study of preclinical subjects.

1.6.2 Assessment of arterial anatomy

The second major group of techniques are those which directly measure the presence of anatomic atherosclerotic plaque. Of these, angiography, most commonly coronary, remains the gold standard. Selective coronary catheters developed by Sones in the
late 1950's (Sones, 1958) allowed the precise delineation of the site and extent of coronary atheroma (Figure 1.6.1) and thus facilitated the successful development of coronary artery bypass surgery. In clinical research serial coronary angiography has been used to assess the effect of interventions on the progression of atherosclerosis (Ludmer et al., 1986), an approach which has been greatly aided by improvements in equipment, contrast media and the development of digital systems which employ edge detection algorithms and videodensitometry to accurately measure plaque size (Jukema et al., 1995). However, coronary angiography has a number of limitations. Firstly its invasive nature and the risk of complications associated with angiography will limit its use to patients with clinically manifest cardiovascular disease. Secondly, in angiography only the lumen of the vessel is visualised and significant atherosclerotic plaque may exist without any apparent reduction in the intraluminal diameter.

The development of intravascular ultrasound over the past decade has further refined the assessment of both coronary anatomy and function (Figure 1.6.2). In this technique a small ultrasound transducer is mounted on the tip of a low profile catheter, which is passed into the coronary artery under radiological control (Liebson and Klein, 1992). As the transducer rotates a circumferential image of the artery from within the lumen is obtained allowing precise depiction of the coronary lumen diameter and area at the level of the catheter tip. The arterial wall at this level can be evaluated for lipid, fibrous tissue, calcification, wall dissections, and intraluminal thrombi. Intravascular ultrasound has been used to assess the severity of coronary disease and the vulnerability of specific plaques to rupture. Plaques containing high levels of calcium are now recognised to be less amenable to percutaneous coronary angioplasty and the success of such procedures and the complete deployment of intravascular stents can be assessed (De, I et al., 1993; Keren et al., 1992). Three-dimensional reconstruction techniques allow depiction of the segment of the artery traversed by the catheter tip and the use of Doppler ultrasound imaging provides information on coronary blood flow velocities through coronary obstructions. Intravascular ultrasound images may provide information that complements coronary angiography and may have an impact on patient care and clinical investigation strategies. In preclinical subjects intravascular ultrasound can identify 'angiographically silent' atherosclerosis (Claessens and Haseldonckx, 1992) but like
Figure 1.6.1 Coronary angiogram. This technique precisely details the intraluminal anatomy of the coronary arteries but gives little information on subclinical atherosclerosis which does not obstruct coronary blood flow.

Figure 1.6.2 Intravascular ultrasound images of coronary arteries. A demonstrating a healthy section of artery with the normal trilaminar morphology. B A large atheromatous plaque. IVUS can define the anatomy and stability of atherosclerotic plaques within the vessel wall.
angiography its use is limited by its invasive nature. In clinical research intravascular ultrasound has been used to assess coronary atherosclerosis and the effects of interventions on plaque structure and size in a longitudinal fashion (Takagi et al., 1997).

1.6.2.1 Non-invasive assessment of atherosclerosis

A number of non-invasive methods are also available for the direct visualisation of atheroma. High-resolution CT scans have recently been used to quantify coronary calcification. High indexes for coronary calcification have been demonstrated in young subjects with risk factors for atherosclerosis (Levenson et al., 1997) and are thought to represent the severity of underlying atheroma. However the significance of coronary calcification to later cardiovascular disease remains unclear (Shemesh et al., 1998). Recently advances in magnetic resonance imaging (MRI) have enabled the detection of coronary atheroma in humans, in vivo (Fayad, 2000) and this represents a promising approach for the future.

More widely available however is the use of external beam ultrasound to directly determine the presence and extent of atherosclerotic plaque in vascular structures. Since its inception in the early 1980s improvements in technology have resulted in higher resolution and “real-time” imaging. The development of Doppler velocimetry and colour flow mapping have further enhanced the sensitivity and specificity for occlusive vascular disease and ultrasound imaging has largely replaced angiography in the clinical assessment of carotid and peripheral vascular disease. However, high frequency transducers, needed to achieve high resolution, penetrate poorly and therefore can only be used on superficial structures and the use of external ultrasound to study deeper arteries, such as the coronaries, is limited by depth and poor resolution. In addition to being able to demonstrate clinically relevant atheroma in major peripheral arteries, ultrasound imaging has been used to examine early changes in vascular wall structure. Increased thickness of the common carotid arterial wall, which is easily scanned and prone to clinical atherosclerotic disease, has been most widely investigated (Salonen and Salonen, 1993). In contrast to assessments of the severity of stenoses, measurement of the intima-media thickness (IMT) (the distance between media/adventitia interface (‘i’ line) and lumen/intima
interface (‘m’ line)) can be determined accurately and reproducibility in almost all subjects. On histological examination an increased IMT may represent reactive adaptive intimal thickening or medial hypertrophy in response to changes in shear stress and wall tension. At a more advanced stage IMT’s of > 1mm are likely to represent atherosclerotic plaque (Bots et al., 1997). Its non-invasive nature has facilitated its use in a wide range of clinical and epidemiological studies and a relationship between IMT and both risk factors (Temelkova-Kurktschiev et al., 1998) and clinical cardiovascular events has been established (Bots et al., 1999; Chambless et al., 1997). The major limitations of IMT as a marker of early atherosclerosis are its lack of sensitivity and specificity for cardiovascular disease, which necessitates the study of large cohorts and, as structural changes progress slowly, their follow up over long periods of time. Despite these caveats a number of studies have demonstrated beneficial effects on IMT after interventions such as cholesterol reduction (Mercuri et al., ) and calcium antagonists (Zanchetti et al., 1998).

1.6.3 Non-invasive assessment of vascular wall function

Healthy arteries are elastic and with each systolic pulse wave they distend and then recoil during diastole (O'Rourke and Mancia, 1999). This results in a distension wave form the characteristics of which will be determined by structural aspects of the arterial wall, including wall thickness, the relative presence of elastic and non-elastic connective tissue, the reflection of pulse waves from the distal vasculature and the release of vasoactive substances. In addition to assessing the physical characteristics of arterial size and structure, these dynamic aspects of vascular function can also be determined. Sophisticated methods which use real time ultrasound have been developed to automatically detect and track movements of arterial walls throughout the cardiac cycle and determine distension waveforms over a number of cycles (Hoeks et al., 1992; Hokanson et al., 1972). The use of high sampling frequencies facilitates accurate tracking of the movement of both the near and far arterial wall. Using this method abnormalities of compliance and distensibility have been demonstrated in patients with risk factors for cardiovascular disease from an early stage (Kool et al., 1995b; Salomaa et al., 1995).
A characteristic of less compliant (stiffer) arteries is that the arterial wall absorbs less kinetic energy and they transmit the pulse wave at a faster rate. A number of techniques have therefore been developed to measure pulse-wave velocity using either Doppler ultrasound or plethysmography and measurements of pulse wave velocity correlate well with the presence and risk of cardiovascular disease (Blacher et al., 1999; Lehmann et al., 1998).

Arterial stiffness is likely to largely represent a passive response of the arterial wall to the pulse wave and, in their greatest part, be determined by structural characteristics of the arterial wall and the distal vasculature. Increased stiffness may be a normal part of ageing (Benetos et al., 1993; Smulyan et al., 1983) but also seems to occur in patients with risk factors for cardiovascular disease including hypertension (Benetos et al., 1993), diabetes (Salomaa et al., 1995), smokers (Kool et al., 1993) and patients with hypercholesterolaemia (Barenbrock et al., 1995; Hopkins et al., 1993). The relation of abnormalities of arterial stiffness to the pathogenesis of atherosclerosis however is not clear and the role of measurements of arterial distensibility in predicting cardiovascular disease and its potential for reversal need to be clarified.
1.7 ASSESSMENT OF ENDOTHELIAL FUNCTION IN HUMANS

IN VIVO

The multifunctional nature of vascular endothelium presents a number of potential markers of endothelial function. To be useful in a clinical environment, the test must be sensitive (i.e., separate normal from abnormal endothelial function) and specific (low rate of false positives), it must be tolerable and of low risk to the subject. Current methods, can be broadly divided into three groups; methods which measure the vasodilator capacity of the endothelium as a direct bioassay of endothelial function; the assessment of vascular permeability; and the measurement of endothelium-derived substances in the plasma.

1.7.1 Assessment of endothelium-dependent dilatation

Techniques for the direct or indirect measurement of endothelial-dependent dilatation have been described for a wide variety of vascular beds ranging from large coronary, pulmonary and systemic conduit arteries to resistance arteries, and veins. All of these methods rely on the same basic principle; contrasting the effect of stimuli which act on the endothelium to cause release of, one or more, endothelium-derived relaxing factors with that of the direct effect of smooth muscle dilators. Structural analogues of L-arginine, which inhibit nitric oxide synthesis, are used to assess the dependence of basal and stimulated vasodilatation on nitric oxide synthesis.

1.7.1.1 Invasive methods in large arteries

An invasive catheter based technique for examining coronary artery endothelial-dependent vasomotion, in patients undergoing diagnostic angiography, was first described by (Ludmer et al., 1986). Quantitative vascular angiography or intravascular ultrasound (Reddy et al., 1994) are used to measure coronary diameter changes in response to serial, incremental, infusions of vasoactive substances (acetylcholine, substance P or bradykinin) or increases in blood flow (induced by pacing or distal infusion of vasodilator substances). The integrity of vascular smooth muscle is assessed by infusing endothelium-independent dilators (glyceryl trinitrate, sodium nitroprusside or calcium antagonists). Basal nitric oxide synthesis can be
determined by infusing analogues of L-arginine. Modifications of this technique have been used to assess endothelial function in the pulmonary circulation (Celermajer et al., 1993b) and in systemic conduit arteries (Liao et al., 1991). Endothelial dysfunction as manifest by paradoxical vasoconstriction to acetylcholine has been demonstrated at sites of atherosclerotic plaque (Ludmer et al., 1986). In patients with clinical coronary vascular disease, abnormal coronary vasodilatation in response to increased coronary blood flow (Nabel et al., 1990), exercise (Gordon et al., 1989) and mental stress has been reported, even in angiographically normal arteries. Improvement in coronary endothelial function, as seen following cholesterol reduction (Anderson et al., 1995; Treasure et al., 1995) and angiotensin-converting enzyme inhibition (Mancini et al., 1996) underlines the role that the assessment of vascular function may have in judging the benefit of such treatments. Whilst assessment of coronary endothelial function is likely to be the gold standard for this approach, the invasive nature of current techniques and potential risks involved, limits its use to patients with symptoms undergoing diagnostic tests. The study of endothelial function in cohorts of young subjects with risk factors, but no clinical evidence of vascular disease, demands safer non-invasive methods for determining vascular function.

1.7.1.2 Non-invasive tests of large vessel endothelium-dependent dilatation

Increased blood flow is the major physiological stimulus for release of vasodilators by the endothelium and this fact has been utilised in the development of a novel non-invasive technique, which uses high-resolution external vascular ultrasound to measure vasoreactivity in large peripheral systemic arteries (Anderson and Mark, 1989; Celermajer et al., 1992). A brief episode of reactive hyperaemia, induced by several minutes of distal ischaemia, is used as the endothelium-dependent stimulus to vasodilatation and this is contrasted with the vasodilator effect of glyceryl-trinitrate administered sublingually. The method is accurate and reproducible (Sorensen et al., 1995) and correlates well with invasive measures of endothelial function in the coronary arteries (Anderson et al., 1995). This method has been used to study brachial, radial and femoral artery vascular function in subjects as young as seven years of age, with a wide range of risk factors for vascular disease (Celermajer et al., 1992; Celermajer et al., 1996; Clarkson et al., 1997). The non-invasive and portable
nature of the technique makes it ideally suitable for applications in population based studies examining the impact of risk factors on vascular function (Leeson et al., 1997) and serial measurements in interventional studies (Clarkson et al., 1996a).

1.7.1.3 Assessment of resistance vessel function.

Vascular endothelium also plays a critical role in the control of resistance vessel tone and blood pressure (Vallance et al., 1989). These small arteries and arterioles (< 200 μM), which are largely found in skeletal muscle, are difficult to visualise directly and techniques for assessing resistance vessel endothelial function have largely relied on measurements of changes in blood flow as an indirect measure of resistance vessel vasodilatation.

In coronary arteries, changes in coronary blood flow in response to infusion of vasoactive agents have been assessed at cardiac catheterisation by Doppler (Kuo et al., 1992; Zeiher et al., 1991). A fine guide wire, with a peizo-electric crystal mounted at its distal end, is inserted into the coronary artery under fluoroscopic control, facilitating the direct measurement of blood flow velocity. Abnormalities of coronary blood flow, which may underlie episodes of myocardial ischaemia, have been demonstrated in patients with risk factors for atherosclerosis (Zeiher et al., 1991) and in patients with microvascular angina (Egashira et al., 1993; Quyyumi et al., 1992). However, as with the assessment of epicardial endothelial function, this technique is limited in its clinical use by its invasive nature. Recently myocardial perfusion defects in response to intra-coronary acetylcholine have been demonstrated, non-invasively, using positron emission tomography (Schwaiger and Hutchins, 1995) and correlate closely with angiographic and Doppler measurements of endothelial function (Hasdai et al., 1997). The development of endothelial-dependent dilators which may be given systemically (e.g., pulmonary inhalation of isoprenaline) might provide a new approach to testing coronary vasomotion.

In the peripheral circulation, resistance vessel function has also been tested using venous occlusion plethysmography (Creager et al., 1990; Panza et al., 1990). The principle of the technique is straightforward: forearm distension, during occlusion of
venous return, is used to determine blood flow into the forearm (Benjamin et al., 1995; Whitney, 1953). The hand, which contains few resistance vessels, is usually excluded from the circulation by inflation of a distal occluding cuff, placed at the wrist, to a suprasystolic pressure (Kerslake, 1949). Venous return is briefly prevented by inflating a pneumatic cuff placed on the upper arm (∼40 mmHg) and the increased tension in a calibrated mercury in-silastic strain-gauge used to determine the rate of forearm distension. To test the direct effect of vasoactive substances, on resistance vessel tone, the brachial artery is canulated, under local anaesthetic, facilitating the local infusion of drugs at subsystemic doses. In view of its minimally invasive nature, this technique has been widely applied to assess the role of endogenous mediators and in the study of the pathophysiology of vascular disease. Drugs can be infused at subsystemic doses and dose response curves determined. The contralateral arm in which no intervention takes place acts as a useful control for changes in blood flow related to neurogenic and cardiac factors outside the forearm. However, the technique is invasive and its use limited to the study of small cohorts of well-defined subjects. Moreover, the relevance of abnormalities of resistance vessel function to atherosclerosis in large arteries is unclear (Liao et al., 1991).

1.7.1.4 Assessment of vasomotor tone in other vascular beds

In the venous circulation, the effect of endothelial-dependent and -independent vasoactive drugs can be assessed in dorsal hand veins (Aellig, 1994; Bhagat et al., 1996). The vein is kept at a constant distending pressure by a proximal ‘congesting cuff’ inflated to 40mmHg. A lightweight magnetized probe is placed over the summit of the vein and passed through the core of a linear variable differential transformer. Displacement of the probe results in a linear change in voltage generated by the transformer which reflects changes in the diameter of the vein. Under constant distension pressure the diameter of a vessel is determined by the state of contraction of the smooth muscle (Laplace's relationship) and increased vasomotor tone results in contraction. Using this method the arteriovenous profile of drugs can be studied and the effect of noxious substances, which could not be infused systemically or into the forearm, on endothelial function in an isolated vein can be assessed (Bhagat et al., 1996).
The vasomotor properties of skin arterioles have been assessed using laser Doppler or dynamic capillaroscopy (Fagrell, 1985). Acetylcholine or other vasoactive agents are infused locally either by injection or iontophoretically. Whilst abnormalities of skin perfusion have been demonstrated which might be particular relevant to microangiopathy as seen in diabetes, the role of the endothelium is not clear (Belcaro et al., 1989; Morris and Shore, 1996; Noon et al., 1998).

1.7.2 Vascular permeability

A critical role of vascular endothelium is the maintenance of a selective barrier between circulating blood and the tissues. Loss of this function may result in oedema and damage to parenchymal tissues. In diabetes, injury to capillary endothelium results in leakage of macromolecules into the glomerular effluent. The detection of microalbuminuria, in these patients, is an important early sign of renovascular disease and associated with an increased risk of large vessel atherosclerosis (Stehouwer et al., 1992). Increased vascular permeability can be assessed directly by examining the leakage of fluorescent dyes from retinal vessels (Chahal et al., 1985; Chalal et al., 1986). Whilst the precise mechanisms which underlie increased permeability are unclear, it is thought to have an important role in the pathogenesis of diabetic retinopathy.

1.7.3 Blood markers of endothelial function

Healthy endothelium is a metabolically active tissue and synthesises a multitude of factors, which might be measured in the blood and act as a marker of endothelial activity. These include vasoactive molecules, thrombotic factors, regulators of fibrinolysis, growth factors and inhibitors and inflammatory mediators (Table 1.7.1). The usefulness of any given substance as a measure of endothelial metabolic activity will depend on its stability and concentration in plasma and the availability of a sensitive assay with which to measure it, and its specificity for the endothelium. The mode of clearance and the plasma half-life are important determinants of the length of time that will be necessary before significant changes related to alterations in endothelial function can be demonstrated.
Table 1.7.1 Partial list of factors synthesised by vascular endothelium

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples</th>
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<tr>
<td>Vasoactive molecules</td>
<td>Nitric oxide</td>
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<tr>
<td></td>
<td>Endothelin</td>
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<tr>
<td></td>
<td>Angiotensin II</td>
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<td>Endothelium-derived hyperpolarization factor</td>
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<td>Arachidonic acid metabolites</td>
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<td>Thrombotic factors</td>
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<td>Von Willebrand factor</td>
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<td>Fibrinolytic factors</td>
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<td>Plasminogen activator inhibitor type-1</td>
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<td>Growth factors</td>
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<td>Leucocyte adhesion molecules</td>
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<td>Monocyte chemotactic peptide-1</td>
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<td>Granulocyte-macrophage colony stimulating</td>
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1.7.3.1 Measurement of nitric oxide metabolism

Nitric oxide concentrations may be determined directly using chemiluminescence in exhaled breath or in the headspace above biological samples (Archer, 1993). The method relies on measuring the luminescence which occurs when NO reacts with ozone and is sensitive and specific. To quantify NO synthesis, nitrite and nitrate can be reduced to NO by exposure to acid and potassium iodide. Recently the development of sensitive electrochemical probes have facilitated the direct in vivo measurement of NO (Vallance et al., 1995) and used to demonstrate the diffusion of NO from the endothelium to smooth muscle (Malinski and Taha, 1992). As with chemiluminescence, the probe detects the electrical signal generated when NO is oxidised at the anode electrode. These may be mounted on an intravenous cannula and potentially used to monitor changes in NO synthesis within vascular beds.

Nitric oxide is synthesised from the amino acid L-arginine, resulting in a single molecule of the nitric oxide radical and L-citruline. Once formed NO is rapidly inactivated resulting in the formation of peroxynitrite and various nitrosothiol compounds but eventually being degraded into nitrite and the stable nitrate radical. The concentration of a number of these intermediate compounds, some of which may have biological activity, can be measured in the plasma (Leone et al., 1995). The half-life of nitrate in the plasma is relatively long and either plasma or 24 hour urinary nitrate levels have been used as a marker of whole body NO synthesis (Baylis and Vallance, 1998; Lyons et al., 1997). Subjects need to avoid a high nitrate intake prior to the assessment and nitrate is measured using high-performance liquid chromatography or high-performance capillary electrophoresis (Leone et al., 1995). Recently the use of radiolabelled L-arginine has facilitated the detection of much smaller fluxes in nitrate and NO metabolism in patients with essential hypertension (Macallan et al., 1997).

Nitric oxide stimulates guanylate cyclase in vascular smooth muscle cells thus increasing cyclic GMP concentrations, some of which escapes from the cell and may be measured in plasma (Moncada et al., 1991). This method however is limited in that a number of molecules other than NO may activate guanylate cyclase and therefore the method lacks specificity and has a high signal to noise ratio.
1.7.3.2 *Thrombotic and fibrinolytic factors*

Von Willebrand factor is thought to largely originate from endothelial cells where it is involved in the binding of platelets and thrombus formation. The potential role of von Willebrand factor as a marker of endothelial cell damage was first proposed by Boneu and colleagues (Boneu *et al.*, 1975). Elevated levels have been demonstrated in patients with diabetes (Greaves *et al.*, 1997; Stehouwer *et al.*, 1995), hypercholesterolaemia (Blann *et al.*, 1997a) and hypertension (Boneu *et al.*, 1978; Kloczko *et al.*, 1995), in smokers (Blann *et al.*, 1997b) and in other clinical settings where endothelial injury is likely to occur (Lip and Blann, 1997). Levels of von Willebrand factor predict the later incidence of cardiovascular morbidity and mortality (Meade *et al.*, 1994). Furthermore, reduction in risk factors reduces the level of von Willebrand factor (Blann *et al.*, 1997a). However, in common with many of the potential blood markers of endothelial function, elevated levels of von Willebrand factor, rather than being a measure of dysfunction are more likely represent enhanced endothelial activation and metabolic activity.

Thrombomodulin is a constitutive membrane protein, which has an important role in cleaving fibrinogen from thrombin and thus converting it from a procoagulant to an anticoagulant (Esmon, 1987). Increased levels of thrombomodulin have been demonstrated in the supernatant of tissue culture after injury to endothelial cells (Ishii *et al.*, 1991). Whilst being almost entirely endothelial in origin, the precise role of circulating soluble forms of thrombomodulin remain unclear. Elevated levels of thrombomodulin have been demonstrated in patients with clinical vascular disease, where they predict the risk of further thrombotic complications (Blann *et al.*, 1997c), though levels are reduced in smokers (Blann *et al.*, 1997b) and the pattern in younger subjects with risk factors is variable (Boffa, 1996).

Plasminogen activator inhibitor type-1 (PAI1) and tissue plasminogen activator have key roles in regulating fibrinolysis and have been associated with the risk of cardiovascular events (Hamsten *et al.*, 1987; Ridker, 1997). Although often cited as endothelial cell markers neither molecule is specific for endothelial cells. The PAI1 gene has a triglyceride responsive site and PAI1 levels are increased in patients with hypertriglyceridaemia.
1.7.3.3 Adhesion molecules

The adhesion of circulating cells to the vascular endothelium is a key step in early atherosclerosis. These processes are mediated by families of endothelial and inflammatory cell adhesion molecules (Adams and Shaw, 1994; Albelda et al., 1994; Cybulsky et al., 1991) of which intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1, P-selectin and E-selectin are the most commonly measured. Of these only E-selectin is specific for endothelial cells. Elevated levels of E-selectin have been demonstrated in diabetes (Cominacini et al., 1995) and hypercholesterolemia (Hackman et al., 1996) and in patients with established coronary and carotid vascular disease (Hwang et al., 1997). However, as with thrombomodulin, levels of E-selectin may be normal in early disease and some risk factor groups (Blann et al., 1997a; DeSouza et al., 1997) and its use as a marker of endothelial activation is unproven. Of the other adhesion molecules, ICAM-1 has consistently been linked with vascular disease. Elevated levels have been demonstrated in hypertension (DeSouza et al., 1997), dyslipidaemia (Hackman et al., 1996), in diabetes (Martin, 1997) and in patients with established atherosclerotic vascular disease (De Caterina et al., 1997; Hwang et al., 1997). Levels of ICAM-1 are an independent risk factor for the myocardial infarction (Ridker et al., 1998). However, whilst elevated levels of ICAM-1 may be a biohumoral correlate of atherosclerosis, they are likely to represent generalised inflammatory activation and their relationship with other more specific markers of endothelial function has not been studied.
1.8 SUMMARY AND AIMS

Cardiovascular disease is a major cause of morbidity and mortality that becomes prevalent from the fourth decade of life. The pathophysiological processes that underlie atherosclerosis however, begin much earlier. The vascular endothelium is now known to perform a wide range of homeostatic functions that help to preserve vascular integrity. Noxious insults damage endothelial cells and result in endothelial dysfunction, either as a protective response or an abnormal reaction, and is thought to be a key event in the initiation of atherogenesis. Assessment of endothelial function might, therefore, provide insight into these early stages of atherosclerosis and allow assessment of the effects of interventions, targeted at retarding atherogenesis form early in its natural history. Many of the different functions of healthy endothelium can be assessed in vivo and provide information on differing aspects of vascular function in different vascular beds. However, the assessment of endothelium-dependent dilatation has been most widely applied. The development of a non-invasive method, which uses high-resolution ultrasound to assess conduit artery responses to flow and exogenous nitrate, has facilitated the study of endothelial function, in vivo, from an age and abnormalities of flow-mediated dilatation have been detected in a wide range of young individuals with risk factors for cardiovascular disease. Understanding the nature of the abnormality which underlies impaired flow-mediated dilatation, its relation to risk factors for atherosclerosis and the cellular events involved will facilitate the development of treatments to retard its progression to clinical cardiovascular disease. The assessment of endothelial function non-invasively will be important in assessing the effects of such treatments.

1.8.1 Aims of this thesis

1. To refine and validate methods for assessing flow-mediated dilatation in vivo.
2. To examine the mechanisms of flow-mediated dilatation and its relation to the dynamics of the flow stimulus.
3. To examine the metabolic and physical determinants of vascular function in a population based cohort, with particular reference to new risk factors.
4. To determine the potential for reversal of endothelial function in individuals at risk of cardiovascular disease in later life.
CHAPTER 2

NON-INVASIVE ASSESSMENT OF FLOW-MEDIATED DILATATION AND ARTERIAL DISTENSIBILITY IN PERIPHERAL CONDUIT ARTERIES: TECHNICAL ASPECTS
2.1 Overview

Although not clinically emergent until later in life the pathophysiological processes which result in vascular disease evolve over a period of many years (Stary, 1989). Early atherosclerotic lesions are dynamic and mutable structures and complete resolution can occur with appropriate modification of risk factors and other proatherogenic influences (Stary, 1979). Intuitively therefore, interventions targeted at preventing cardiovascular disease might have their greatest effect if instituted at this early stage. In the short term, the impact of this approach will have to be tested against non-clinical markers of disease progression rather than cardiovascular morbidity and mortality. Such tests will need to reflect sensitively and specifically the presence of early atherosclerosis and its progression or regression over time and following intervention. They will need to be safe, relatively painless and ideally non-invasive and applicable to population studies. As discussed in Section 1.4, abnormalities of vascular function, and particularly those relating to the endothelium, are an early event in atherogenesis and are detectable using tests that satisfy many of these criteria. The work presented in this thesis focuses on the non-invasive assessment of endothelial function. In addition, in a number of studies arterial distensibility is measured. This chapter describes the general protocol for assessing flow-mediated dilatation and arterial distensibility in peripheral conduit arteries and discusses technical aspects and recent developments in the techniques that might influence their use in clinical studies.

2.2 Non-invasive assessment of endothelial function

The assessment of flow-mediated dilatation (FMD) in peripheral conduit arteries, using high-resolution ultrasound, is a non-invasive technique that has been developed at Gt. Ormond St. Hospital and been widely adopted in the assessment of endothelial function. The technique is painless, safe and totally non-invasive and has been used in children as young as 5 years of age. Previous studies have demonstrated the accuracy and reproducibility of this methodology (Sorensen et al., 1995). Endothelial function changes with age in men and women and shows a pattern of decline, which
mirrors cardiovascular risk profiles (Celermajer et al., 1994a). Classical risk factors disturb endothelial dysfunction from the first decade of life. Abnormal FMD has been demonstrated in active (Celermajer et al., 1993) and passive cigarette smokers (Celermajer et al., 1996), children with hypercholesterolaemia (Sorensen et al., 1994), diabetes (Clarkson et al., 1996b; Goodfellow et al., 1996) and hypertension, with a dose dependent influence and interaction of risk factors comparable to that seen in epidemiological studies of later cardiovascular events (Celermajer et al., 1994b). Novel influences are also associated with impaired FMD, including hyperhomocysteinaemia (Celermajer et al., 1993a), systemic inflammation (Hingorani et al., 2000) and childhood vasculitis (Dhillon et al., 1996). There is a relationship between birth weight and FMD, which manifests by the end of the first decade of life (Leeson et al., 1997). There is a genetic influence on endothelial function based on demonstration of abnormal FMD in offspring in families with a history of premature atherosclerosis even in the absence of environmental risk factors (Clarkson et al., 1997). Transient impairment of endothelial function occurs in response to systemic inflammation (Hingorani et al., 2000), mental stress (Ghiadoni et al., 2000) and metabolic challenges (Bellamy et al., 1998; Vogel et al., 1997). Recently FMD has been used as a surrogate endpoint in interventional clinical trials. Improvement has been demonstrated after l-arginine therapy (Clarkson et al., 1996), anti-oxidant vitamins (Hornig et al., 1998), exercise training (Clarkson et al., 1999) and cholesterol reduction (Mullen et al., 2000). As endothelial dysfunction is an early event in atherosclerosis it has been argued that such improvements represent a beneficial effect on the pathogenesis of vascular disease (Mullen et al., 1997).

2.2.1 Description of general protocol

This method follows the general protocol described by Celermajer et al., (1992) and developed within the Vascular Physiology Unit at Gt. Ormond St. Hospital. High-resolution ultrasound is used to assess conduit artery dilator responses to endothelium dependent (local hyperaemia) and independent (glyceryl trinitrate) stimuli. All studies are performed in a quiet temperature controlled laboratory. Subjects lay at rest for at least 10 minutes prior to the beginning of the study and remain supine throughout. Blood pressure and the electrocardiogram are monitored.
continuously. The target artery (brachial or radial) is imaged in longitudinal section using a standard ultrasound system (Acuson 128XP/10 with a 7 MHz linear array transducer). The operator selects a straight segment of the artery so that it is clearly visible over a 2-3cm length. The image is then magnified using a resolution box function and the operating parameters set to optimise the lumen/arterial wall interfaces (Figure 2.2.1). The ultrasound focus is normally set to the level of the anterior wall in view of the greater difficulty in imaging this interface. Once a stable and clear image is achieved the transducer position is fixed using a stereotactic clamp. Fine adjustments, in the coronal and sagittal planes, to correct for small translational movements can thereafter be made by turning micrometer screws on the clamp base. The transducer is normally placed along the artery such that a longitudinal image is seen. However, in this plane translational movements of the artery relative to the transducer may introduce errors. Theoretically, this can be counteracted by using a skewed plane, whereby, after small translational movements the artery will remain clearly visible, but at the expense of some vessel wall information (Figure 2.2.2). The reproducibility of arterial measurements might be improved by this technique (Stadler et al., 1996). In practice however, the use of a stereotactic clamp, with adjustments in more than one plane, allows accurate tracking of small translational movements whilst maintaining maximum vessel wall information. In the experiments described in this thesis therefore arteries were all imaged in a longitudinal plane.

Blood flow velocity is estimated using pulsed wave Doppler with the cursor set at $70^\circ$ to the longitudinal axis of the artery and the range gate [1.5 mm] in the center of the artery. Resting volumetric blood flow can be calculated by multiplying the velocity time integral of a single pulse wave (corrected for angle) by the heart rate and vessel cross sectional area. Scans are recorded onto Super VHS videotape for archiving purposes and the later measurement of flow.
Figure 2.2.1 B-mode image of the brachial artery in a longitudinal plane. With the transducer held by a rigid clamp, adjustable by micrometer screws, high quality images can be maintained throughout studies.

Figure 2.2.2 Different planes for imaging arteries. Imaged in longitudinal section (upper panel), the most data on the arterial wall is acquired but the image is relatively sensitive to translational motion. In a skew plane (lower panel) arterial wall data is reduced but with small movements the maximal arterial diameter should still be clearly defined.
2.2.1.1 Flow mediated dilatation (FMD)

Brachial artery diameter is determined at rest and after a brief period of reactive hyperaemia induced by inflating a pneumatic tourniquet placed around the distal limb to 300mmHg and its rapid release after a prespecified length of time (normally 5 minutes) (Figure 2.2.3). The increase in blood flow during reactive hyperaemia is determined using pulsed wave Doppler at predefined intervals (see below). Hardcopy images of the artery are taken and notes made of the subjects arm and the transducer position enabling precise reproduction of conditions and measurement of the same segment of artery at subsequent visits.

2.2.1.2 Endothelium-independent dilatation

At least 5 minutes rest following the assessment of FMD is allowed to facilitate vessel recovery. A further baseline scan is performed and then a single dose (normally 400 μg) of glyceryl trinitrate (GTN), as a pump action spray, is administered sublingually and the response to this endothelium-independent dilator assessed after three to four minutes (Figure 2.2.3).

2.2.2 Choice of artery

The artery should be accessible and relatively straight such that it can be imaged with the subject in a comfortable position over prolonged periods of time. The distal limb should be accessible to allow positioning of the occluding cuff. The size of the artery is also important as small arteries can be difficult to image whilst the normal changes in arterial diameter seen in large arteries approach the limits of resolution of the ultrasound system (Clermajer et al., 1992). In practice therefore, arteries between 2-5 mm internal diameter are acceptable and only the brachial, radial and femoral artery generally satisfy these criteria. The brachial artery is the most appropriate artery in the majority of adult subjects, whilst in younger children the femoral artery can be used. In large adults and when the proximal infusion of drugs is necessary the radial artery may be used. When assessing arterial elasticity the carotid artery (Hoeks et al., 1990; Hokanson et al., 1972) or the brachial artery (Leeson et al., 2000) have most frequently been used.
2.2.3 Measurement of arterial diameter

The accuracy of ultrasonic measurement of arterial diameter is determined by factors related to the characteristics of the image and the limitations of the ultrasound system. Good quality images, which precisely define the anterior and posterior vessel walls, will generally provide the most accurate measurements. The accuracy and reproducibility of this technique in humans has previously been established (Sorensen et al., 1995). The resolution of the ultrasound system is determined by the wavelength of the ultrasound beam. Using a 7 MHz probe and presuming the speed of sound through the tissues is $\approx 1540$ metres per second, the wavelength of the ultrasound beam is $\approx 0.2$ mm and the axial resolution (ability to differentiate two juxtapositioned objects) is approximately 0.1 mm. However, for the assessment of arterial dilatation, other factors including television line width, and the echo pattern are important. A number of different techniques have been used for the measurement of arterial diameter (Figure 2.2.4).

2.2.3.1 B-Mode calipers

This method which was described by Celermajer and colleagues (1992) uses the ultrasonic calipers incorporated into the ultrasound system to manually determine arterial diameter. A single point on the artery is chosen and measurements made on consecutive end-diastolic frames. The artery is measured between the opposing M-lines of the anterior and posterior wall. This method has the advantage of not requiring any specialised equipment and as measurements are made directly from the B-mode image allows the observer to visually correct for any translational movements. However, to avoid introducing bias it is necessary to blind the observer to the timing of the scans, and the method is time consuming and labour intensive limiting the frequency with which repeated measurements can be made. As a result researchers have usually chosen a specific time point i.e. 55 – 65 seconds after release of the occluding cuff at which vasodilatation is assessed (Celermajer et al., 1992). Thus maximal dilatation may be missed if it occurs either earlier or later than the selected time window. Furthermore a single measurement at a single site on the artery is likely to be less accurate than taking the average of a number of measurements especially if taken over a length of the artery. The use of B-mode
callipers has therefore been largely superseded by semi-automatic analytical techniques.

2.2.3.2 A-mode wall tracking

In addition to assessing arterial distensibility (section 2.5), A-mode wall tracking devices may be used to determine end-diastolic arterial diameter. The resolution of these systems is determined by the pulse repetition frequency (the number of pulses per second) and not by the frequency of the ultrasound transducer. With radio frequency (RF) signals this can be as high as 300 per second and give a theoretical resolution of as little as 3 μm. The accuracy and reproducibility of these methods has been reported by a number of groups with coefficients of variation of diameter measurements in the order of 2.5 to 4.5% and distensibility of 8% in peripheral arteries (Kool et al., 1994a). This method has many advantages over using ultrasound callipers to assess artery diameter. Firstly it is semi-automated in that the observer is only required to place the sample volume cursors at the arterial wall interfaces and might reduce the chances of observer bias. The RF data may also be stored off line and archived for later analysis. The methodology however remains limited in that data can only be acquired over relatively short time spans (about 5 seconds) and thus the whole time course of vascular reactivity in response to stimuli can not be assessed. Secondly serial studies require very accurate reproduction of the image and placement of the sample cursors at the same position in relation to the arterial interfaces. Whilst with B-mode visual checks can be made the RF data has relatively few visual clues as to the presence of artefact particularly related to phase interference from closely placed reflectors.

2.2.3.3 Quantitative vascular ultrasound

The intrinsic limitations of using either ultrasound callipers or a wall tracking device to measure arterial diameter has led to the development of a number of automated computer based technologies to enhance the assessment of vascular reactivity (Stadler et al., 1996). Sequential B-mode images of the target artery are digitised and stored on a personal computer for off line analysis. The acquisition rate can be altered to acquire images at a fixed time interval (normally 3 - 5 seconds) or gated with the ECG to acquire an end diastolic image for each heartbeat. The rate at which
images can be stored depends on the speed of the interface (PCI vs. ISA) and processor and the amount of memory available in which to cache data. Images are stored on the hard disk of the personal computer along with data on the source of the image and the time of acquisition in relation to the first frame. Pulsed wave Doppler is used to continuously measure blood flow and the ultrasound system set to update the B-mode image with each QRS complex.

Each frame is analysed semi-automatically using an edge detection algorithm, similar to that developed for assessing coronary artery diameter at angiography. The operator initially identifies a 2 - 3 cm segment of the artery and selects preliminary edge points near the anterior and posterior walls. The true edges of the arterial walls are then located by gradient-based edge detection in proximity to the user selected preliminary edge points. The arterial diameter is estimated from the detected edges by a least-squared-error model fit, where the anterior and posterior walls are modelled as parabolas with the same curvature but independent vertices (Stadler et al., 1996). The operator selected preliminary edge points are reused for subsequent images in the time series, or can be adjusted as a group to compensate for small translational movements of the artery.

This method has a number of advantages over previous methods. Firstly as a 2 - 3 cm section of artery is analysed the measurements are more likely to be representative of the whole artery than if measurements at a single point are made and will be less sensitive to small changes in the image at any one site. Secondly a continuous assessment of arterial diameter can be made throughout the experimental protocol and allow a more complete assessment of arterial function and description of abnormalities. Baseline diameter can be calculated on the basis of a mean diameter over a prolonged period (normally 1 - 2 minutes ≈ 12 to 40 frames) and take into account any natural variability in arterial tone over this time. For the work presented in this thesis two different programs have been used (BHFacq [courtesy of Dr Lees and Stadler, Boston Heart Foundation; Information Integrity, Boston USA] though both rely on the same edge detection and image analysis algorithms.
Figure 2.2.3 Schematic diagram of the general protocol for studying flow mediated dilatation and dilatation to GTN.
Figure 2.2.4 Methods for measuring arterial diameter.
2. A-mode tracking device: tracks arterial movement along a single A-mode beam and measures the distension waveform and diameter (middle panel).
3. Edge detection system: This system automatically measures arterial diameter over a length of artery and allows the full time course of vasoreactivity to be assessed (lower panel).
2.2.4 Data analysis and presentation

2.2.4.1 Flow-mediated dilatation

Dilatation is usually presented as a ratio of the baseline measurement (Celermajer et al., 1992). When ultrasound callipers or a wall-tracking device have been used normally only a single measurement at baseline and in response to the stimulus is made, at a predefined time point. With continuous acquisition of data both the baseline measurement and the measurements of arterial response can be based on an average of 2 or more consecutive images (normally 20 frames over a 1 minute baseline period and 3 - 4 frames over 12 - 15 second intervals thereafter). Dilatation at each time point can then be calculated relative to the preceding baseline.

Thus, FMD can be calculated as:

\[
\text{Flow mediated dilatation} = \frac{D2 - D1}{D1} \times 100\% 
\]

Where \(D1\) = baseline diameter and \(D2\) = the diameter of the artery in response to reactive hyperaemia.

Alternatively diameter might be expressed as an absolute change from baseline i.e.

\[
\text{Flow mediated dilatation} = D2 - D1\ (mm)
\]

The response to GTN can be calculated in a similar fashion.

In previously published studies, FMD has been calculated on the basis of only 2 measurements at baseline and at a fixed time point (usually 55 – 65 seconds) after release of the cuff (Celermajer et al., 1992). Whilst this method has been sensitive enough to detect significant differences between risk factor groups from a young age, it remains unclear whether additional physiological measurements of FMD would enhance these results. With continuous measures of arterial diameter the complete time course of FMD can be assessed so that additional parameters including the maximal dilatation (\(\text{FMD}_{\text{max}}\)), the time to peak dilatation (\(t_{\text{max}}\)), the gradient of the initial dilatation response (\(\text{Ddil}/\text{Dt}\)) and the area under the curve of the whole
dilatation time course (FMD\textsubscript{AUC}) can be calculated using a triangulation method (Figure 2.2.5). Further research will be necessary to determine which measure most precisely define abnormal endothelial function and the mechanisms that underlie these defects.

2.2.4.2 Blood flow and reactive hyperaemia

Blood flow is calculated from the videotaped pulsed Doppler waveform. The profile of the velocity waveform over a single beat is traced, at each of a number of predetermined time points (normally 1 or 2 at baseline, 1 during cuff inflation and at 15 second intervals during reactive hyperaemia) and the velocity time integral (VTI) for each beat is automatically calculated (Figure 2.2.6). Volume blood flow may be calculated by correcting the VTI for the angle of incidence (70°) and heart rate and blood vessel cross sectional area.

\[
\text{Blood flow (mls/min)} = \text{VTI (m/s)} \times \cos 70^\circ \times \text{heart rate} \times \pi \times \left(\frac{\text{diameter}}{2}\right)^2
\]

This is likely to overestimate blood flow by as much as 30% as lower velocity flows at the periphery of arteries are not accounted for. However, the error is likely to remain constant and relative changes in blood flow in an individual are likely to be accurate, as will comparisons between individuals and different groups.
Figure 2.2.5 Using an edge detection system the complete time course of flow-mediated dilatation can be defined. This facilitates the assessment of multiple different parameters of the dilatation response including the maximum dilatation ($FMD_{\text{max}}$), the time to maximum dilatation ($t_{\text{max}}$), the gradient of the dilatation response ($Ddil/ Dt$) and the area under the curve ($FMD_{\text{AUC}}$) of the time dilatation curve.
Figure 2.2.6 Measurement of blood flow using pulsed wave Doppler. At rest (upper panel) blood flow is largely during systole returning to baseline during diastole. During reactive hyperaemia (lower panel) maximum blood flow velocity is only slightly increased but a marked increase in diastolic flow occurs. The velocity time integral (VTI) for a single beat is measured during the different conditions.
2.3 Relationship between flow and dilatation

2.3.1 Introduction

In determining the blood flow stimulus to the endothelium, reactive hyperaemia has generally been reported as a ratio of baseline blood flow.

\[
\text{Reactive hyperaemia (\%) = } \frac{\text{Peak blood flow}}{\text{Baseline blood flow}} \times 100
\]

However, using this approach a significant relationship between reactive hyperaemia and the subsequent dilatation has not established (Leeson et al., 1997) and this has led some observers to question whether flow is indeed the stimulus for vasodilatation (Bhagat et al., 1997). For such a relationship to be established a temporal and dose dependent association between changes in blood flow and dilatation will need to be demonstrated. A number of factors might be responsible for the apparent discrepancy between measures of reactive hyperaemia and arterial dilatation. Reactive hyperaemia and subsequent conduit artery dilatation are both highly dynamic responses with a time shift of approximately one minute in their peak action. The data for both responses can be presented in a number of different ways, which might, in itself, alter relationships. For instance, reactive hyperaemia is normally presented as the ratio of peak blood flow to baseline blood flow. However, this method is potentially flawed, as baseline blood flow is highly variable and therefore percentage reactive hyperaemic blood flow will be largely determined by the baseline blood flow. The mechanism(s) whereby the endothelium senses blood flow are not understood but might involve sensing of volume blood flow, the rate of change in blood flow or the integral of blood flow over time. Furthermore, the stimulus to the endothelium is likely to be related to an increase in shear stress (as opposed to volume blood flow) which is largely determined by blood flow velocity (Brands et al., 1995). As shear stress is inversely proportional to the cubed root of the radius of the vessel, the reduction in shear stress which occurs as a result of physiological arterial dilatation will be of relatively small size. Thus for a 10% increase in diameter, shear stress will only be reduced by \( \approx 2.5\% \). In contrast, a doubling in blood flow velocity will proportionally increase shear stress by the same factor. Thus
a ratio of the peak increase in volume blood flow might, in itself, be a poor
determinant of the subsequent dilatation. Similarly, although FMD is normally
presented as the maximum increase in diameter as a ratio of the baseline, other
parameters of the flow envelope might more accurately reflect the endothelial
response to blood flow and NO bioavailability.

To address these issues the relationship between parameters of reactive hyperaemic
blood flow with differing durations and intensities and the complete time course of
the subsequent brachial artery dilatation was assessed in healthy volunteers.

2.3.2 Specific methods

Eight healthy volunteers were recruited. Brachial artery dilatation was assessed in
response to reactive hyperaemic blood flow induced by cuff occlusion times of 2, 3,
5 and 8 minutes. Each scan was performed by the same operator but in a random
order using an edge detection algorithm to measure the complete time course of
FMD (section 2.2.4.3).

For each scan maximal FMD (FMD_{max}), absolute FMD (FMD_{abs}) the time to
maximal dilatation (t_{max}), the area under the curve of dilatation (FMD_{AUC}) and the
slope of the dilatation/time curve (Ddil/Dt) were calculated. Blood flow velocity was
measured at baseline at 5 and 10 seconds after release of the occluding cuff and
thereafter at 15 second intervals to a maximum of 90 seconds. The peak blood flow
velocity \( V_{Tl_{max}} \) and the ratio of blood flow for each time point was calculated with
reference to baseline blood flow, the AUC of absolute blood flow velocity (VTI_{AUC})
and of the ratio of blood flow velocity was calculated for each scan. The slope of the
degradation of blood flow velocity was calculated (DVTI/Dt). Pearson bivariate
correlation coefficients were used to assess which flow variables were most closely
associated with parameters of FMD.
2.3.3 Results

Complete data was available for all scans on all subjects. The reactive hyperaemia flow envelopes are shown in Figure 2.3.1. Reactive hyperaemic blood flow was maximal at 5 seconds after the cuff release 2 or 3 minutes of distal forearm ischemia nad at 10 seconds after 5 or 8 minutes. Increasing duration of forearm ischemia, above 3 minutes, did not significantly increase peak reactive hyperaemic blood flow (Figure 2.3.2). However, there was a dose dependent increase in the duration of hyperaemic blood flow such that blood flow velocity was increased up to 75 seconds after cuff release and the AUC of the flow envelope was significantly increased (P = 0.01) and the slope of flow degradation significantly decreased (P = 0.001) (Figure 2.3.2).

Similarly increased occlusion time was associated with a stepwise increase in $FMD_{max}$ ($P < 0.001$) (Figure 2.3.3), $Ddil/dt$ ($P < 0.001$) and $FMD_{AUC}$ ($P < 0.001$) but had little influence on the $t_{max}$ ($P = 0.3$) or the $FMD_{abs}$ ($P = 0.4$).

The correlation coefficients between flow variables and parameters of dilatation are shown in Table 2.3.1. $FMD_{max}$ was significantly correlated with VTI at 10 seconds after cuff release ($r = 0.4$, $P = 0.02$), $DVTI/Dt$ ($r = -0.4$, $P = 0.04$), $VTI_{AUC}$ ($r = 0.3$, $P = 0.03$). Although $FMD_{max}$ was not associated with baseline blood flow or $VTI_{max}$, there was a significant association with the percentage peak reactive hyperaemia ($r = 0.4$, $P = 0.03$). Similar associations were seen between $FMD_{AUC}$ and reactive hyperaemia. (Table 2.3.1) but there was no association between $t_{max}$ and any flow variable. Correction of VTI for subjects heart rate did not significantly improve the relationship between the assessment of blood flow and FMD.
Figure 2.3.1 Flow envelope of reactive hyperaemia during different lengths of forearm ischaemia.
Figure 2.3.2 Effect of different lengths of forearm ischaemia on peak reactive hyperaemia (upper panel) and the area-under the time/VTI curve (AUC) (lower panel). Beyond two minutes there was little increase in peak reactive hyperaemia but a significant increase in the AUC of time/VTI curve.
Figure 2.3.3 Effect of different lengths of forearm ischaemia on maximum flow-mediated dilatation.
### Table 2.3.1 Pearson correlation coefficients for reactive hyperaemic and dilatation parameters in 8 healthy subjects.

<table>
<thead>
<tr>
<th></th>
<th>$\text{xFMD}_{\text{abs}}$</th>
<th>$\text{xFMD}_{\text{max}}$</th>
<th>$\text{FMD}_{\text{AUC}}$</th>
<th>$\text{Ddil/Dt}$</th>
<th>$t_{\text{max}}$</th>
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<tbody>
<tr>
<td>$\text{FMD}_{\text{max}}$</td>
<td>0.97 $\dagger$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{FMD}_{\text{AUC}}$</td>
<td>0.95 $\dagger$</td>
<td>0.97 $\dagger$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{Ddil/Dt}$</td>
<td>0.88 $\dagger$</td>
<td>0.89 $\dagger$</td>
<td>0.84 $\dagger$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$t_{\text{max}}$</td>
<td>0.11</td>
<td>0.14</td>
<td>0.17</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>$\text{VTI}_{\text{bl}}$</td>
<td>-0.18</td>
<td>-0.15</td>
<td>-0.25</td>
<td>-0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>$\text{VTI}_{\text{max}}$</td>
<td>0.25</td>
<td>0.26</td>
<td>0.16</td>
<td>0.30</td>
<td>0.24</td>
</tr>
<tr>
<td>$\text{VTI}_{10}$</td>
<td>0.41</td>
<td>0.48 $\dagger$</td>
<td>0.40 $\star$</td>
<td>0.43 $\star$</td>
<td>0.21</td>
</tr>
<tr>
<td>$\text{VTI}_{\text{AUC}}$</td>
<td>0.42 $\star$</td>
<td>0.39 $\star$</td>
<td>0.32</td>
<td>0.42 $\star$</td>
<td>0.32</td>
</tr>
<tr>
<td>$\text{DVTI/Dt}$</td>
<td>-0.46 $\dagger$</td>
<td>-0.44 $\star$</td>
<td>-0.41 $\star$</td>
<td>-0.32</td>
<td>-0.07</td>
</tr>
<tr>
<td>$\text{RH%}$</td>
<td>0.49 $\dagger$</td>
<td>0.49 $\star$</td>
<td>0.52 $\dagger$</td>
<td>0.30</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* $P < 0.05$; $\dagger P < 0.01$; $\dagger P < 0.001$

Abbreviations: $\text{xFMD}_{\text{abs}}$ = absolute FMD; $\text{FMD}_{\text{max}}$ = maximal flow mediated dilatation; $\text{FMD}_{\text{AUC}}$ = area under the curve for dilatation; $\text{Ddil/Dt}$ = gradient of the time/dilatation curve; $t_{\text{max}}$ = time to maximum dilatation after release of the cuff; $\text{VTI}_{\text{max}}$ = maximal blood flow velocity after release of the cuff; $\text{VTI}_{10}$ = blood flow velocity at 10 seconds after release of the cuff; $\text{VTI}_{\text{AUC}}$ = area under the curve of the blood flow envelope; $\text{DVTI/Dt}$ = gradient of flow degradation during reactive hyperaemia; $\text{RH\%}$ = peak percentage increase in reactive hyperaemic blood flow from baseline.

### 3.4 Discussion

These data demonstrate a significant dose dependent relationship between parameters of reactive hyperaemic blood flow and subsequent brachial artery dilatation. Increased blood flow was associated with enhanced $\text{FMD}_{\text{max}}$, an increased $\text{Ddil/Dt}$ gradient and the AUC of FMD. These parameters were closely related and further studies will be required to determine which most sensitively differentiates between risk factor groups. Increasing duration of forearm ischaemia had relatively little effect on the peak intensity of blood flow following release of the cuff and
consequently $VTI_{\text{max}}$ did not significantly predict FMD. In contrast, the duration of hyperaemic blood flow and the AUC were significantly enhanced. This is consistent with the poor correlation between peak blood flow and FMD that was seen and has previously been reported (Leeson et al., 1997) and suggests that endothelial activation is dependent, at least in part, on the integral of blood flow over time rather than detecting rapid changes in shear stress as occur with reactive hyperaemia. From a physiological point of view this might be appropriate as high gradients in shear stress which occur with the transmission of a pulse wave would not be sufficient to stimulate the endothelium to synthesise vasodilators. The mechanisms whereby the endothelium integrates the flow stimulus are unknown however. Although there were no significant correlations between any parameter of FMD and either baseline blood flow or $VTI_{\text{max}}$ there was a significant association between $\text{FMD}_{\text{max}}$ and $\text{FMD}_{\text{AUC}}$ and the percentage increase in blood flow (i.e. the ratio of $VTI_{\text{max}}$ and baseline VTI). This suggests that the relative increase in blood flow might also be an important stimulus to the endothelium and it is possible that a number of different mechanisms are operating.

These findings have practical implications for the study of endothelial function. Firstly, they indicate that differences in FMD between individuals and risk factor groups might be mediated by differences in the blood flow stimulus to the endothelium. Factors such as the size and musculature of the arm might be important in determining such responses. In the experimental setting, therefore, it will be necessary to adjust FMD for any inter-individual or inter-cohort differences in the reactive hyperaemic stimulus in order to correctly assess differences in endothelial function. Secondly, these data indicate that peak hyperaemic blood flow itself is a poor determinant of the subsequent dilatation and adequate assessment of the flow stimulus to the endothelium will require description of the hyperaemic flow envelope over a longer period of time.
2.4 Exogenous nitrates and smooth muscle sensitivity

As part of the general protocol glyceryl trinitrate (GTN) is administered to demonstrate the integrity of the smooth muscle response to nitrates. In the majority of experiments a dose of 400 µg has been administered, and this is occasionally associated with unpleasant side-effects including flushing and headache which has limited its use in children. This dose of GTN results in a much larger dilatation than that seen following reactive hyperaemia and although abnormalities of the GTN response have previously been reported in young subjects with hypercholesterolaemia (Clarkson et al., 1996b), it remains unclear (Sorensen et al., 1994) whether these represent changes in the arterial structure limiting further dilatation or decreased smooth muscle sensitivity to nitrates. To examine this lower doses of nitrate should be administered such that a dose response curve can be determined.

2.4.1 Specific methods

The possibility of determining smooth muscle sensitivity by administering incremental submaximal doses of GTN sublingually was investigated. In six healthy subjects GTN solution (2 mg/ml) was diluted in saline to give separate vials containing different concentrations of GTN such that a single 40 µL aliquot contained 12.5, 25, 50, 100, 200 or 400 µg of GTN. This was administered sublingually using a micropipette and the response of the brachial artery determined over a 10 minute period.

2.4.2 Results

Administrations of lower doses (12.5 to 100 µg) of GTN had no significant side effects and specifically were not associated with headache in any subject. Brachial artery dilatation increased in a dose dependent fashion (Figure 2.4.1). Doses up to 100 µg were associated with an initial peak after which the effect was gradually lost.
Figure 2.4.1 Dose response curves for sublingual glyceryl-trinitrate (GTN)

2.4.3 Discussion

These data demonstrate that low dose GTN can be administered sublingually to produce reliable endothelium-independent dilatation in the brachial artery and is not associated with any side effects. Moreover, the level of dilatation more closely resembles that seen in response to reactive hyperaemia and is likely to be a more accurate assay of smooth muscle sensitivity to exogenous nitrates. The effect of low doses of GTN is short lived and is therefore suitable for serial studies over short periods of time. A single 400 µg dose of GTN is likely to result in a supramaximal dilatation, the magnitude of which will be determined by the structural constraints of the arterial wall.
2.5 Assessment of arterial distensibility

The ability of an artery to distend in response to pressure is likely to be largely determined by the structural characteristics of the arterial wall and the relative distribution of elastic and tensile proteins (Roach and Burton, 1957). Such elastic properties of arteries might have an important role in reducing high levels of shear stress on the arterial wall and storing energy, which can be released in diastole thus maintaining a constant perfusion of peripheral tissues. Parameters of arterial elasticity can be assessed non-invasively using reproducible high-resolution ultrasound techniques (Hokanson et al., 1972; Kool et al., 1994a; Reneman et al., 1996). Decreased distensibility has been noted in elastic arteries of patients with increasing age (Smulyan et al., 1983; Benetos et al., 1993) hypertension (Benetos et al., 1993; Reneman and Hoeks, 1995) and a range of other risk factors for atherosclerosis (Kool et al., 1993) and might represent an additional marker of vascular disease in young preclinical subjects (Leeson et al., 2000).

In addition to assessing the local blood velocity, Doppler signal processing can be employed to assess, non-invasively, the displacement of the arterial walls during the cardiac cycle (distension waveform) and hence, the time-dependent changes in arterial diameter relative to its initial diameter at the start of a cardiac cycle (Hoeks et al., 1990; Hokanson et al., 1972). The displacement of the arterial wall is obtained by processing radio-frequency signals within a sample volume coinciding with the arterial wall. In this method the position of vascular interfaces are calculated from the Doppler or radio-frequency signal derived from a fixed ultrasound beam, perpendicular to the artery of interest (Figure 2.5.1). Once a stable image is achieved, a short 5 – 10 second segment of A-mode signal is routed to a wall tracking device within a personal computer and the data stored on the hard disk for later analysis. The initial A-mode signal is displayed on the screen and volume sample cursors are manually placed at the echo peaks corresponding to the anterior and posterior arterial wall interfaces. The precise position of arterial interfaces are automatically determined by examining the change in phase of the returning signal. The system locks on to these peaks and tracks their movement throughout the cardiac cycle. A distension waveform is subsequently displayed and derived measures including arterial end-diastolic diameter, distension and distensibility of the vessel reported.
Figure 2.5.1 Assessment of arterial distensibility. A Doppler signal with an angle of insonation perpendicular to the artery is used to acquire an A-mode signal (upper panel). Volume sample cursors are placed over the echoes which correspond to the near and far arterial walls (middle panel). Movements of the near and far walls are tracked throughout the cardiac cycle for a 5 second period and the distension wave form determined (lower panel).
2.5.1 Data analysis

A number of parameters of arterial elasticity have been previously reported. These include the distensibility coefficient, compliance, the stress/strain elastic modulus and Youngs modulus. The distensibility coefficient (DC) is calculated as the percent change in volume of the artery per unit change in pressure. Assuming the target vessel is straight, of equal calibre along its length and has a circular cross section then DC can be calculated as the percent change in arterial diameter:

\[ DC = \frac{\Delta D}{D \times \Delta P} \times 100 \text{ mmHg} \]

Where \( D \) = arterial diameter, \( \Delta D \) = change in diameter and \( \Delta P \) = change in pressure

Arterial compliance (CC) is defined as the absolute volume change per unit change in pressure and can similarly be defined as:

\[ CC = \frac{\Delta D}{\Delta P} \text{ mm/mmHg} \]

Whilst these measures give important information on arterial elasticity they are influenced by both vessel size and blood pressure. Alternative measures include Youngs elastic modulus, which reflects arterial wall elasticity independently of these parameters but requires an accurate measure of arterial wall thickness. In practice with current ultrasound technology there is often great difficulty in identifying the interface between the adventitia and surrounding tissues in view of their similar ultrasound properties and therefore this derivative has not been used.

Blood pressure is normally measured in the brachial artery and the assumption is made that this is representative of blood pressure in the carotid artery. In fact, because of transformation of the pressure wave in the arterial tree, blood pressure is usually higher in peripheral arteries than in the central vessels (Nichols and O’Rourke, 1990). However, a linear relationship between brachial and carotid artery blood pressure has been demonstrated in dogs (Reneman et al., 1992) and the
validity of this method has been demonstrated previously (Imura et al, 1986; Stefa douros et al, 1973; Stefanadis et al, 1990).

2.6 Summary

The non-invasive techniques for measuring vascular function described in this chapter are safe and relatively easy to perform. The assessment of FMD, using high-resolution ultrasound, has been widely adopted and its accuracy and reproducibility has facilitated its use in serial studies examining the effect of interventions (Mullen et al., 1998b). Although brachial artery endothelial dysfunction is associated with the presence of coronary endothelial dysfunction and atherosclerosis (Anderson et al., 1995), to date no long-term follow up studies have demonstrated a link between endothelial dysfunction and later cardiovascular morbidity and mortality.

In these preliminary studies a dose dependent relationship between dilatation and the intensity of reactive hyperaemic blood flow, as determined by measures of duration of blood flow or the AUC has been established. The precise relationship between the blood flow signal and dilatation however, remains unclear and whether the response is entirely mediated by NO synthesis is not known.

The relevance of reduced distensibility to the pathological processes involved in atherogenesis is not clear and particularly the role of the endothelium and nitric oxide synthesis in modulating arterial elasticity remains unknown (Joannides et al., 1997; Sudhir et al., 1995).
CHAPTER 3

ROLE OF VASOACTIVE MEDIATORS IN CONDUIT ARTERY

DISTENSION AND DILATATION:

RELEVANCE TO ENDOTHELIAL DYSFUNCTION IN

HYPERCHOLESTEROLAEMIA
3.1 Introduction

The observation that canine femoral arteries dilate in response to increases in blood flow was first noted by Schretzenmayr (1933). The mechanism for this was originally thought to be due to ascending dilatation from the distal vasculature (Fleisch, 1935). Lie and colleagues (1970) however, subsequently observed that transection of the artery, to separate it physically from the distal vasculature, failed to abolish the response, and proposed that local mechanisms were responsible for regulating flow-mediated dilatation (FMD). In 1980 Furchgott and Zawadzki demonstrated the central role of the endothelium in vasodilatation to acetylcholine and proposed the existence of an endothelium-derived relaxing factor, which has subsequently been identified as nitric oxide (NO) (Palmer et al., 1987). The dependence of FMD on an intact endothelium was demonstrated in experiments in dogs, in vitro (Smiesko et al., 1985) and in vivo (Pohl et al., 1986). In humans FMD was also observed in peripheral conduit arteries (Anderson and Mark, 1989; Sinoway et al., 1989) and in the coronary circulation (Cox et al., 1989), and has become a paradigm for the assessment of endothelial function in vivo (Section 2.2).

Interpreting the results of endothelial function tests which use flow as a signal will depend on a precise knowledge of the mechanisms that underlie FMD in humans in vivo. Endothelial cells, in culture, are sensitive to shear stress, pressure and stretch (Davies and Tripathi, 1993; Traub and Berk, 1998), responding by altering their structure and alignment and synthesising vasoactive substances which contribute to the regulation of vascular smooth muscle tone. Those that have been identified include the dilators nitric oxide, and prostacyclin, and the existence of an endothelium dependent hyperpolarizing factor has been proposed (Vanhoutte and Mombouli, 1996). The mechanisms by which endothelial cells sense and respond to blood flow however have not been well characterized. Activation of potassium channels has been proposed (Cooke et al., 1991a). Recent studies have demonstrated a specific potassium channel which varies in magnitude and duration as a function of shear stress, desensitises slowly and recovers rapidly and fully on cessation of flow (Olesen et al., 1988). Whatever the mechanism by which the endothelium transduces the flow signal, vasodilatation is mediated by release of one or more mediators.
Studies in humans, using specific inhibitors of NO, have demonstrated that in resistance vessels, NO is synthesised continuously and contributes to the regulation of basal tone, systemic vascular resistance and blood pressure (Vallance et al., 1989). In conduit arteries however, the role of NO as a mediator is less clear and specifically, data on the role of NO as the mediator of FMD in vivo, is contradictory. In humans, a number of groups have demonstrated abolition of FMD in the brachial artery during local intra-arterial infusion of the specific NO synthesis antagonist, N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), following short episodes of reactive hyperaemia (Joannides et al., 1995; Lieberman et al., 1996). In conscious dogs, coronary FMD in response to rapid atrial pacing can be inhibited by co-infusion of L-NMMA (Canty, Jr. and Schwartz, 1994). In contrast, FMD following infusion of adenosine, which results in a more sustained hyperaemic response is resistant to the effects of NO inhibition. In humans similar responses might also occur, with pacing induced coronary FMD being NO dependent (Egashira et al., 1996; Quyyumi et al., 1995; Tousoulis et al., 1997), whilst FMD in response to a sustained flow stimulus is NO independent (Shiode et al., 1996). These data suggest heterogeneity of the endothelial response to blood flow, whereby the physical or dynamic characteristics of the flow stimulus might be important in determining the mechanism of the subsequent dilatation. Determining the nature of these responses will have important implications for the interpretation of endothelial function tests and understanding the pathogenesis of endothelial dysfunction.

In contrast arterial distensibility is likely to be largely a passive response determined by the structural characteristics of the arterial wall and the relative distribution of elastic and tensile proteins (Roach and Burton, 1957). The role of the endothelium in mediating arterial elasticity is unclear and particularly studies which have examined the role of NO synthesis in conduit artery distensibility have not reached a consensus (Joannides et al., 1997; Sudhir et al., 1995).

These investigations sought to further examine the role of NO synthesis in conduit artery distensibility and dilatation in response to a flow stimulus. Secondly, this study examined the relationship between the dynamic characteristics of the flow stimulus and the mechanisms that modulate conduit artery tone. Finally this study examined whether FMD in response to different flow stimuli is altered in patients at
risk of atherosclerosis. Local infusion of drugs was used to probe the mechanisms of vascular function and specific methods of causing transient and sustained increases in blood flow in the conduit arteries of the arm were developed to assess arterial response under different physiological conditions.

3.2 General methods

All studies were approved by the local ethics committee and all subjects gave informed written consent.

3.2.1 Arterial cannulation

To facilitate the local infusion of drugs the brachial artery, in the non-dominant arm, was cannulated at the antecubital fossa (Figure 3.2.1). A sterile 27 gauge needle was inserted into the artery after infusion of 2 mls of local anaesthetic (lignocaine 1% w/v). The needle was fixed with tape and connected by fine epidural catheter tubing to a high pressure Harvard pump, which had previously been calibrated. Saline (0.9% w/v) or drugs (see below) dissolved in saline were infused intra-arterially at 0.5 ml/min.

Figure 3.2.1 To facilitate the local infusion of drugs, a 27 gauge needle was inserted into the brachial artery at the antecubital fossa, under local anesthesia, and the radial artery imaged distal to this site.
3.3 Effect of nitric oxide inhibition on arterial distensibility

3.3.1 Specific methods

In 5 healthy subjects the non-dominant brachial artery was cannulated, at the antecubital fossa as outlined above. A 15 minute rest period was observed, during which saline was infused continuously, prior to commencement of the study. The radial artery was imaged in longitudinal section and magnified using the resolution box function. A segment of the artery was selected and once a stable and clear image was achieved, 5 seconds of radio-frequency signal from this segment routed to an A-mode tracking device (Ingenious Systems, Netherlands) where the data was stored for later analysis of the distension wave form (Section 2.5). Blood pressure was measured in the contralateral arm on three occasions, using an automatic oscillometric sphygmomanometer, during each condition. The protocol was repeated in the presence of intra-arterial noradrenaline (240 pmol/min; pre-infused for 10 minutes, [Antigen Pharmaceuticals Ltd, Ireland]) to determine the effects of a non specific vasoconstrictor followed by L-NMMA (intra-brachial infusion of 4 μmol/min; pre-infused for 10 minutes [Clinalfa Switzerland]). Each cycle of the protocol was separated by a 10 minute rest period during which saline was infused and each drug was preinfused for at least 10 minutes prior to data acquisition (Figure 3.3.1).

3.3.2 Data analysis

On completion of each study, the stored radio frequency data for each scan was analysed by placement of volume sample cursors, at the near and far vessel wall interfaces (Section 2.5). Arterial distension was automatically tracked on a beat by beat basis. The mean values of three separate measurements were used during each condition. The mean pulse pressure (systolic minus diastolic blood pressure) was determined during each condition. The distensibility coefficient (DC) was determined as the percentage increase in arterial volume during the pulse wave per mmHg change in pressure (Section 2.5.1). Compliance (CC) was determined as the absolute volume change per mmHg change in pressure. Vascular responses during the different conditions were compared using two-way analysis of variance.
Figure 3.3.1 Role of nitric oxide in radial artery distensibility: study protocol. Saline, noradrenaline and L-NMMA were infused via the brachial artery at the antecubital fossa and their effects on radial artery distensibility coefficient assessed.
3.3.3 Results

Intra-arterial noradrenaline and nitric oxide synthase inhibition with L-NMMA had no significant effect on radial artery diameter, blood flow or systemic blood pressure compared to saline.

There was no change in radial artery distensibility or compliance during inhibition of NO synthesis or infusion of the non-specific smooth muscle vasoconstrictor noradrenaline (Figure 3.3.2)

3.3.4 Discussion

These data suggest that the endothelial release of nitric oxide does not directly contribute to conduit artery compliance and distensibility. This is consistent with the physiological response of conduit arteries to an intense stimulus such as reactive hyperaemia, when arterial dilatation is delayed for up to 60 seconds after the increase in blood flow. The absence of an effect of noradrenaline might also indicate that smooth muscle tone does not directly influence arterial elasticity.

The ability of an elastic artery to distend during the passage of a pulse wave is likely to be an important physiological process that helps to maintain an efficient blood supply to peripheral tissues. This is likely to be largely a passive process determined by the structure of the arterial wall and the relative distribution and alignment of elastic and tensile proteins (Roach, 1957). Increased stiffness of conduit arteries has been demonstrated with increasing age (Benetos et al., 1993; Smulyan et al., 1983) in patients with hypertension (Benetos et al., 1993), diabetes (Salomaa et al., 1995), smokers (Kool et al., 1993) and patients with hypercholesterolaemia (Barenbrock et al., 1995; Hopkins et al., 1993). The nature of these abnormalities is not clear but our data suggest that reduced compliance and distensibility do not directly represent endothelial dysfunction and impaired NO bioavailability. Although, these data do not exclude a chronic effect of endothelial dysfunction on the vascular wall, the recent finding that distensibility may be impaired from as early as the first decade of life in relation to cholesterol levels, and is not related to endothelial function (Leeson et al., 1999), would be more consistent with parallel pathophysiological processes.
Figure 3.3.2 Effect of noradrenaline and nitric oxide synthesis inhibition on radial artery distensibility.
3.4 Mechanisms of regulation of conduit artery tone at rest and during hyperaemia

3.4.1 Specific methods

3.4.1.1 Subjects

Healthy volunteers, aged between 18 – 45 years, were recruited from amongst colleagues and staff at Gt. Ormond St. Hospital and University College London. All subjects were non-smokers and on no vasoactive medication. The effect of NO and prostanoid inhibition on FMD in response to reactive hyperaemia and hand warming were assessed.

3.4.1.2 Measurement of conduit artery diameter and blood flow

The radial artery was scanned in longitudinal section, magnified using a resolution box function and gated with the R wave of the ECG. End-diastolic images of the artery were acquired every 3 to 5 seconds using data-acquisition software (Information Integrity, Boston, USA) and stored in digital format off-line for later analysis. Arterial diameter over a 1-2 cm segment was determined for each image using a semi-automatic edge detection algorithm (Stadler et al., 1996). Blood flow in the radial or brachial artery, at the same site at which vessel diameter was being assessed, was recorded continuously throughout the study using pulsed wave Doppler.

3.4.1.3 Protocol 1; role of endothelial mediators in the dilatation of the radial artery to reactive hyperaemia

In 8 healthy subjects the non-dominant brachial artery was cannulated as outlined above. A 15 minute rest period was observed, during which saline was infused continuously, prior to commencement of the study. Once completed, radial artery diameter and blood flow were measured continuously for 1 min of baseline, during 5 minutes of reduced blood flow, induced by inflation of a pneumatic cuff (to 300 mmHg) placed at the wrist, distal to the segment of artery being analysed, and for a further 5 min during a brief episode of reactive hyperaemia after release of the cuff. This protocol was repeated in the presence of intra-arterial noradrenaline (240
pmol/min; pre-infused for 10 minutes [Antigen Pharmaceuticals Ltd, Ireland]) to determine the effects on baseline flow and diameter followed by L-NMMA (intra-brachial infusion of 4 μmol/min; pre-infused for 10 minutes [Clinalfa Switzerland]) (Figure 3.4.1). Each cycle of the protocol was separated by a 10 minute period to allow radial artery blood flow and diameter to re-equilibrate.

In a separate experiment, to assess the role of vasoactive prostanoids in radial artery dilatation to transient increases in blood flow, the same protocol was performed, in 6 subjects, before and 2 hours after inhibition of cyclo-oxygenase by administration of aspirin (600 mg po).

3.4.1.4 Protocol 2; mechanisms of radial artery dilatation to sustained increases in blood flow caused by hand warming

In 6 subjects, continuous measurements of radial artery blood flow and diameter were made after the ipsilateral hand had been immersed in cold water (22 °C) for at least 15 minutes and then for 10 minutes following exchange for warm water (42 - 45 °C) (Figure 3.4.2). After a further 15 minute period during which the hand was again cooled to 22 °C (to allow return to basal parameters), arterial diameter and blood flow were measured during a 10 min infusion of L-NMMA (4 μmol/min) and for 10 minutes when the hand was re-immersed in warm water. In 3 subjects, radial artery diameter during hand warming was assessed before and 2 hours after administration of aspirin (1200 mg PO).

3.4.2 Data analysis

The velocity time integral (VTI) was determined at baseline (mean of at least 2 measurements during the first minute of each study) and at prespecified time points for each study. VTI was measured during the last minute of cuff inflation and at 15 second intervals during reactive hyperaemia. Peak VTI and the area under the curve of VTI over 45 seconds were determined for each study. During hand warming VTI was measured every minute for the duration of the study.
Figure 3.4.1 Method for assessing FMD in the radial artery. Protocol 1: assessment of FMD in response to reactive hyperaemia. Drugs (saline, noradrenaline and L-NMMA) were infused via the brachial artery at the antecubital fossa and their effects on radial artery FMD in response to reactive hyperaemia assessed.
Figure 3.4.2 Method for assessing FMD in the radial artery. Protocol 2: assessment of FMD in response to hand warming. Hand warming, in a purpose built bath, results in a gradual increase in blood flow over minutes, which is maintained at a steady state. The effects of saline □ and L-NMMA ■, on flow-mediated dilatation were assessed sequentially to determine the role of nitric oxide in dilatation to this flow stimuli.
Baseline vessel diameter was determined as the mean diameter of all measurements (12-20 measurements) during the first minute of each study and subsequent dilatation expressed as a percentage change from the baseline diameter. During cuff inflation, arterial constriction in response to reduced blood flow was determined as the mean vessel constriction from all measurements for 1 minute prior to cuff release and the AUC during the whole of cuff inflation. Three measures of FMD in response to reactive hyperaemia were determined for each scan; the maximal dilatation that occurred in response to reactive hyperaemia (FMD_{max}); the time to maximal dilatation (t_{max}) and the area under the curve for vessel dilatation over 3 minutes following cuff release (FMD_{AUC}). Following hand warming, arterial dilatation during the last minute of recording (when flow and vessel diameter had reached steady state; average of 12 - 15 images) were used for analysis. In addition, the initial response of the radial artery was assessed by calculating the AUC of blood flow from 5 - 10 minutes after hand warming and dilatation from 6 - 12 minutes. The gradient of the initial response was calculated using linear regression in which dilatation was the dependent variable and blood flow velocity the independent variable.

3.4.2.1 Statistical analysis

All results are expressed as mean ± standard error and compared using Students t test for paired or unpaired observations as appropriate or by analysis of variance for repeated measures (ANOVA).

3.4.3 Results

3.4.3.1 Effect of noradrenaline and L-NMMA on basal blood flow and tone

During infusion of L-NMMA and noradrenaline there was no significant change in heart rate or blood pressure (measured in the contralateral arm), indicating that any systemic effects of these drugs were negligible. Both L-NMMA (4 umol/min) and noradrenaline (240 pmol/min) infusion resulted in small but non-significant reductions in radial artery blood flow velocity compared to baseline, however there was no significant change in arterial diameter (Table 3.4.1).
3.4.3.2 Blood flow velocity and reactive hyperaemia

During inflation of the occluding cuff, radial artery blood flow was almost completely abolished and there was no further incremental reduction during infusion of noradrenaline or L-NMMA. Following release of the cuff, there was a peak 3.6 ± 0.5 fold (maximum VTI 0.23 ± 0.01 m) increase in blood flow velocity which was maximum within 15 seconds and rapidly attenuated thereafter, such that by 1 minute baseline levels were achieved. Neither peak VTI nor the area under the curve for the first 45 seconds of reactive hyperaemia were significantly affected by noradrenaline or L-NMMA (Figure 3.4.3a).

3.4.3.3 Effect of noradrenaline and L-NMMA on FMD following reactive hyperaemia

During saline infusion, inflation of the occluding cuff was associated with a constriction of 3.9 ± 1.1% in radial artery diameter. This constriction was significantly enhanced during infusion of noradrenaline but was not significantly affected by L-NMMA (Table 3.4.1). In response to reactive hyperaemia, \( \text{FMD}_{\text{max}} \) was 5.3 ± 1.2% (\( t_{\text{max}} \) 69.3 ± 8.1 secs, \( \text{FMD}_{\text{AUC}} \) 438 ± 163 AU) during infusion of saline. Infusion L-NMMA (\( \text{FMD}_{\text{max}} \) 0.7 ± 0.71%, \( P < 0.001, \text{FMD}_{\text{AUC}} \) -251 ± 102 AU, \( P < 0.001 \)), but not noradrenaline, significantly attenuated \( \text{FMD}_{\text{max}} \) and \( \text{FMD}_{\text{AUC}} \) in response to reactive hyperaemia (Figure 3.4.3b). Neither noradrenaline or L-NMMA had any significant effect on \( t_{\text{max}} \) or \( D_{\text{dil}}/D_t \).

3.4.3.4 Role of vasoactive prostanoids in FMD following reactive hyperaemia

In 6 subjects, aspirin (600 mg PO) had no significant effect on radial artery blood flow velocity or diameter at rest, the constriction during cuff inflation (-4.86 ± 1.19% vs -4.43 ± 0.93%, \( P = \text{NS} \)), reactive hyperaemia or \( \text{FMD}_{\text{max}} \) (4.65 ± 0.49% vs 4.86 ± 0.50%, \( P = \text{NS} \)) respectively.
Table 3.4.1 Effect of nitric oxide inhibition on radial artery FMD to reactive hyperaemia.

<table>
<thead>
<tr>
<th></th>
<th>Saline (0.9% w/v)</th>
<th>Noradrenaline (240 pmol/min)</th>
<th>L-NMMA (4 μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal VTI (m/s)</td>
<td>0.046 ± 0.006</td>
<td>0.039 ± 0.012</td>
<td>0.034 ± 0.013</td>
</tr>
<tr>
<td>Basal diameter (mm)</td>
<td>2.91 ± 0.23</td>
<td>2.83 ± 0.21</td>
<td>2.93 ± 0.22</td>
</tr>
<tr>
<td>Cuff-up VTI (m)</td>
<td>0.008 ± 0.001</td>
<td>0.008 ± 0.002</td>
<td>0.017 ± 0.009</td>
</tr>
<tr>
<td>Constriction (%)</td>
<td>-3.9 ± 1.1</td>
<td>-8.1 ± 1.2 †</td>
<td>-5.5 ± 1.3</td>
</tr>
<tr>
<td>Constriction AUC</td>
<td>-887 ± 207</td>
<td>-1921 ± 367 †</td>
<td>-1431 ± 304</td>
</tr>
<tr>
<td>Peak VTI (m/s)</td>
<td>0.230 ± 0.012</td>
<td>0.253 ± 0.016</td>
<td>0.222 ± 0.016</td>
</tr>
<tr>
<td>RH\text{AUC}</td>
<td>8.91 ± 1.12</td>
<td>9.93 ± 1.21</td>
<td>8.01 ± 1.36</td>
</tr>
<tr>
<td>FMD\text{max} (%)</td>
<td>5.3 ± 1.2</td>
<td>5.5 ± 1.3</td>
<td>0.7 ± 0.7 †</td>
</tr>
<tr>
<td>t_{\text{max}} (sec)</td>
<td>69.3 ± 8.1</td>
<td>82.4 ± 11.7</td>
<td>74.3 ± 11.4</td>
</tr>
<tr>
<td>FMD\text{AUC}</td>
<td>438 ± 163</td>
<td>150 ± 191</td>
<td>-251 ± 102 †</td>
</tr>
<tr>
<td>Ddil/Dt (%/s)</td>
<td>0.25 ± 0.05</td>
<td>0.34 ± 0.04</td>
<td>0.26 ± 0.07</td>
</tr>
</tbody>
</table>

AUC = area under the curve (arbitrary units); RH = reactive hyperaemia; FMD = flow-mediated dilatation. Data is mean ± S.E.M, † P < 0.05 compared to saline (ANOVA).
3.4.3.5 Role of nitric oxide and vasoactive prostanoids in radial artery dilatation to sustained blood flow

In 6 subjects, hand warming was used to stimulate sustained increases in blood flow in the radial artery. Immersion of the hand in cold water resulted in decreased radial artery blood flow compared to that normally seen in subjects at room temperature. With hand warming to between 42 °C and 45 °C radial artery blood flow increased from 0.016 ± 0.001 m to 0.113 ± 0.012 m during infusion of saline (mean 8.9 ± 1.5 fold increase) (Figure 3.4.4). This resulted in a mean radial artery dilatation of 11.7 ± 1.8%. Infusion of L-NMMA (4 μmol/min) had no significant effect on radial artery blood flow or dilatation during hand warming (Figure 3.4.4). In 3 of these subjects, a further 10 minute infusion of L-NMMA at 16 μmol/min (to compensate for the dilutional effect of increased forearm blood flow) had no additional effect on dilatation associated with hand warming than that seen during L-NMMA 4 μmol/min (radial dilatation was 13.3 ± 2.5% before and 13.1 ± 1.3% in the presence of L-NMMA).

Aspirin (1200 mg), had no significant effect on radial artery dilatation following hand warming (6.9 ± 1.4% before vs. 7.4 ± 1.0% after, P = NS) as seen with FMD following reactive hyperaemia.

To determine whether dilatation following hand warming was mediated by increased blood flow or through other mechanisms, the direct effects of hand warming on radial artery dilatation were assessed in 5 subjects. When radial artery blood flow was maintained at baseline levels, by inflating a wrist cuff to between diastolic and systolic blood pressure, the dilatation during hand warming was significantly attenuated compared to when hyperaemia was unopposed (2.7 ± 1.1% vs 8.1 ± 1.6% respectively, P = 0.01).
Figure 3.4.3 Effect of L-NMMA and noradrenaline on reactive hyperaemia and flow-mediated dilatation (FMD). (a) Neither noradrenaline or L-NMMA had any significant effect on the reactive hyperaemic flow envelope. (b) In contrast L-NMMA but not noradrenaline significantly inhibited FMD compared to saline.
Figure 3.4.4 Effect of L-NMMA on hyperaemia (velocity-time integral (VTI)) in response to hand warming and flow-mediated dilatation (FMD). Hand warming caused a gradual but sustained increase in blood flow (upper panel) which was associated with a large and sustained dilatation of the radial artery (lower panel). The hyperaemic response was not significantly different during infusion of L-NMMA ■ from that seen with saline □.
3.4.4 Discussion

This study demonstrates that under normal physiological conditions, NO synthesis is important in mediating conduit artery dilatation in response to intense and transient flow increases, but that under basal conditions or when the flow increase is maintained, alternative mechanisms of vasodilatation predominate. Understanding the mechanisms of these heterogeneous responses of the endothelium to flow has implications for the design and interpretation of endothelial function tests and the treatment of early atherosclerosis.

Under normal physiological conditions both conduit and resistance arteries maintain a constant state of dilatation, the degree of which is determined by the relative balance between vasodilator and constrictor influences. In the present study, local infusion of the NO synthase inhibitor L-NMMA, noradrenaline and oral aspirin were used to probe the mechanisms of regulation of conduit artery tone under different conditions of blood flow. Reduced blood flow, during cuff inflation, resulted in significant radial artery constriction, confirming the presence of tonic flow-mediated dilatation under normal resting conditions. If NO is important in maintaining basal conduit artery dilatation, then during infusion of L-NMMA, constriction of the artery should occur under resting conditions and not be enhanced by a further reduction in flow. In this study, L-NMMA had no effect on radial artery diameter under resting conditions and there was a trend to increased radial artery constriction during periods of reduced flow indicating minimal basal NO mediated dilatation in conduit arteries.

Previous reports have demonstrated a role for NO in conduit artery dilatation in response to a brief episode of reactive hyperaemia (Joannides et al., 1995; Lieberman et al., 1996). This finding was confirmed in the present study in which FMD following reactive hyperaemia was almost completely abolished during infusion of L-NMMA, an effect that was not explained by any change in the flow stimulus. In contrast, L-NMMA did not significantly alter the dilator response to a sustained flow stimulus caused by local hand warming. Radial artery dilatation during hand warming was negligible, if radial artery blood flow was maintained at basal values excluding the possibility that the dilatation was a non-specific response to the stimulus used (i.e. hand warming).
These findings have physiological, pathophysiological and therapeutic implications. These findings suggest that a physiological role of the NO pathway is to provide a mechanism to limit the degree to which shear stress is elevated in response to rapid changes in blood flow (Vallance et al., 1989) and imply that there is adaptation of the response of the NO pathway. Whether this occurs because of reduced NO production or desensitisation to the effects of NO is unclear, but understanding how the pathway is switched off might have implications for understanding how activity of the NO pathway is reduced in cardiovascular disease.

The mechanism(s) of dilatation in response to sustained flow is at present unclear, but might involve endothelial or non-endothelial dependent pathways. Inhibition of cyclooxygenase with aspirin had no effect on basal radial artery diameter or dilatation in response to either flow stimulus, implying that prostanoids are not important determinants of tone in this vessel under these conditions (Joannides et al., 1995). One possibility is that neuronal components are involved, although in animal experiments, FMD was preserved following surgical or pharmacological denervation (Lie et al., 1970; Hilton, 1959; Fleisch, 1935). This is an area for investigation in the future. An alternative explanation for these findings is that other vasodilator mechanisms are able to compensate for reduced NO synthesis, but that their rate of response to intense and rapidly changing stimuli is limited compared to the NO pathway. Further, experiments with blockade of multiple pathways will be needed to test this hypothesis.

In summary, these data indicate that whilst NO is an important regulator of conduit artery tone in response to brief increases in blood flow, the maintenance of arterial dilatation under basal conditions or during sustained hyperaemia is mediated by NO-independent mechanisms. These data have important scientific implications for the interpretation of tests of endothelial function and the development of therapeutic strategies to replace or enhance endogenous NO activity. Further studies to elucidate the mechanism of this phenomenon will help to clarify its role and that of the NO pathway in physiological and pathophysiological conditions.
3.5 Impact of hypercholesterolaemia on FMD to sustained flow

3.5.1 Introduction

Previous studies have demonstrated impaired FMD, in response to reactive hyperaemia, in young patients with hypercholesterolaemia (Sorensen et al., 1994) consistent with an abnormality of nitric oxide bioavailability. In section 3.3 it has been demonstrated that FMD in response to a sustained blood flow stimulus, in contrast to that seen following a brief episode of reactive hyperaemia, is not attenuated by the nitric oxide synthase inhibitor L-NMMA suggesting an NO independent mechanism. It is not known whether the abnormality of FMD in patients with risk factors for atherosclerosis would also extend to this pathway, consistent with a generalized endothelial dysfunction or whether a more specific abnormality of NO metabolism might occur. The aim of this study was therefore to examine FMD in response to flow stimuli with different dynamic characteristics in patients with hypercholesterolaemia and matched controls.

3.5.2 Specific methods

Patients with hypercholesterolaemia (n = 8) were recruited from outpatient clinics and control subjects (n = 8) from amongst colleagues and friends. All subjects were non-smokers and taking no regular vasoactive medication. Hypercholesterolemic patients were asked to stop cholesterol-lowering medication for at least 2 weeks before being studied.

All subjects attended in a fasting state for venesection to assess their lipid profile. Brachial artery diameter and blood flow were measured continuously for one minute before, during 5 minutes of mid-forearm cuff inflation, and for 5 min after cuff deflation, to document responses to transient increases in blood flow. Subsequently, the skin was marked to allow accurate repositioning of the transducer and the brachial artery cannulated as above. After 10 - 15 min to allow parameters to return to resting values, during which saline was infused continuously, acetylcholine was infused into the distal brachial artery at cumulative doses of 10, 100 and 1000 nmol/min (each dose for 5 minutes), to allow the increase in blood flow to reach a
sustained plateau at each dose of acetylcholine (Figure 3.5.1). The effect of this sustained increase in blood flow on the brachial artery, upstream from the point of infusion, was assessed continuously.

To ensure that the infusion of acetylcholine did not have a systemic effect, in three of the subjects brachial artery flow and diameter was continuously monitored in the contralateral arm.

3.5.2.1 Data analysis and statistical methods

The velocity time integral was determined at baseline (mean of at least 2 measurements during the first minute of each study) and at prespecified time points for each study. VTI was measured during the last minute of cuff inflation and at 5 second intervals for the first 15 seconds after cuff deflation and at 15 second intervals thereafter during reactive hyperaemia. Peak VTI and the area under the curve of VTI over 60 seconds were determined for each study. FMD_{max}, t_{max}, FMD_{AUC}, and Ddil/Dt in response to reactive hyperaemia were determined as before.

During infusion of acetylcholine the VTI was assessed at 1 minute intervals throughout the study. To determine the relationship between the graded increase in flow and brachial artery dilatation, mean dilatation (average of 12 measurements) and mean VTI (average of 2 measurements) were calculated for the last minute at each concentration. For each subject, dose response curves for steady state dilatation were constructed using linear regression analysis. The slope of the dilatation/VTI curve was interpolating and the VTI at which 7% dilatation of the brachial artery occurred calculated.

All results are expressed as mean ± standard error. and compared using Students t test for unpaired observations or by analysis of variance for repeated measures (ANOVA) as appropriate. Significance was inferred when P < 0.05.
Figure 3.5.1 Assessment of FMD in response to reactive hyperaemia and sustained blood flow. A stepwise increase in brachial artery flow was induced by infusion of acetylcholine, at an incremental dose, via the brachial artery at the antecubital fossa (downstream of the segment of artery being examined).
3.5.3 Results

The baseline characteristics of the hypercholesterolemic and control groups are given in Table 3.5.1. There were significant differences in total cholesterol levels between the 2 groups but other parameters were comparable.

3.5.3.1 Response to reactive hyperaemia

Detailed comparison of flow and dilatation parameters are shown in Table 3.5.1. There were no significant differences in peak reactive hyperaemia or the AUC of reactive hyperaemia between the two groups. Brachial artery FMD$\text{max}$ following reactive hyperaemia, caused by a 5 min forearm cuff inflation, was 7.4 ± 0.7% in controls, and 3.9 ± 0.8% in patients with hypercholesterolaemia (Figure 3.5.2a; $P = 0.006$). There was a significant difference in Ddil/Dt (0.32 ± 0.02%/s vs. 0.21 ± 0.03%/s, $P = 0.03$) and a borderline significant difference in FM$D_{\text{auc}}$ between the groups but no difference in t$\text{max}$. Within the group studied there was a significant negative correlation between FMD in response to reactive hyperaemia and cholesterol ($r = 0.7, P < 0.05$; Figure 3.5.2b).

3.5.3.2 Response to sustained hyperaemic blood flow

Sustained increases in blood flow in the brachial artery were caused by infusion of acetylcholine (10, 100, and 1000 nmol/min each dose for 5 min) downstream to dilate the resistance vasculature. Brachial artery VTI increased in a dose-dependent manner, in both groups reaching a sustained plateau at each increment. This was associated with a step-wise dilatation of the brachial artery in both groups (Figure 3.5.3). There was no change in blood flow or dilatation of the contralateral brachial artery. There was no difference in blood flow or radial artery dilatation to this sustained flow stimulus, at any level, between the hypercholesterolaemic and control groups. There was no significant difference in the gradient of the dose responses between the two groups (73.8 ± 16.6%/m vs. 59.8 ± 18.0%/m, $P = 0.4$) or in the calculated blood flow which would result in a 7% dilatation (0.18 ± 0.02 m vs. 0.19 ± 0.02 m, $P = 0.4$). There was no correlation between the slope of the FMD/VTI curves and cholesterol level. On multiple linear regression analysis, brachial artery...
dilatation was significantly associated with VTI but addition of cholesterol level to this model did not significantly influence this relationship.

Table 3.5.1 Baseline demographic and vascular function in control and hypercholesterolaemic subjects

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>HC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (% male)</td>
<td>8 (76)</td>
<td>8 (88)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>33 ± 1.7</td>
<td>38 ± 1.7</td>
<td>0.08</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.35 ± 0.28</td>
<td>6.96 ± 0.45</td>
<td>0.0002</td>
</tr>
<tr>
<td>Basal VTI (m)</td>
<td>0.058 ± 0.010</td>
<td>0.075 ± 0.014</td>
<td>0.32</td>
</tr>
<tr>
<td>Cuff up VTI (m)</td>
<td>0.015 ± 0.007</td>
<td>0.014 ± 0.002</td>
<td>0.82</td>
</tr>
<tr>
<td>Peak VTI (m)</td>
<td>0.406 ± 0.038</td>
<td>0.333 ± 0.017</td>
<td>0.78</td>
</tr>
<tr>
<td>Peak RH (%)</td>
<td>800 ± 163</td>
<td>562 ± 111</td>
<td>0.25</td>
</tr>
<tr>
<td>RH AUC</td>
<td>12.61 ± 1.72</td>
<td>12.47 ± 1.23</td>
<td>0.95</td>
</tr>
<tr>
<td>Baseline diameter (mm)</td>
<td>4.11 ± 0.16</td>
<td>4.45 ± 0.28</td>
<td>0.34</td>
</tr>
<tr>
<td>Constriction (%)</td>
<td>-0.24 ± 0.43</td>
<td>-0.74 ± 0.27</td>
<td>0.34</td>
</tr>
<tr>
<td>FMD max (%)</td>
<td>7.4 ± 0.7</td>
<td>3.9 ± 0.8</td>
<td>0.01</td>
</tr>
<tr>
<td>t max (sec)</td>
<td>54.4 ± 11.5</td>
<td>58.8 ± 11.7</td>
<td>0.46</td>
</tr>
<tr>
<td>FMD AUC</td>
<td>637 ± 107</td>
<td>298 ± 127</td>
<td>0.06</td>
</tr>
<tr>
<td>Ddil/Dt (%/s)</td>
<td>0.32 ± 0.02</td>
<td>0.21 ± 0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

VTI = velocity time integral, RH = reactive hyperaemia, RH AUC = area under the curve of the reactive hyperaemic flow envelope, FMD max = maximal flow-mediated dilatation, t max = time to maximum dilatation, FMD AUC = area under the curve of flow-mediated dilatation, Ddil/Dt = gradient of the dilatation/time curve. Data is mean ± S.E.M. Groups were compared using independent students t-tests.
Figure 3.5.2 Flow-mediated dilatation in response to reactive hyperaemia in hypercholesterolaemic subjects and controls. FMD was significantly attenuated in subjects with hypercholesterolaemia (a) and there was a strong inverse correlation between total cholesterol and FMD (b).
Figure 3.5.3 Flow-mediated dilatation in response to sustained flow, induced by distal infusion of an incremental dose acetylcholine, in hypercholesterolaemic subjects and controls. The velocity time integral (VTI) of blood flow increased in a stepwise fashion to reach steady state at each concentration (upper panel). This was associated with a contemporaneous and dose-dependent dilatation of the brachial artery. Neither the flow response to acetylcholine or dilatation to this sustained flow stimulus were altered by infusion of the nitric oxide inhibitor L-NMMA.
3.5.4 Discussion

These data demonstrate that FMD in response to reactive hyperaemia and sustained blood flow are differentially affected in hypercholesterolaemia suggesting that hypercholesterolaemia selectively affects the NO pathway with sparing of the dilator mechanisms which underlie dilatation to sustained flow increases.

In healthy volunteers, there was a linear relationship between flow and brachial artery dilatation. The acetylcholine stimulus itself was not responsible for dilatation, because acetylcholine was infused distal to the site at which brachial artery diameter was measured and it has a very short half-life in vivo.

FMD, following reactive hyperaemia, was significantly impaired in subjects with hypercholesterolaemia compared to controls and a close correlation between FMD and plasma cholesterol was seen. These data are in accord with previous studies (Sorensen et al., 1994). However, following a sustained flow stimulus, similar brachial artery dilatation was seen in hypercholesterolaemic subjects and controls. There was no significant difference in the flow/dilatation response curves in these groups and no correlation between the slope of the flow/dilatation curves and plasma cholesterol level. This study did not specifically investigate the dependence of dilatation in response to sustained flow, induced by infusion of acetylcholine, on NO synthesis. Further experiments will be required to establish this and whether abnormalities of FMD in other risk factor groups and patients with established cardiovascular disease are also limited to the response to reactive hyperaemia.

These data have implications for the understanding of vascular damage in hypercholesterolemia and for the future design of studies to examine endothelial function in risk factor groups, which will need to concentrate on functions related to the NO pathway. Although a large number of effects of hypercholesterolaemia on endothelial function have been reported, including impaired vasomotion, expression of adhesion molecules increased oxidative stress and growth, a specific abnormality of NO metabolism might be important in view of its antiatherogenic properties. It is possible that such actions are mediated by the phasic release of NO in response to
rapidly changing shear stress and therefore therapeutic interventions to replace NO therapy may need to mimic this action.
CHAPTER 4

DETERMINANTS OF VASCULAR FUNCTION AND

STRUCTURE IN A POPULATION OF NON-INDUSTRIAL CIVIL

SERVANTS
4.1 INTRODUCTION

4.1.2 Glucose intolerance and insulin resistance as a risk factor for cardiovascular disease

A number of large longitudinal population based studies have reported a positive association between elevated plasma glucose and insulin levels and cardiovascular morbidity and mortality. (Despres et al., 1996; Fontbonne et al., 1991; Fuller et al., 1980; Gerstein and Yusuf, 1996; Perry et al., 1996; Singer et al., 1992). In the Honolulu Heart study nearly 80% of men, of Japanese ancestry, had evidence of impaired glucose metabolism after an oral glucose load (Donahue et al., 1987). Over 12 years of follow-up the attributable risk of coronary artery disease was equivalent to that of diabetes but, because of its high prevalence, on a population basis glucose intolerance was associated with nearly three times as many deaths as frank diabetes and therefore represents a major public health issue.

Fasting hyperglycaemia is thought to represent decreased insulin sensitivity and is associated with compensatory hyperinsulinemia (Ferrannini et al., 1991), central obesity, raised systolic blood pressure (Ferrannini et al., 1987) and an atherogenic lipoprotein profile (Grundy, 1997); the latter characterised by elevated levels of triglycerides, a predominance of small dense low-density-lipoprotein (LDL) particles and reduced high-density lipoprotein (HDL) cholesterol levels (Grundy, 1997). This clustering of risk factors, first described by Reaven (1988), has been termed the metabolic syndrome or insulin resistance syndrome (DeFronzo and Ferrannini, 1991) and appears to effect up to 10% of healthy working individuals (Brunner et al., 1997).

The mechanisms which underlie this clustering of metabolic abnormalities and the nature of the association of insulin resistance with later cardiovascular disease is not clear and remains controversial (Pinkney et al., 1997). Insulin resistance as reflected by high fasting insulin levels or glucose intolerance might itself be involved in the pathogenesis of atherosclerosis or alternatively it might be related to atherosclerosis
only through association with established risk factors as part of the metabolic syndrome. In such subjects cardiovascular risk might represent the cumulative effects of the multiple risk factors acting on the vascular wall or synergy between components of metabolic syndrome might enhance risk further, whereby the presence of metabolic syndrome itself represents an independent risk factor.

Alternatively insulin resistance might have a direct role in promoting vascular disease and a number of potential mechanisms exist. Hyperinsulinaemia and/or hyperglycaemia might have a direct effect on the vascular wall altering both structure and function. Insulin and its precursors are potent growth factors and the hyperinsulinaemia associated with insulin resistance might be an important factor that promotes medial hypertrophy and development of the fibroproliferative plaque (Stout, 1992). Similarly, hyperglycaemia and the formation of advanced glycosylation end products result in increased oxidative stress (Williams et al., 1998), enhanced lipid peroxidation (Bucala et al., 1993), nitric oxide inactivation and direct cellular damage (Bucala et al., 1991).

Insulin, in addition to its metabolic actions on glucose uptake, is a vasodilator and stimulates synthesis of nitric oxide by the endothelium (Baron et al., 1990; Scherrer et al., 1994) and at physiological levels reduces large artery stiffness (Westerbacka et al., 1999). These actions are likely to be important physiological mechanisms for regulating the distribution of hormone and substrate to peripheral tissues. In insulin resistance states reduced insulin mediated NO synthesis and increased arterial stiffness might be important mechanisms that facilitate atherogenesis (Baron, 1996; Taddei et al., 1995) and contribute to reduced glucose uptake and thus glucose intolerance (Baron, 1996; Pinkney et al., 1997). However the association between glucose uptake and insulin mediated vasodilatation has not been universally reported (Utriainen et al., 1996) and whether insulin acts directly on endothelial cells to stimulate NO release is not known.

Finally insulin resistance and atherosclerosis might represent two distinct parallel pathophysiological processes with a common antecedent aetiological factor. Endothelial dysfunction in this situation might occur as a consequence of associated hypertension (Suzuki et al., 1996), or an atherogenic lipoprotein profile (Steinberg et
al., 1997). One such common denominator might be low birth weight which has been associated with hypertension (Law and Barker, 1994) and insulin resistance (Phillips et al., 1994), impaired endothelial function (Goodfellow et al., 1998; Leeson et al., 1997) and cardiovascular morbidity and mortality in later life (Barker, 1993).

Recent reports have demonstrated a positive association between components of the metabolic syndrome, particularly obesity, and inflammatory mediators including C-reactive protein and interleukin-6 (Festa et al., 1999; Festa et al., 2000; Yudkin et al., 1999) and deficiencies of coagulation and fibrinolysis (Agewall, 1999; Bastard et al., 2000; Meigs et al., 2000). Such inflammatory responses are known to impair endothelial function (Bhagat and Vallance, 1997; Hingorani, 2000) and elevated levels of acute phase reactants have been linked to the presence of atherosclerosis and to the later incidence of cardiovascular morbidity and mortality (Ridker et al., 2000a; Ridker et al., 2000b). The mechanism of the association between inflammation and the metabolic syndrome remains unclear but it is possible that this mediates the increased risk of cardiovascular disease. The generation of IL6 is increased following a carbohydrate load (Mohamed-Ali et al., 1997) and Yudkin and colleagues (1999) have proposed that this might be a unifying mechanism that underlies both the genesis of an acute phase response that promotes atherogenesis and the development insulin resistance.

Determining the impact of the MS and its component parts on the vascular wall before the emergence of clinical vascular disease, will be important in advancing understanding of the mechanisms of increased cardiovascular risk in this large section of the population who are at risk of premature cardiovascular disease.

4.1.2.1 Insulin resistance and vascular dysfunction.

A number of previous studies have looked for evidence of endothelial dysfunction in patients with insulin resistance, with contradictory findings. Petrie et al., 1996 reported an association between insulin sensitivity and basal (Petrie et al., 1996), but not stimulated, NO production in forearm resistance vessels a finding that was supported by the results of others (Tack et al., 1998; Utriainen et al., 1996). Steinberg and colleagues found reduced leg blood flow in response to infraradial
infusions of metacholine in obese, insulin resistant subjects (Steinberg et al., 1996). However, in this small study, patients and controls were not comparable for obesity or blood pressure and it is possible that the effects of insulin resistance were confounded by the effects of these associated risk factors or differences in the metabolism of metacholine related to subjects' physical characteristics (Chowienczyk et al., 1994). In a recent study, in patients with hypertension, no difference in vascular function was found in matched insulin sensitive or resistant subjects (Natali et al., 1997). Furthermore, the recent finding that enhanced insulin sensitivity following treatment with the insulin sensitising drug troglitazone did not improve endothelial function (Tack et al., 1998) does not support an association between insulin resistance and endothelial function. Few studies have examined the impact of insulin resistance or glucose tolerance on conduit artery endothelial function, which is likely to be more relevant to progression of clinical atherosclerosis.

A number of studies have also examined the relationship between insulin resistance and conduit artery elasticity. Elevated pulse wave velocity, as an indirect marker of arterial stiffness, has been demonstrated in patients with diabetes mellitus (Lehmann et al., 1992). Arterial distensibility is associated with plasma insulin levels (Neutel et al., 1992) and increased in patients with hypertension (Benetos et al., 1993). In the “Atherosclerosis in the Community Study” significant associations between fasting insulin and glucose levels and carotid artery distensibility were apparent though it is not clear whether these associations were independent of the effects of other risk factors including lipid levels and hypertension (Salomaa et al., 1995). In non-diabetic women, van Popele and colleagues (2000) demonstrated significant associations between carotid artery distensibility and a number of metabolic variables related to insulin resistance including insulin levels, obesity, triglyceride levels and HDL cholesterol levels, which were independent of blood pressure.

### 4.1.2 Assessment of insulin resistance

Insulin sensitivity is defined as the change in glucose elimination rate for a given change in insulin concentration. A number of experimental techniques have been developed to determine insulin sensitivity in vivo, however in clinical practice the
precise quantification of insulin resistance remains difficult (Stevenson and Godsland, 1997). The hyperinsulinaemia euglycaemic clamp method uses a continuous infusion of insulin at a predetermined rate. Glucose is infused and the rate adjusted until steady state is achieved. At this point the rate of infusion will be equivalent to the glucose uptake and when divided by the insulin level will reflect the sensitivity of tissues to insulin at that concentration. However the level of insulin required is non-physiological and this method tends to overestimate true insulin sensitivity. Alternatively an intravenous glucose tolerance test can be used to estimate insulin sensitivity using the minimal model of glucose disappearance (Swan et al., 1994). This method uses complex mathematical modelling to relate change in glucose levels to change in insulin. It has the advantage of being physiological in that it uses the bodies own pancreatic response to hyperglycaemia but like the hyperinsulinaemic clamp is time consuming. In large studies a single fasting measure of plasma insulin concentration has been used to give an approximation of insulin sensitivity (Folsom et al., 1999) though the response to an oral glucose load is probably a better reflection of true insulin sensitivity (Eschwege and Fontbonne, 1992). Recently Matsuda and colleagues have described a method for estimating whole insulin sensitivity, based on results of an oral glucose tolerance test which correlates closely to “true” insulin sensitivity measured by hyperinsulinaemic euglycaemic clamp (Matsuda and DeFronzo, 1999).

4.1.3 Study aims

1. To examine the impact of the metabolic syndrome on vascular function in middle aged men and women with no prior history of cardiovascular disease.
2. To examine whether insulin resistance and glucose intolerance directly impact on vascular function, independently of associated risk factors.
3. To examine associations between markers of chronic inflammation and vascular function.
4. To use a multivariate model to examine clustering of metabolic variables and their relationship with vascular function.
4.2 METHODS AND DATA COLLECTION

4.2.1 The Whitehall II study

The Whitehall study began in 1967 and originally recruited 18,403 British civil servants, examining the relationship between cardiovascular risk factors and subsequent morbidity and mortality (Fuller et al., 1980). A steep inverse association between social class and mortality from a wide range of diseases was one of the main findings (Marmot et al., 1984). The Whitehall II study was designed to investigate the causes of the social gradient in a new cohort of 10,304 civil servants recruited in 1985 (Marmot et al., 1991). Between November 1985 and March 1988 participants completed a detailed questionnaire and attended a screening examination (phase 1, baseline). The questionnaire included details on the prevalence of concurrent disease, socio-economic status and lifestyle. A range of anthropometric measurements were recorded and risk factors determined at the screening examination.

During 1991-1993, 8355 (83%) of participants took part in the third phase of data collection (phase 3) when additional risk factors including glucose tolerance and insulin were measured. Phase 5 of the Whitehall II study commenced in 1997, when all participants were invited back for a further detailed assessment.

4.2.2 Study design

Participants who attended the phase 5 follow-up examination (1997 – 1998) with no history of clinical cardiovascular disease who were non-smokers and not treated diabetics were identified. From this large group three separate study groups were selected.

1. Subjects with the metabolic syndrome (MS), defined as being in the adverse quintile for 3 out of 5 of waist-hip ratio, 2 hour post OGTT glucose concentration, systolic blood pressure, plasma triglyceride and HDL cholesterol level, were identified (Brunner et al., 1997).

2. A large unselected (other than no history of clinical cardiovascular disease, non-smokers and not treated diabetics) group individuals with a range of risk factors
but who did not satisfy the criteria for diagnosis of the MS were recruited as a “control” (C) sample of the general study population.

3. To examine the effect of glucose tolerance and insulin resistance on vascular function. Individuals, with a phase 3 (1991-1993), 2 hour post OGTT glucose concentration in the lowest quintile or highest quintile, were defined as glucose tolerant (GT) and glucose intolerant (GI) respectively. Subjects whose HDL-cholesterol level, fasting triglyceride level, systolic blood pressure and waist-hip ratio were in the adverse quintile were excluded to avoid the confounding effects of these risk factors.

4.2.2.1 Blinding and selection

All subjects who attended the phase 5 examination and satisfied the criteria for one of the groups above were invited, by letter, to attend for further detailed assessment of vascular function. Selection of subjects was performed independently such that investigators having further contact with the subjects were blinded to the subjects group. The local research ethics committee approved this study and all subjects gave written informed consent.

4.2.3 Power calculations

Previous studies have demonstrated wide differences in endothelial function between risk factor groups and healthy controls. On the basis of these studies power calculations determined that 60 subjects in each group would be necessary to detect a half standard deviation difference between the GT and GI groups.

4.2.4 Data collection

Participants in the Whitehall II study, had a detailed assessment at each stage of the study including a questionnaire and screening examination. The questionnaire included details on current civil service employment grade (divided into 6 levels) which was used as a measure of socio-economic status. Smoking was categorised (never, ex and current) and quantified by packyears.
4.2.4.1 Blood pressure and anthropometry

At each assessment blood pressure was measured twice, in the sitting position after 5 minutes rest, with the Hawksley random-zero sphygmomanometer. Hypertension was defined as systolic blood pressure ≥ 160mmHg and / or diastolic blood pressure ≥ 90mmHg or on antihypertensive medication. Height and weight were measured, and body mass index (BMI) was calculated as weight (Kg) / height (m)^2. Waist circumference (smallest circumference at or below the costal margin) and hip circumference (at the level of the greater trochanter of the right femur) were recorded with subjects in the standing position, unclothed using a fiberglass tape measure at 600 g tension. Waist-hip ratio was calculated as the ratio of the two circumference measurements.

4.2.4.2 Assessment of insulin resistance

Glucose tolerance was defined on the basis of a 75g oral glucose tolerance test (OGTT) (Brunner et al., 1997). Fasting and 2 hour post OGTT plasma glucose (electrochemical glucose oxidase) and insulin (radioimmunoassay using polyclonal guinea pig antiserum) levels were measured. Whole body insulin sensitivity was inferred by calculation of an insulin sensitivity index (ISI) as recently described (Matsuda and DeFronzo, 1999). ISI = 10,000/square root of ([fasting glucose x fasting insulin] x [mean glucose x mean insulin during OGTT])

A fasting venous sample of blood was drawn from each subject. Cholesterol concentration was determined by cholesterol oxidase-peroxidase colorimetric method (Boehringer Mannheim) and triglycerides by enzymatic colorimetry. High-density lipoprotein (HDL) cholesterol was determined after dextran sulphate-magnesium chloride precipitation of non-HDL cholesterol. LDL cholesterol was calculated using the Friedewald formula (Friedwald et al., 1972).

4.2.4.4 Inflammatory and coagulation factors

Levels of von Willebrand factor (vWF), plasminogen activator inhibitor type-1 (PAI1) C-reactive protein (CRP), interleukin-6 (IL6) and serum amyloid A (SAA)
were measured using ELISA. Fibrinogen levels were measured using a modification of the clotting method of Clauss (Oosting and Hoffmann, 1997).

4.2.4.5 Risk factors and cardiovascular risk index (CVRI)

To analyse overall differences in risk factors and metabolic measurements between the groups, a ‘z-score’ was calculated for each variable and the risk burden for each subject, in relation to the population as a whole, determined as the mean z-score for the risk factors (age, total, HDL and LDL cholesterol level, triglyceride level and systolic and diastolic blood pressure) and insulin resistance (fasting and two-hour post OGTT glucose and insulin level and the ISI) calculated.

In addition, a composite cardiovascular risk index (CVRI) (including age, gender, systolic blood pressure, presence of diabetes, and smoking status) was calculated for each subject, for phase 3 and phase 5 data, using an algorithm derived from the Framingham database (Anderson et al., 1991). Whilst this algorithm does not take into account many of the more recently recognised risk factors it has proven a reliable predictor of risk and risk reduction following intervention in previous studies (Haq et al., 1999).

4.2.5 Assessment of vascular function

Brachial artery flow-mediated dilatation (FMD) and carotid artery distensibility and intima media thickness were measured non-invasively using high-resolution external ultrasound as previously described. All studies were performed in a temperature controlled laboratory after subjects had been resting for at least 10 minutes.

4.2.5.1 Assessment of brachial artery endothelial function

Brachial artery flow-mediated dilatation and response to glyceryl trinitrate were assessed in the right arm using the standard protocol. Brachial artery reactivity was measured over the whole time course of the experiment using the digital edge detection software. A 5 minute period of forearm ischemia was used to stimulate reactive hyperaemia and GTN (400 μg sublingually) used as the stimulus for endothelium-independent dilatation. Blood flow velocity was estimated using pulsed
wave Doppler at rest, prior to release of the cuff and at 15 second intervals following its release.

Baseline brachial diameter was determined as the average of all measurements made over the first minute of rest prior to cuff inflation or administration of GTN. Following release of the tourniquet images acquired over 12 second intervals (4 frames) were averaged whilst after administration of GTN the mean vessel diameter between 180 and 240 seconds was used as the measure of endothelium-independent dilatation. The maximal flow-mediated dilatation (FMD) and dilatation to GTN were expressed as a percentage in relation to their respective baselines. Peak reactive hyperaemia (VTI\text{peak}) and the AUC of reactive hyperaemia (VTI\text{AUC}) over the first 60 seconds were calculated as a measure of the stimulus to the endothelium.

4.2.5.2 Assessment of carotid artery distensibility coefficient (DC)

The common carotid artery was imaged in longitudinal section. A segment of the artery, 0.5 to 1 cm below the carotid bulb, was selected and once a stable and clear image was achieved, 5 seconds of radio-frequency signal from this segment routed to an A-mode tracking device (Ingenious Systems, The Netherlands) where the data were stored for later analysis. Three measurements were acquired from each common carotid artery. Scans in which a satisfactory distensibility waveform was not achieved were rejected and repeated. On completion of each study, the stored radio frequency data for each scan were analysed by placement of volume sample cursors, at the anterior and posterior vessel wall interfaces. Arterial wall movement was tracked and vessel diameter and distension determined on a beat by beat basis. Blood pressure was determined in the left arm, contemporaneously with the measurements of carotid distension, using an automatic oscillometric sphygmomanometer.

Mean left and right carotid artery distension were calculated from the three measurements made on each side of the neck and the carotid artery distensibility coefficient (DC) expressed as the percentage change in cross-sectional area per mmHg change in blood pressure (Section 2.2.5.3) (Reneman et al., 1996).
4.2.5.2 Measurement of carotid intima-media thickness

Longitudinal and short axis images of the common carotid artery, one centimetre proximal to the carotid bulb, in which the far wall intima-media interface (M-line) was clearly defined were magnified and recorded on video tape for later analysis. For each image the distance between the leading edge of the intima and the adventitia was measured at four separate sites, using ultrasonic calipers, and the results averaged. In view of the greater propensity to underestimate IMT, the greatest measurement of the short axis and long axis views was taken as previously described (Lee et al., 1995). IMT was measured in both the right and the left common carotid artery and the results meaned.

4.2.7 Statistical methods

All data were analysed using SPSS version 8.0.2. Variables with a normal distribution are expressed as the mean ± standard deviation. Skewed data were normalised by logarithmic transformation, prior to statistical analyses and are expressed as median (range). Significance was inferred at P values less than 0.05. Specific statistical methods pertinent to the study hypotheses are detailed below in the relevant section and are not repeated here.

Independent student t-tests were used to compare differences in metabolic variables and vascular function between MS subjects and controls and between the GT and GI groups. Analysis of variance was used to examine differences between GI and GT subjects and control and MS subjects.

Multiple regression analyses were used to explore relationships between risk variables, measured during phase 3 (1991 – 1993) and during phase 5 (1997 – 1998) and the main dependent variables of brachial artery FMD, carotid artery distensibility and IMT. Analyses were performed initially in the C subjects and in GT and GI subjects (as a single group) separately and then in the whole cohort including subjects with MS. In the case of FMD, initial exploratory analyses were performed to determine which flow variable best accounted for FMD and then this variable was entered into the model to correct for any differences in reactive hyperaemia between
subjects. Age and subject gender were entered into each model and the independent variables added one at a time to determine their influence on the dependent variable.

Bivariate Pearson correlation coefficients were used to examine relations between levels of inflammatory mediators and other contemporaneously measured risk variables. Analysis of variance was used to examine differences in levels of inflammatory mediators between the separate study groups. Multiple regression analyses similar to those above were then used to examine the relationship between individual inflammatory factors and vascular function. The CVRI was added to the models to test whether significant associations were independent of the influence of classical risk factors.

Finally because of the complex interrelationships which exist between metabolic, physical and inflammatory variables, factorial analysis was used to identify major factors which underlie these interrelationships and these were related to the measures of vascular function and structure. Specific details of these methods are given in section 4.3.5.

4.3 RESULTS

4.3.1 Recruitment

480 eligible participants were identified of which 290 agreed to take part (65 GT, 50 GI, 34 MS and 141 C). There were no differences in the demographic or risk factor profile of subjects who declined to take part from the study group. The majority of subjects were Caucasian (88%), 8% Asian and 4% Afro-Caribbean.

Thirty-four subjects with the metabolic syndrome were studied. Metabolic syndrome was classified on the basis of having 3 out of 5 adverse risk factors (Brunner et al., 1997). Table 4.3.1 gives the incidence of each factor as a criterion for inclusion within this group. Four subjects were in the adverse quintile for all 5 criteria, 9
subjects satisfied 4 criteria and the remainder had only 3 criteria. There was a predominance of women in this group, in contrast to the control group.

Table 4.3.1 Frequency of adverse criterion in metabolic syndrome subjects

<table>
<thead>
<tr>
<th>Metabolic syndrome criterion</th>
<th>No of subjects in adverse quintile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>31</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>9</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>31</td>
</tr>
<tr>
<td>2hr OGTT glucose</td>
<td>15</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>15</td>
</tr>
</tbody>
</table>

4.3.2 Risk profiles

The subjects characteristics from the phase 3 (1991 – 1993) and phase 5 (1997 – 1998) assessments are given in Table 4.3.2 and Table 4.3.3 respectively. There were marked differences in risk factor parameters between MS subjects and control subjects at both assessments with significantly higher mean z-scores for both risk factors and insulin resistance and the calculated CVRI in MS subjects (Figure 4.3.1).

Between the GT and GI groups there were significant differences in the phase 3 fasting glucose and post OGTT glucose and insulin level, the ISI and z-score for insulin resistance but not fasting insulin level (Table 4.3.2). Phase 3 levels of classical risk factors were similar between the GT and GI groups though when taken together there was a borderline significant difference in the mean z-score for risk factors ($P = 0.05$). On analysis of the contemporary phase 5 data, significant differences in both the post OGTT glucose and insulin level and ISI remained evident but there were no significant differences in levels of any other of the measured risk factors, the CVRI or the mean risk factor z-score between the GT and GI subjects indicating that these two groups were comparable other than for glucose tolerance and insulin resistance (Table 4.3.3).
Compared to the control population GI subjects were more insulin resistance and glucose intolerant whilst the reverse was true for GT subjects (Figure 4.3.1). However, by design GT and GI subjects generally had lower levels of classical cardiovascular risk factors, with significant differences in the phase 3 systolic (P < 0.001) and diastolic (P = 0.001) blood pressure and the CVRI (P = 0.02) (Table 4.3.3). The risk factor z-score was significantly lower in GT and GI subjects than in the controls (P = 0.01) (Figure 4.3.1).

4.4.3.1 Tracking of risk factors.

The Pearson correlation coefficient between risk factors measured at phase 3 and phase 5 are outlined in Table 4.3.4. Levels of all the parameters from phase 3 were significantly associated with those at phase 5 indicating tracking of individual risk factors. In addition there was a close association between the overall assessment of risk profile using either z-scores or the CVRI. However the strength of the relationship varied being strongest for anthropometric measures (BMI r = 0.93) and weakest for measures of glucose metabolism and insulin resistance (r values 0.43 – 0.57).

4.3.3 Vascular function.

4.3.3.1 General results

Complete flow data, for reactive hyperaemia, was available on 285 out of the 290 scans. Using Pearson correlation coefficients, FMD\textsubscript{max} was most closely associated with VTI\textsubscript{peak} (r = 0.31, P = 0.001), with a progressively decreasing association with VTI’s measured at 15 second intervals from cuff release. There was no association with percent reactive hyperaemia.

Measurements of vascular function in the right and left carotid artery were significantly correlated with each other (DC r = 0.59, P < 0.001; IMT r = 0.51, P < 0.001), supporting the accuracy of these methods and the use of averaged results in analyses. However, overall IMT was low (0.70 ± 0.16 mm) compared to previous population studies and this is likely to reflect the healthy nature of this group.
Table 4.3.2 Subject characteristics phase 3 (1991 - 1993)

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MS</th>
<th>P</th>
<th>GT &amp; GI</th>
<th>GT</th>
<th>GI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>141</td>
<td>34</td>
<td></td>
<td>115</td>
<td>65</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.9 ± 5.2</td>
<td>46.8 ± 6.1</td>
<td>0.05</td>
<td>44.3 ± 5.2</td>
<td>43.4 ± 5.5</td>
<td>45 ± 4.5</td>
<td>0.06</td>
</tr>
<tr>
<td>% male</td>
<td>60</td>
<td>32</td>
<td>0.001</td>
<td>66</td>
<td>67</td>
<td>64</td>
<td>0.7</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.21 ± 0.45</td>
<td>5.44 ± 0.59</td>
<td>0.04</td>
<td>5.17 ± 0.54</td>
<td>5.08 ± 0.55</td>
<td>5.29 ± 0.51</td>
<td>0.04</td>
</tr>
<tr>
<td>Fasting insulin (µU/ml)</td>
<td>5.1 (1.2 - 20.9)</td>
<td>10.1 (2.2 - 30.5)</td>
<td>&lt; 0.001</td>
<td>5.1 (1.0 - 22.1)</td>
<td>5.0 (1.0 - 22.1)</td>
<td>5.4 (1.0 - 20.9)</td>
<td>0.7</td>
</tr>
<tr>
<td>2hrOGTT glucose (mmol/l)</td>
<td>5.18 (2.71 -10.0)</td>
<td>6.40 (2.44 -11.8)</td>
<td>&lt; 0.001</td>
<td>4.2 (1.90 -13.50)</td>
<td>3.70 (1.90 -5.10)</td>
<td>7.22 (6.60 -13.5)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2hr OGTT insulin (µU/ml)</td>
<td>37.4 (2.6 -304)</td>
<td>72.7 (7.2 -213)</td>
<td>&lt; 0.001</td>
<td>33.2 (2.5 -181.2)</td>
<td>15.5 (2.50 -173.7)</td>
<td>57.8 (23.0 -181.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>1.3 (0.2 -11.6)</td>
<td>0.6 (0.2 -4.6)</td>
<td>0.008</td>
<td>1.6 (0.3 -15.4)</td>
<td>2.6 (0.7 -15.4)</td>
<td>0.88 (0.3 -2.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.26 ± 1.87</td>
<td>6.87 ± 1.07</td>
<td>0.006</td>
<td>6.36 ± 1.0</td>
<td>6.26 ± 1.02</td>
<td>6.49 ± 0.96</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.49 ± 0.41</td>
<td>1.09 ± 0.27</td>
<td>&lt; 0.001</td>
<td>1.58 ± 0.35</td>
<td>1.58 ± 1.03</td>
<td>1.57 ± 0.32</td>
<td>0.9</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>4.21 ± 1.12</td>
<td>4.55 ± 1.08</td>
<td>0.10</td>
<td>4.32 ± 0.95</td>
<td>4.25 ± 0.98</td>
<td>4.42 ± 0.92</td>
<td>0.3</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.04 (0.34 -3.53)</td>
<td>2.23 (0.79 -6.2)</td>
<td>&lt; 0.001</td>
<td>0.93 (0.33 -2.05)</td>
<td>0.90 (0.42 -1.77)</td>
<td>1.03 (0.33 -2.05)</td>
<td>0.1</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>120.2 ± 13.0</td>
<td>123.8 ± 13.1</td>
<td>0.2</td>
<td>113.0 ± 10.5 *</td>
<td>112.0 ± 10.5</td>
<td>114.2 ± 10.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>79.4 ± 9.3</td>
<td>80.9 ± 9.0</td>
<td>0.4</td>
<td>75.5 ± 7.8 *</td>
<td>74.5 ± 7.7</td>
<td>76.7 ± 7.9</td>
<td>0.1</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>24.5 ± 3.4</td>
<td>29.2 ± 3.7</td>
<td>&lt; 0.001</td>
<td>24.3 ± 3.2</td>
<td>24.2 ± 2.6</td>
<td>24.3 ± 3.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.85 ± 0.09</td>
<td>0.96 ± 0.1</td>
<td>&lt; 0.001</td>
<td>0.84 ± 0.08</td>
<td>0.84 ± 0.09</td>
<td>0.85 ± 0.10</td>
<td>0.9</td>
</tr>
<tr>
<td>CVRI (%)</td>
<td>6.8 ± 5.6</td>
<td>11.2 ± 6.1</td>
<td>&lt; 0.001</td>
<td>5.4 ± 3.4 *</td>
<td>5.1 ± 3.6</td>
<td>5.8 ± 3.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Mean risk factor z score</td>
<td>0.02 ± 0.62</td>
<td>0.76 ± 0.58</td>
<td>&lt; 0.001</td>
<td>-0.26 ± 0.45 *</td>
<td>-0.33 ± 0.44</td>
<td>-0.16 ± 0.44</td>
<td>0.05</td>
</tr>
<tr>
<td>Mean IR z score</td>
<td>-0.03 ± 0.61</td>
<td>0.60 ± 0.75</td>
<td>&lt; 0.001</td>
<td>-0.08 ± 0.45</td>
<td>-0.60 ± 0.59</td>
<td>0.40 ± 0.46</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Data is mean ± standard deviation except in the case of skewed data when median and range are presented. The main comparisons were between metabolic syndrome (MS) subjects and controls and glucose tolerant (GT) and intolerant (GI) subjects (independent t-tests). Analysis of variance with Bonferroni adjustments for multiple tests was used for all other comparisons (* P < 0.05 compared to the control population).
<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MS</th>
<th>P</th>
<th>GT &amp; GI</th>
<th>GT</th>
<th>GI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>141</td>
<td>34</td>
<td>0.05</td>
<td>115</td>
<td>65</td>
<td>50</td>
<td>0.06</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.9 ± 5.4</td>
<td>52.0 ± 6.2</td>
<td>0.05</td>
<td>50.3 ± 5.2</td>
<td>49.5 ± 5.5</td>
<td>51.2 ± 4.5</td>
<td>0.06</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>5.06 ± 0.61</td>
<td>5.33 ± 0.59</td>
<td>0.05</td>
<td>5.21 ± 0.49</td>
<td>5.16 ± 0.45</td>
<td>5.28 ± 0.54</td>
<td>0.22</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>120 (60 - 700)</td>
<td>190 (90 - 420)</td>
<td>&lt; 0.001</td>
<td>108 (40 - 370)</td>
<td>110 (60 - 370)</td>
<td>110 (40 - 301)</td>
<td>0.94</td>
</tr>
<tr>
<td>2hr OGTT glucose (mmol/l)</td>
<td>5.78 (2.89 - 11.0)</td>
<td>6.71 (3.29 - 12)</td>
<td>0.004</td>
<td>5.68 (3.40 - 11.1)</td>
<td>5.23 (3.40 - 7.54)</td>
<td>6.17 (4.02 - 11.1)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2hr OGTT insulin (pmol/l)</td>
<td>480 (100 - 1940)</td>
<td>820 (360 - 1770)</td>
<td>&lt; 0.001</td>
<td>382 (60 - 1300)</td>
<td>300 (600 - 1300)</td>
<td>460 (70 - 1280)</td>
<td>0.04</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>2.39 (0.50 - 9.44)</td>
<td>1.19 (0.58 - 2.33)</td>
<td>&lt; 0.001</td>
<td>5.13 (1.53 - 19.4)</td>
<td>6.73 ± 3.58</td>
<td>5.21 ± 3.27</td>
<td>0.01</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.75 ± 1.06</td>
<td>6.54 ± 1.12</td>
<td>0.003</td>
<td>5.62 ± 0.77</td>
<td>5.52 ± 0.76</td>
<td>5.75 ± 0.77</td>
<td>0.12</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.58 ± 0.43</td>
<td>1.15 ± 0.24</td>
<td>&lt; 0.001</td>
<td>1.45 ± 1.35</td>
<td>1.41 ± 0.35</td>
<td>1.51 ± 0.35</td>
<td>0.15</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.73 ± 1.04</td>
<td>4.35 ± 0.9</td>
<td>0.02</td>
<td>3.72 ± 0.76</td>
<td>3.70 ± 0.73</td>
<td>3.76 ± 0.81</td>
<td>0.69</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.9 (0.4 - 2.1)</td>
<td>2.5 (0.9 - 3.9)</td>
<td>&lt; 0.001</td>
<td>0.9 (0.4 - 2.4)</td>
<td>0.80 (0.4 - 2.4)</td>
<td>0.90 (0.40 - 2.1)</td>
<td>0.17</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>122.3 ± 16.3</td>
<td>128.9 ± 15.9</td>
<td>0.07</td>
<td>113.7 ± 14.0</td>
<td>111.7 ± 12.0</td>
<td>115.7 ± 15.6</td>
<td>0.18</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76.7 ± 10.1</td>
<td>80.6 ± 9.5</td>
<td>0.08</td>
<td>74.3 ± 9.0</td>
<td>73.7 ± 8.3</td>
<td>75.0 ± 9.8</td>
<td>0.46</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>25.5 ± 3.9</td>
<td>30.1 ± 3.9</td>
<td>&lt; 0.001</td>
<td>24.9 ± 3.3</td>
<td>25.1 ± 3.1</td>
<td>24.7 ± 3.6</td>
<td>0.58</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.86 ± 0.09</td>
<td>0.92 ± 0.07</td>
<td>&lt; 0.001</td>
<td>0.84 ± 0.08</td>
<td>0.85 ± 0.09</td>
<td>0.84 ± 0.08</td>
<td>0.66</td>
</tr>
<tr>
<td>CVRI (%)</td>
<td>12.9 ± 7.7</td>
<td>17.4 ± 6.1</td>
<td>0.005</td>
<td>11.2 ± 5.9</td>
<td>10.8 ± 6.0</td>
<td>11.8 ± 5.8</td>
<td>0.38</td>
</tr>
<tr>
<td>Mean risk factor z score</td>
<td>-0.02 ± 0.64</td>
<td>0.80 ± 0.48</td>
<td>&lt; 0.001</td>
<td>-0.22 ± 0.45</td>
<td>-0.26 ± 0.43</td>
<td>-0.16 ± 0.48</td>
<td>0.53</td>
</tr>
<tr>
<td>Mean IR z score</td>
<td>0.04 ± 0.83</td>
<td>0.67 ± 0.64</td>
<td>&lt; 0.001</td>
<td>-0.15 ± 0.63</td>
<td>-0.30 ± 0.58</td>
<td>0.06 ± 0.66</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Data is mean ± standard deviation except in the case of skewed data when median and range are presented. The main comparisons were between metabolic syndrome (MS) subjects and controls and glucose tolerant (GT) and intolerant (GI) subjects (independent t-tests). Analysis of variance with Bonferroni adjustments for multiple tests was used for all other comparisons (* P < 0.05 compared to the control population)
Figure 4.3.1 Mean z-scores for risk factors (upper panel) and insulin resistance (lower panel) in glucose tolerant (GT), glucose intolerant (GI), metabolic syndrome (MS) and control (C) subjects. Data is mean ± SD. Differences between groups are assessed by two-way ANOVA with post hoc Bonferroni correction for multiple tests.
Table 4.3.4 Tracking of risk factors from phase 3 (1991 – 93) to phase 5 (1997 – 98).

<table>
<thead>
<tr>
<th>Variable</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>0.43</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>0.44</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2hr OGTT glucose (mmol/l)</td>
<td>0.43</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2hr OGTT insulin (pmol/l)</td>
<td>0.46</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>0.57</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>0.78</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HDLc (mmol/l)</td>
<td>0.77</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.80</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>0.67</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>0.53</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>0.93</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Pearson correlation coefficient between risk factor variables from phase 3 to phase 5. These results demonstrate close tracking of risk factors between the different assessments.
The intensity of reactive hyperaemia was significantly associated with carotid artery DC ($r = 0.2$, $P = 0.003$). After correcting for this there was no association between FMD and DC. There was a weak correlation between carotid artery IMT and DC ($r = 0.12$, $P = 0.03$) but not FMD.

### 4.3.3.2 Metabolic syndrome and controls

There were no significant differences in resting vessel size, blood flow or reactive hyperaemic blood flow between any of the groups (Table 4.3.5). However, both FMD and GTN-MD were significantly impaired in the MS subjects compared to the control population (Figure 4.3.2). Similarly carotid artery DC was significantly impaired in MS subjects compared to both controls and GT and GI subjects (Figure 4.3.3). IMT levels were generally low, emphasising the healthy nature of this population, and there was no difference in carotid artery IMT between MS subjects and controls.

### 4.3.3.3 Glucose tolerance and vascular function

In contrast to the effects of the metabolic syndrome on vascular function, there were no significant differences in FMD, endothelium-independent dilatation to GTN or carotid artery DC or IMT between the GT and GI groups (Figure 4.3.2 and Figure 4.3.3).
### Table 4.3.5 Brachial artery reactivity, carotid distensibility and intima-media thickness

<table>
<thead>
<tr>
<th>Brachial artery</th>
<th>Controls</th>
<th>MS</th>
<th>P</th>
<th>GT</th>
<th>GI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline diameter (mm)</td>
<td>4.6 ± 0.8</td>
<td>4.7 ± 0.7</td>
<td>0.37</td>
<td>4.6 ± 0.7</td>
<td>4.5 ± 0.7</td>
<td>0.48</td>
</tr>
<tr>
<td>Baseline VTI (m/s)</td>
<td>0.07 ± 0.04</td>
<td>0.08 ± 0.04</td>
<td>0.40</td>
<td>0.07 ± 0.04</td>
<td>0.08 ± 0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>RH VTI_{peak} (m/s)</td>
<td>0.33 ± 0.09</td>
<td>0.31 ± 0.09</td>
<td>0.34</td>
<td>0.33 ± 0.10</td>
<td>0.33 ± 0.09</td>
<td>0.65</td>
</tr>
<tr>
<td>RH VTI_{AUC}</td>
<td>2.72 ± 0.93</td>
<td>2.83 ± 0.80</td>
<td>0.54</td>
<td>3.15 ± 0.88</td>
<td>2.80 ± 0.80</td>
<td>0.20</td>
</tr>
<tr>
<td>FMD_{max} (%)</td>
<td>5.1 ± 2.7</td>
<td>4.0 ± 2.3</td>
<td>0.02</td>
<td>4.7 ± 3.3</td>
<td>4.5 ± 3.5</td>
<td>0.68</td>
</tr>
<tr>
<td>GTN-MD (%)</td>
<td>15.4 ± 5.4</td>
<td>13.0 ± 4.1</td>
<td>0.02</td>
<td>14.8 ± 4.4</td>
<td>16.1 ± 6.4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

### Carotid artery measurements

<table>
<thead>
<tr>
<th>Carotid artery measurements</th>
<th>Controls</th>
<th>MS</th>
<th>P</th>
<th>GT</th>
<th>GI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (mm)</td>
<td>7.0 ± 1.1</td>
<td>7.5 ± 0.9</td>
<td>0.005</td>
<td>6.5 ± 0.7</td>
<td>6.6 ± 0.7</td>
<td>0.56</td>
</tr>
<tr>
<td>DC (mmHg)</td>
<td>0.31 ± 0.12</td>
<td>0.21 ± 0.06</td>
<td>&lt; 0.001</td>
<td>0.35 ± 0.11</td>
<td>0.34 ± 0.09</td>
<td>0.48</td>
</tr>
<tr>
<td>IMT (mm)</td>
<td>0.70 ± 0.16</td>
<td>0.72 ± 0.16</td>
<td>0.45</td>
<td>0.68 ± 0.16</td>
<td>0.72 ± 0.18</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Abbreviations. FMD = flow-mediated dilatation, GTN-MD = glyceryl trinitrate mediated dilatation, VTI = velocity time integral, DC = distensibility coefficient, IMT = intima-media thickness. The main comparisons were between metabolic syndrome (MS) subjects and controls and glucose tolerant (GT) and intolerant (GI) subjects (independent t-tests).
Figure 4.3.2 Flow-mediated dilatation and dilatation to glyceryl trinitrate (mean ± SD) in glucose tolerant (GT), glucose intolerant (GI), metabolic syndrome (MS) and control (C) subjects.
Figure 4.3.3 Distensibility coefficient (DC, mean ± SD) in glucose tolerant (GT), glucose intolerant (GI), metabolic syndrome (MS) and control (C) subject.
4.3.4 Determinants of vascular function

4.3.4.1 Flow-mediated dilatation

Linear regression analyses were used to assess the relationships between metabolic parameters and vascular function. FMD was significantly associated with subjects' age ($\beta = -0.18$, $P = 0.002$) the intensity of reactive hyperemia ($\beta = 0.25$, $P < 0.001$) but not with subjects' gender ($\beta = 0.04$, $P = 0.5$). After adjusting for these, of the phase 3 parameters, FMD was significantly associated only with total cholesterol level ($\beta = -0.13$, $P = 0.04$) and the CVRI ($\beta = -0.13$, $P = 0.03$) (Table 4.3.6). In contrast the contemporaneously measured phase 5 systolic ($\beta = -0.14$, $P = 0.01$) and diastolic ($\beta = -0.12$, $P = 0.03$) blood pressure and the CVRI ($\beta = -0.16$, $P = 0.007$) were significantly associated with FMD and a borderline association with total cholesterol ($\beta = -0.12$, $P = 0.06$) remained. Associations between blood pressure and the CVRI and FMD were linear and occurred at levels that would be considered acceptable in the normal population (Figure 4.3.4). There was no association between FMD and any single parameter of glucose metabolism, the ISI or the mean z-score for insulin resistance, in either group or the entire cohort.

4.3.4.2 Distensibility

Mean DC was significantly associated with subjects' age ($P < 0.001$) but not with subjects' gender ($P = 0.5$). After adjusting for these, significant associations with the phase 3 fasting glucose ($P = 0.02$) and insulin level ($P < 0.001$), the two-hour post OGTT insulin level ($P = 0.003$) and the ISI ($P = 0.04$) were found (Table 4.3.7) In addition significant associations between DC and HDL cholesterol level ($P < 0.001$) triglyceride level ($P < 0.001$), systolic ($P < 0.001$) and diastolic ($P < 0.001$) blood pressure, BMI ($P < 0.001$) and WHR ratio ($P < 0.001$) were found. Carotid artery DC was also significantly associated with the CVRI ($P < 0.001$). In contrast to FMD, a very similar pattern of relationships was apparent between the contemporaneous phase 5 measurements and DC, except that in addition a significant association with total cholesterol level was apparent. Although significant associations between DC and parameters of glucose tolerance and insulin resistance were apparent, after adjustment for the effects of HDL cholesterol, triglycerides, blood pressure and body mass index these effects were diminished to a non significant level. On multiple stepwise regression analysis only BMI ($\beta = -0.20$, $P = 0.002$), age ($\beta = -0.36$, $P <
0.001), triglyceride level ($\beta = -0.16$, $P = 0.03$) and systolic blood pressure ($\beta = -0.16$, $P = 0.03$) had a significant independent effect on DC. Previous studies have suggested differences in men and women (Salomaa et al., 1995). Although subjects gender did not significantly predict carotid artery DC, analyses, using the phase 5 data, were repeated for both men and women separately. Similar associations were found in each group with the exception that total cholesterol and post OGTT insulin were significantly associated with DC only in men and fasting glucose only in women (Table 4.3.8).

### 4.3.4.3 Intima-media thickness

Common carotid IMT was significantly associated with age ($\beta = 0.15$, $P = 0.003$) but not gender ($P = 0.1$). After adjusting for these, there were no significant associations between any individual phase 3 parameter and IMT but a significant association between IMT and the CVRI was apparent ($\beta = 0.20$, $P = 0.001$) (Table 4.3.9). Phase 5 measurements which were significantly associated with IMT were systolic blood pressure ($\beta = 0.12$, $P = 0.05$), WHR ($\beta = 0.21$, $P = 0.001$) and the CVRI ($\beta = 0.20$, $P = 0.001$) (Table 4.3.9). No parameter of glucose metabolism was independently associated with IMT.
Table 4.3.6 Determinants of flow mediated dilatation

<table>
<thead>
<tr>
<th></th>
<th>Phase 3</th>
<th>Phase 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta</td>
<td>P</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>0.01</td>
<td>0.85</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>-0.04</td>
<td>0.55</td>
</tr>
<tr>
<td>2hr OGTT glucose (mmol/l)</td>
<td>-0.02</td>
<td>0.76</td>
</tr>
<tr>
<td>2hr OGTT insulin (pmol/l)</td>
<td>0.05</td>
<td>0.46</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>-0.06</td>
<td>0.38</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.02</td>
<td>0.78</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>-0.12</td>
<td>0.27</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.06</td>
<td>0.36</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>-0.04</td>
<td>0.60</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>-0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>-0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>CVRI (%)</td>
<td>-0.13</td>
<td>0.03</td>
</tr>
</tbody>
</table>


Abbreviations: FMD = flow-mediated dilatation, GT = glucose tolerant, GI = glucose intolerant, OGTT = oral glucose tolerance test, HDL = high-density lipoprotein, LDL = low-density lipoprotein, CVRI = cardiovascular risk index
Table 4.3.7 Determinants of carotid artery distensibility

<table>
<thead>
<tr>
<th></th>
<th>Phase 3</th>
<th></th>
<th></th>
<th>Phase 5</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta</td>
<td>P</td>
<td>Beta</td>
<td>P</td>
<td>Beta</td>
<td>P</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>-0.15</td>
<td>0.02</td>
<td>-0.13</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>-0.35</td>
<td>&lt; 0.001</td>
<td>-0.29</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2hr OGTT glucose (mmol/l)</td>
<td>-0.10</td>
<td>0.12</td>
<td>-0.11</td>
<td>0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2hr OGTT insulin (pmol/l)</td>
<td>-0.20</td>
<td>0.003</td>
<td>-0.18</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>-0.19</td>
<td>0.003</td>
<td>-0.25</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.07</td>
<td>0.30</td>
<td>-0.13</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.35</td>
<td>&lt; 0.001</td>
<td>0.30</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>-0.06</td>
<td>0.40</td>
<td>-0.11</td>
<td>0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.38</td>
<td>&lt; 0.001</td>
<td>-0.38</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.27</td>
<td>&lt; 0.001</td>
<td>-0.31</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>-0.35</td>
<td>&lt; 0.001</td>
<td>-0.30</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>-0.45</td>
<td>&lt; 0.001</td>
<td>-0.49</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>-0.41</td>
<td>&lt; 0.001</td>
<td>-0.45</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CVRI (%)</td>
<td>-0.38</td>
<td>&lt; 0.001</td>
<td>-0.39</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: FMD = flow-mediated dilatation, GT= glucose tolerant, GI = glucose intolerant, OGTT = oral glucose tolerance test, HDL = high-density lipoprotein, LDL = low-density lipoprotein, CVRI = cardiovascular risk index
<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta</td>
<td>P</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>-0.09</td>
<td>0.27</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>-0.30</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2hr OGTT glucose (mmol/l)</td>
<td>-0.09</td>
<td>0.29</td>
</tr>
<tr>
<td>2hr OGTT insulin (pmol/l)</td>
<td>-0.23</td>
<td>0.005</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>0.23</td>
<td>0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>-0.15</td>
<td>0.04</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.27</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>-0.08</td>
<td>0.35</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>-0.43</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.26</td>
<td>0.001</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>-0.26</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>-0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>-0.42</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CVRI (%)</td>
<td>-0.44</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Linear regression model for distensibility coefficient in men and women adjusted for age. A consistent pattern of associations was seen in men and women, though total cholesterol and post OGTT insulin level were only significant in men and fasting glucose level was only significant in women.

Abbreviations: OGTT = oral glucose tolerance test, HDL = high-density lipoprotein, LDL = low-density lipoprotein, CVRI = cardiovascular risk index
<table>
<thead>
<tr>
<th></th>
<th>Phase 3</th>
<th>Phase 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta</td>
<td>P</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>-0.06</td>
<td>0.32</td>
</tr>
<tr>
<td>Fasting insulin (pmol/l)</td>
<td>-0.06</td>
<td>0.39</td>
</tr>
<tr>
<td>2hr OGTT glucose (mmol/l)</td>
<td>0.06</td>
<td>0.32</td>
</tr>
<tr>
<td>2hr OGTT insulin (pmol/l)</td>
<td>0.03</td>
<td>0.67</td>
</tr>
<tr>
<td>Insulin sensitivity index</td>
<td>-0.02</td>
<td>0.44</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>0.11</td>
<td>0.08</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>0.01</td>
<td>0.87</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>0.09</td>
<td>0.14</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.08</td>
<td>0.19</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>0.05</td>
<td>0.40</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>0.05</td>
<td>0.38</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>0.03</td>
<td>0.63</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>-0.02</td>
<td>0.80</td>
</tr>
<tr>
<td>CVRI (%)</td>
<td>0.20</td>
<td>0.001</td>
</tr>
</tbody>
</table>


Abbreviations: FMD = flow-mediated dilatation, GT= glucose tolerant, GI = glucose intolerant, OGTT = oral glucose tolerance test, HDL = high-density lipoprotein, LDL = low-density lipoprotein, CVRI = cardiovascular risk index
Figure 4.3.4 Relation between flow-mediated dilatation (FMD) and quintiles of systolic blood pressure and the calculated 10 year cardiovascular risk index (CVRI). Data is mean ± SD.
4.3.4 Inflammation and coagulation

There were significant correlations between all of the inflammatory and coagulation factors measured and parameters related to the metabolic syndrome reflecting the complex inter-relationships that are known to exist between these variables (Table 4.3.10). Particularly strong relationships between measures of obesity and CRP and PAI1 were apparent (Figure 4.3.5). There were no significant differences in any of the inflammatory markers and coagulation factors measured between the GT and GI groups (Table 4.3.11). In contrast CRP (P < 0.001), fibrinogen (P < 0.001) and PAI1 (P < 0.001) levels were significantly elevated in MS subjects compared to all the other groups. In addition, IL6 (P = 0.02), and SAA (P = 0.04) were significantly elevated in the MS group compared to GT subjects only. There were no significant differences between the groups in levels of VWF.

After adjusting for age, gender and reactive hyperaemia, FMD was significantly associated with SAA and PAI1 levels (Table 4.3.12). There were no significant associations with CRP, IL6 levels or any of the other inflammatory markers. On stepwise multiple regression analysis including all the inflammatory variables and the CVRI, FMD was independently associated with levels of PAI1 (β = -0.14, P = 0.04) and SAA (β = -0.14, P = 0.03).

Carotid artery DC was significantly associated with CRP (β = -0.25, P < 0.001) and PAI1 (β = -0.39, P < 0.001) (Figure 4.3.6). After adjusting for classical risk factors in the CVRI, the relationships between DC and CRP (β = -0.25, P < 0.001) and PAI1 (β = -0.26, P < 0.001) remained significant and in addition the relationship between DC and SAA reached statistical significance (β = -0.16, P = 0.02). A similar pattern of associations was seen when men and women were analysed separately but all associations were reduced to a non-significant level after adjustment for BMI. Carotid artery IMT was not significantly related to any of the inflammatory or coagulation variables.
Table 4.3.10 Correlation between metabolic, inflammatory and haemostatic variables

<table>
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<tr>
<th></th>
<th>Fasting glucose</th>
<th>OGGTT glucose</th>
<th>Fasting insulin</th>
<th>OGGTT insulin</th>
<th>ISI</th>
<th>TG</th>
<th>BMI</th>
<th>WHR</th>
<th>SBP</th>
<th>IL6</th>
<th>CRP</th>
<th>SAA</th>
<th>Fibrinogen</th>
<th>PIA1</th>
</tr>
</thead>
<tbody>
<tr>
<td>OGGTT glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Fasting insulin</td>
<td>0.27***</td>
<td>0.05</td>
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<td></td>
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</tr>
<tr>
<td>OGGTT insulin</td>
<td>0.27**</td>
<td>0.05</td>
<td>0.51***</td>
<td>0.51***</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>ISI</td>
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<td>-0.50***</td>
<td>-0.74***</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.05</td>
<td>0.28***</td>
<td>0.45***</td>
<td>0.37***</td>
<td>-0.40***</td>
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<tr>
<td>BMI</td>
<td>0.23***</td>
<td>0.11</td>
<td>0.42***</td>
<td>0.21***</td>
<td>-0.23***</td>
<td>0.45***</td>
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<tr>
<td>WHR</td>
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<td>0.11</td>
<td>0.33***</td>
<td>0.22***</td>
<td>-0.32***</td>
<td>0.49***</td>
<td>0.47***</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systolic BP</td>
<td>0.14*</td>
<td>0.16**</td>
<td>0.25***</td>
<td>0.18***</td>
<td>-0.24***</td>
<td>0.22***</td>
<td>0.40***</td>
<td>0.32***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>0.07</td>
<td>0.15*</td>
<td>0.16*</td>
<td>0.19**</td>
<td>-0.15*</td>
<td>0.19**</td>
<td>0.23***</td>
<td>0.16*</td>
<td>0.19***</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CRP</td>
<td>0.06</td>
<td>0.16*</td>
<td>0.28***</td>
<td>0.23***</td>
<td>-0.24***</td>
<td>0.35***</td>
<td>0.50***</td>
<td>0.34***</td>
<td>0.38***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SAA</td>
<td>-0.01</td>
<td>0.19***</td>
<td>0.14*</td>
<td>0.11</td>
<td>-0.13</td>
<td>0.11</td>
<td>0.22***</td>
<td>-0.02</td>
<td>0.11</td>
<td>0.24***</td>
<td>0.60***</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
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<td>0.07</td>
<td>0.23***</td>
<td>0.17*</td>
<td>-0.18**</td>
<td>0.19***</td>
<td>0.32***</td>
<td>0.09</td>
<td>0.22***</td>
<td>0.37***</td>
<td>0.58***</td>
<td>0.41***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIA1</td>
<td>0.22***</td>
<td>0.15*</td>
<td>0.42***</td>
<td>0.32***</td>
<td>-0.37***</td>
<td>0.42***</td>
<td>0.50***</td>
<td>0.47***</td>
<td>0.24***</td>
<td>0.22***</td>
<td>0.24***</td>
<td>0.02</td>
<td>0.17**</td>
<td></td>
</tr>
<tr>
<td>VWF</td>
<td>0.12*</td>
<td>-0.04</td>
<td>0.12*</td>
<td>0.06</td>
<td>-0.08</td>
<td>-0.09</td>
<td>-0.03</td>
<td>-0.08</td>
<td>0.03</td>
<td>0.11</td>
<td>0.07***</td>
<td>0.03</td>
<td>0.17**</td>
<td>0.17**</td>
</tr>
</tbody>
</table>

Pearson correlation coefficients between metabolic, physical and inflammatory variables. * P < 0.05, ** P < 0.01, *** P < 0.001. Abbreviations: ISI = insulin sensitivity index, TG = triglycerides, BMI = body mass index, WHR = waist/hip ratio, SBP = systolic blood pressure, IL6 = interleukin-6, CRP = C reactive protein, SAA = serum amyloid A, PIA1 = plasminogen activator inhibitor type 1, VWF = vonWillebrand factor.
### Table 4.3.11 Inflammatory and haemostatic variables

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>MS</th>
<th>GT</th>
<th>GI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL6 (pg/ml)</strong></td>
<td>1.18 (38 – 9.56)</td>
<td>1.78 (0.52 – 4.09) §</td>
<td>0.93 (0.43 – 9.61)</td>
<td>1.23 (0.44 – 10.13)</td>
</tr>
<tr>
<td><strong>CRP (mg/l)</strong></td>
<td>1.27 (0.16 – 18.49) §</td>
<td>2.90 (0.63 – 22.69) §†</td>
<td>0.79 (0.21 – 8.24)</td>
<td>1.11 (0.07 – 30.76)</td>
</tr>
<tr>
<td><strong>SAA (mg/ml)</strong></td>
<td>2.4 (0.7 – 19.2)</td>
<td>3.05 (0.9 – 31.9) §</td>
<td>1.9 (0.9 – 15.2)</td>
<td>2.6 (0.70 – 68.3)</td>
</tr>
<tr>
<td><strong>Fibrinogen (g/l)</strong></td>
<td>3.05 ±0.58</td>
<td>3.46 ± 0.63 §†</td>
<td>2.90 ±0.47</td>
<td>3.11 ±0.52</td>
</tr>
<tr>
<td><strong>PAI1 (ng/ml)</strong></td>
<td>7.7 ± 3.5</td>
<td>10.4 ± 2.5 §†</td>
<td>7.2 ± 3.1</td>
<td>7.3 ± 3.0</td>
</tr>
<tr>
<td><strong>VWF (IU/l)</strong></td>
<td>113 ± 43</td>
<td>104 ± 37</td>
<td>113 ± 36</td>
<td>122 ± 40</td>
</tr>
</tbody>
</table>

† = P < 0.05 compared to glucose tolerant subjects, § = P < 0.05 compared to controls (Post hoc 2 way ANOVA with Bonferroni correction for multiple tests).

Abbreviations: IL6 = interleukin-6, CRP = C reactive protein, SAA = serum amyloid A, PAI1 = plasminogen activator inhibitor type 1, VWF = vonWillebrand factor.

### Table 4.3.12 Inflammatory determinants of vascular function and structure

<table>
<thead>
<tr>
<th></th>
<th>FMD</th>
<th>DC</th>
<th>IMT</th>
</tr>
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<tr>
<td><strong>Beta</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>-0.07</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>-0.08</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>SAA</td>
<td>-0.14</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>-0.06</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>PAI1</td>
<td>-0.12</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td>0.04</td>
<td>0.58</td>
<td></td>
</tr>
</tbody>
</table>

Linear regression models for vascular function adjusted for age, subject gender and the intensity of the reactive hyperaemic stimulus (FMD only).

Abbreviations: IL6 = interleukin-6, CRP = C reactive protein, SAA = serum amyloid A, PAI1 = plasminogen activator inhibitor type 1, VWF = vonWillebrand factor.
Figure 4.3.5 Relation between body mass index and plasminogen activator inhibitor type 1 levels (PAI1).
Figure 4.3.6 Relation between carotid artery distensibility coefficient (DC) and quintiles of C reactive protein (CRP) and plasminogen activator inhibitor type 1 levels (PAI1). Data is mean ± SD.
4.3.5 Multivariate analysis

4.3.5.1 Introduction

Although the clustering of metabolic abnormalities associated with insulin resistance has been proposed as an independent cardiovascular risk factor the complex interrelationships that exist between variables limits the use of conventional statistical techniques in determining the biological relevance of associations. As demonstrated in section 4.3.4, factors such as inflammation are correlated with vascular function but are confounded by co-variance with other risk factors, such as measures of obesity. Factor analysis, however, is a technique that allows inclusion of interrelated variables in the statistical model and has been applied to investigate clustering of cardiovascular risk factors (Donahue et al., 1997; Lempiainen et al., 1999; Meigs et al., 1997). Using this technique Lempiainen (1999) and colleagues identified several factors that were thought to represent insulin resistance, glucose intolerance and hypertension and have demonstrated a significant association between a number of these factors and subsequent cardiovascular disease. However, none of these studies have included inflammatory and haemostatic indices and the impact of this cluster of metabolic abnormalities on vascular physiology at an earlier stage in the disease process has not been studied.

In this study factor analysis was used to identify major components of the MS, classical cardiovascular risk factors, inflammatory and haemostatic mediators and determine the relationships between these factors and vascular function.

4.3.5.2 Statistical methods

Factor analysis attempts to identify underlying variables, or ‘factors’, that explain the pattern of correlations within a set of observed variables (Kleinbaum et al., 1988; Stevens, 1986). It is often used in data reduction, by identifying a small number of factors that explain most of the variance observed in a much larger number of manifest variables (Figure 4.3.7). Factor analysis can also be used to generate hypotheses regarding causal mechanisms or to screen variables for subsequent analysis (for example, to identify collinearity prior to a linear regression analysis). Factor analysis is a three-step process as outlined below.
4.3.5.2.1 Principal component analysis

Principal component analysis is a factor extraction method used to form uncorrelated linear combinations of the observed variables. The first principal component is the combination of variables that accounts for the most variance in the dataset. Successive components explain progressively smaller portions of the variance and are all uncorrelated with each other. Principal component analysis is used to obtain the initial factor solution. Generally only components with an eigenvalue (sum of the squared factor loadings which represents the amount of variance attributable to each component) greater than 1.0 are retained in the analysis for rotation.

4.3.5.2.2 Rotation of the principal components

Rotation is a general method for making a factor solution easier to interpret. There are several different methods for rotation. Varimax rotation is an orthogonal rotation method that minimizes the number of variables that have high loadings on each factor, thus simplifying the interpretation of the factors and maintains the independence between the factors. Once rotated principal components are referred to as factors and the amount of variance accounted for by each factor is recalculated.
4.3.5.2.3 Interpretation of factors.

Interpretation involves assessing which factors load high on a particular factor and naming the factor accordingly. Factor loadings, equivalent to a Pearson’s correlation coefficient between the variable and the factor greater than 0.40 are considered significant, though loadings greater than or equal to 0.30 should also be taken into account (Stevens, 1986). Variables may load significantly on more than one factor and these might represent biologically relevant links between physiological processes.

In this study, analyses were performed in men and women separately and then in the whole group. Age, parameters of glucose tolerance and insulin resistance, obesity, blood pressure, lipid levels and inflammatory and haemostatic variables were the initial variables. For each analysis the factor scores were saved as a separate variable and then multiple regression analyses were used to explore which factors determined the main dependent variables of FMD, carotid artery distensibility and IMT. Standardised regression coefficients were used to allow comparison between variables.

4.3.5.3 Results

One hundred and forty two subjects (68 males, 74 women) had a complete dataset and were included in the analysis.

4.3.5.3.1 Principal-component analyses

Principal component analyses reduced the 20 interrelated variables to 4 newly defined uncorrelated factors. Consistent with previous reports (Edwards et al., 1994; Lempiainen et al., 1999; Meigs et al., 1997), factor 1 was a distinct factor which is likely to represent the central metabolic syndrome, was identified. This factor contained positive loadings for fasting insulin level, BMI, WHR and triglycerides and a negative loading for HDL cholesterol level. In addition a significant positive loading for PAI1 level was found in both men and women consistent with it being a legitimate part of the MS. In women, levels of IL6, CRP and SAA also loaded significantly on factor 1, whilst in men the loading for CRP was only of borderline significance. However, in both men and women a separate inflammatory factor
(factor 3 in men and factor 4 in women) with positive loadings for IL6, CRP, SAA and fibrinogen was apparent. Consistent with the study of Meigs et al., (1997) separate factors representing blood pressure (factor 4 in men and factor 2 in women) were found and these were linked to the central metabolic syndrome by co-associations with PAI1. In men a separate factor for glucose tolerance (factor 2) was found as previously reported (Meigs et al., 1997) whilst in women measures of glucose tolerance loaded on factor 1. In women a separate factor with high loadings for lipid levels was apparent (Factor 3). These four factors accounted for 57% and 63% of the total variance in men and women respectively.

A similar pattern of loadings was found when men and women were considered together. Factor 1 was interpreted as representing the MS, factor 2 as an inflammatory factor, factor 3 as a lipid factor and factor 4 as a blood pressure factor. (Table 4.3.13).

4.3.5.3.2 Relation to vascular function

On multiple regression analyses, after adjustment for reactive hyperaemia, FMD was independently associated only with factor 4, which was interpreted as representing blood pressure in men and with factor 2 (blood pressure) and factor 4 (inflammation) in women (Table 4.3.14). In contrast in whole group, FMD was significantly associated with the metabolic syndrome but not with blood pressure (Figure 4.3.8).

In similar analyses, DC was significantly associated with factor 1 (metabolic syndrome) and factor 4 (blood pressure) in men. In contrast, in women DC was associated with factor 2 (blood pressure) and factor 4 (inflammation) but not the factor representing the central metabolic syndrome. In the whole group FMD was associated only with the factor representing the MS. In contrast, DC was associated with the metabolic syndrome factor and blood pressure with borderline associations with lipids and inflammation (Table 4.3.14). None of the factors were associated with IMT in men or women.
Table 4.3.13 Factors and factor loadings (phase 5)

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th></th>
<th></th>
<th></th>
<th>Women</th>
<th></th>
<th></th>
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<td>0.05</td>
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<td>-0.11</td>
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<td>2H OGTT glucose</td>
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<td>0.75</td>
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<td>-0.15</td>
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<td>0.11</td>
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<td>0.44</td>
<td>0.62</td>
<td>0.24</td>
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<td>0.79</td>
<td>0.22</td>
<td>0.05</td>
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<tr>
<td>Waist/hip ratio</td>
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<td>0.22</td>
<td>0.02</td>
<td>0.42</td>
<td>0.61</td>
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<td>0.75</td>
<td>-0.01</td>
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<td>-0.09</td>
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<td>-0.05</td>
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<td>0.95</td>
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<td>0.92</td>
<td>0.01</td>
<td>-0.05</td>
<td>0.07</td>
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<td>0.28</td>
<td>0.74</td>
<td>0.07</td>
<td>0.87</td>
<td>0.08</td>
<td>0.03</td>
<td>0.34</td>
<td>0.00</td>
<td>0.16</td>
<td>0.66</td>
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<td>0.09</td>
<td>0.23</td>
<td>0.80</td>
<td>0.04</td>
<td>0.87</td>
<td>-0.08</td>
<td>0.05</td>
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<td>-0.09</td>
<td>-0.01</td>
<td>0.67</td>
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<td>Interleukin-6</td>
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<td>0.54</td>
<td>0.16</td>
<td>0.49</td>
<td>-0.06</td>
<td>0.02</td>
<td>0.39</td>
<td>0.27</td>
<td>0.52</td>
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<td>C reactive protein</td>
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<td>0.84</td>
<td>0.11</td>
<td>0.45</td>
<td>0.42</td>
<td>0.11</td>
<td>0.53</td>
<td>0.45</td>
<td>0.68</td>
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<td>Serum amyloid A</td>
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<td>-0.02</td>
<td>0.82</td>
<td>-0.15</td>
<td>0.51</td>
<td>0.09</td>
<td>0.01</td>
<td>0.53</td>
<td>-0.04</td>
<td>0.74</td>
<td>-0.10</td>
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<td>Fibrinogen</td>
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<td>0.64</td>
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<td>0.16</td>
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<td>PAI1</td>
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<td>-0.11</td>
<td>0.42</td>
<td>0.52</td>
<td>0.42</td>
<td>0.21</td>
<td>0.26</td>
<td>0.74</td>
<td>0.10</td>
<td>-0.02</td>
<td>0.19</td>
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<tr>
<td>VWF</td>
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<td>-0.26</td>
<td>-0.04</td>
<td>0.22</td>
<td>-0.13</td>
<td>-0.08</td>
<td>-0.27</td>
<td>0.62</td>
<td>-0.06</td>
<td>0.19</td>
<td>-0.31</td>
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Variance explained (%)

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<tr>
<th></th>
<th>18</th>
<th>14</th>
<th>13</th>
<th>12</th>
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<td>51</td>
<td>63</td>
<td>20</td>
<td>33</td>
<td>45</td>
<td>57</td>
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</tbody>
</table>

Extraction Method: Principal Component Analysis. Rotation Method: Varimax with Kaiser Normalization
Abbreviations: PIA1 = plasminogen activator inhibitor type 1, VWF = vonWillebrand factor
Table 4.3.14 Determinants of vascular function (phase 5)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Interpretation</th>
<th>FMD</th>
<th>DC</th>
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<tr>
<td></td>
<td></td>
<td>beta</td>
<td>P</td>
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<tr>
<td>Men</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Metabolic syndrome</td>
<td>-0.13</td>
<td>0.28</td>
</tr>
<tr>
<td>2</td>
<td>Lipids</td>
<td>0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>3</td>
<td>Inflammation</td>
<td>-0.07</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>Blood pressure</td>
<td>-0.25</td>
<td>0.05</td>
</tr>
<tr>
<td>Women</td>
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<td></td>
<td></td>
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<tr>
<td>1</td>
<td>Metabolic syndrome</td>
<td>-0.02</td>
<td>0.83</td>
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<tr>
<td>2</td>
<td>Blood pressure</td>
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<td>0.04</td>
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<td>3</td>
<td>Lipids</td>
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<td>0.27</td>
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<tr>
<td>4</td>
<td>Inflammation</td>
<td>-0.28</td>
<td>0.02</td>
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<tr>
<td>All</td>
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<tr>
<td>1</td>
<td>Metabolic syndrome</td>
<td>-0.25</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>Inflammation</td>
<td>-0.14</td>
<td>0.12</td>
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<tr>
<td>3</td>
<td>Lipids</td>
<td>-0.03</td>
<td>0.70</td>
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<tr>
<td>4</td>
<td>Blood pressure</td>
<td>0.03</td>
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Linear regression models for vascular function adjusted for the intensity of the reactive hyperaemic stimulus (FMD only).
Figure 4.3.8 Relation between FMD and carotid artery distensibility coefficient and the metabolic syndrome cluster Factor 1.
4.4 DISCUSSION

This study shows that the metabolic syndrome is associated with abnormal vascular function before the onset of clinical vascular disease but that glucose intolerance and insulin resistance are not major contributors to this process. In contrast, established risk factors such as age, blood pressure and hyperlipidaemia are significantly associated with vascular dysfunction. In addition, this study has demonstrated a significant association between inflammatory factors and vascular dysfunction, supporting a role for inflammation in the pathogenesis of atherosclerosis.

The clustering of metabolic abnormalities associated with glucose intolerance and insulin resistance is now widely recognised as an important risk factor for atherosclerosis although definitive data to support this is lacking and the mechanisms remain unclear. This study was designed to explore the impact of the risk factors associated with the metabolic syndrome and particularly glucose tolerance and insulin resistance on vascular function before the emergence of clinical vascular disease in healthy middle-aged men and women. Initial studies demonstrated significant abnormalities of brachial artery FMD, dilatation to GTN and carotid artery distensibility in subjects with the MS compared to a large control population, consistent with a pathological role for this cluster of risk factor abnormalities in the pathogenesis of atherosclerotic vascular disease. There was no significant abnormality of carotid artery IMT in this group, however, IMT levels overall were low compared to that reported in other studies (Chambless et al., 1997), reflecting the relatively healthy nature of this population and the early stage in atherosclerotic vascular disease. These data suggest a widespread abnormality of vascular function in the MS that extends beyond the endothelium.

The mechanisms which underlie vascular dysfunction in the MS remain unclear. As expected, MS subjects were significantly more insulin resistant and glucose intolerant than controls but, in addition, there were also differences in a wide range of other metabolic and physical parameters and, therefore, it is not possible to determine the impact of individual risk parameters on vascular dysfunction. To determine whether insulin resistance has a direct effect on vascular function, two separate groups of glucose tolerant and intolerant subjects were studied. Insulin
sensitivity was not formally assessed by hyperinsulinaemic clamp for logistic reasons. However, the OGTT has been widely used as a surrogate marker of insulin sensitivity and the ISI is more closely related to true insulin sensitivity than any other derived measure (Matsuda and DeFronzo, 1999). These subjects were selected to minimise the potentially confounding effects of other risk factors associated with the MS and the overall risk burden in these subjects was significantly less than in control or MS groups. In this study, there were no significant differences in FMD or carotid artery DC between GT or GI subjects indicating that insulin resistance and carbohydrate metabolism had little impact on these measures of vascular function. These results, in mainly white Caucasians, are consistent with the recent finding of Chambers and colleagues, (1999) in which impaired FMD in Indian Asians was not explained by differences in insulin resistance and those of Natali and colleagues (1997), in patients with hypertension. They suggest that the abnormalities in endothelial function found in previous studies (Petrie et al., 1996; Steinberg et al., 1996) are mediated by associated obesity and hypertension.

The GT and GI subjects were selected on the basis of risk data collected during the phase 3 examination of the Whitehall II study (1991 - 1993) and, therefore, this result implies that glucose intolerance and insulin resistance are not related to the development of vascular dysfunction over a number of years. Whilst changes in risk factors might diminish the effect over time, there was a close correlation between measures of glucose tolerance and insulin resistance between the phase 3 and phase 5 examination and a significant difference in the post OGTT glucose level and ISI remained apparent in measurements made contemporaneously with the assessment of vascular function. Moreover, the ability of a risk factor to predict vascular abnormalities at a later date is likely to be of greater clinical significance in a preclinical population. The absence of a more temporal association between glucose tolerance and endothelial function was supported by the multiple regression analyses in which no association between FMD and any contemporary measure of glucose metabolism or insulin resistance was seen. There were, however, significant associations between FMD and both systolic and diastolic blood pressure, the calculated cardiovascular risk index and a borderline association with total cholesterol level even after adjusting for the effects of age and the intensity of the reactive hyperaemic stimulus. The relationships between FMD and systolic blood
pressure and the CVRI were linear and were apparent at levels of blood pressure that would be considered satisfactory in the general population. Furthermore they were not diminished by the addition of other risk factor parameters or parameters of glucose tolerance and insulin resistance to the model, none of which had a significant effect.

In contrast to the relationships apparent with the contemporary phase 5 data, of the phase 3 parameters measured, FMD was associated significantly only with total cholesterol level. This difference in the pattern of associations is likely to reflect the dynamic and mutable nature of endothelial function which will reflect the impact of both chronic risk factors (Mullen et al., 1997) and acute influences that injure the vascular wall (Bellamy et al., 1998; Ghiadoni, 2000; Hingorani, 2000; Lundman et al., 1997). Therefore, whilst measurements of endothelial function are likely to be a useful marker of temporal vascular health and reflect recent changes in risk factor burden they are unlikely to be predictive of vascular disease over long periods of time. In contrast, consistent associations between risk variables measured during both phase 3 and phase 5 and carotid artery distensibility were apparent suggesting that distensibility is a more enduring marker of vascular dysfunction associated with cardiovascular risk. Carotid artery DC was significantly associated with fasting glucose and insulin levels, the ISI but not glucose tolerance as indicated by the two-hour post OGTT glucose level. In addition strong associations were found between DC and BMI, WHR, triglyceride level and HDL cholesterol levels, blood pressure and the calculated CVRI. Only the contemporary measure of total cholesterol was associated with DC and on further subanalysis this relationship was only apparent in men. These results are broadly similar to those of the ARIC study in which significant associations between indices of arterial stiffness and fasting glucose and insulin levels were found (Salomaa et al., 1995). As expected however, significant interactions were found between the measures of glucose homeostasis and levels of obesity, blood pressure, and fasting plasma lipid levels. After adjusting for the potential confounding effects of these covariate risk factors the effects of glucose tolerance and insulin resistance were reduced to non-significant levels. These data suggest that the relationship between carotid artery DC and insulin resistance is largely mediated through the effects of adiposity, hypertension and dyslipidemia,
though the limitations of multiple regression analyses in the context of interrelated variables should be noted.

Accumulating data from clinical trials has indicated a link between the presence of a low grade chronic inflammatory state and later cardiovascular disease (Libby and Ridker, 1999). Although this may represent a direct effect of inflammation on the vascular wall, levels of inflammatory and haemostatic variables are also closely associated with each other and with physical and metabolic parameters associated with insulin resistance (Yudkin et al., 1999). These findings were confirmed in the current study in which multiple interrelationships between variables occurred. Of these, BMI was more closely associated with the inflammatory variables than any other physical or metabolic variable. This is consistent with adipose tissue being an important generator of the acute phase response (Mohamed-Ali et al., 1997) and suggests that total body fat as opposed to fat distribution (as reflected in the WHR) is the major determinant of this response. In this study, levels of serum amyloid-A and PAI1 were significantly associated with FMD, and these relationships were independent of the effect of classical risk factors embodied in the CVRI. Similarly, levels of CRP and PAI1 predicted carotid artery DC with a borderline association with SAA. Interestingly, levels of von Willebrand factor, which are thought to be a biochemical correlate of endothelial function were not significantly associated with either measure of vascular function or IMT. However, von Willebrand is likely to be largely released from smaller vessels and post capillary venules where different influences may impact on the vascular wall. In addition, no significant associations between IL6 levels and FMD or DC were apparent. This is likely to reflect the short half-life of cytokines compared to that of the acute phase proteins and differences in the hepatic response to cytokines. These data indicate that vascular function in healthy middle aged men and women is associated with markers of inflammation and fibrinolytic activity, and support a role for cytokine-mediated inflammation in the early stages of atherogenesis.

The multiple regression analyses used to explore relationships between metabolic and inflammatory variables and vascular function used in this study are limited by the complex interrelationships which exist between the variables. As a result it is not possible to exclude the possibility that positive relationships are mediated by
covariance with other variables or that biologically important relationships are diminished by confounding. However it is likely that only a relatively small number of discrete and unrelated physiological processes underlie the complex interaction between risk variables. Identifying these will offer new insight into the metabolic syndrome and their physiological relevance to vascular function can be tested. Such data reduction using principal component analysis has previously been applied to the metabolic syndrome (Donahue et al., 1997; Lempiainen et al., 1999; Meigs et al., 1997). Lempiainen et al., (1999), have recently demonstrated a significant association between a factor corresponding to the central metabolic syndrome and subsequent cardiovascular morbidity and mortality. The data presented here differs from previous reports in that, in addition to metabolic parameters, a range of inflammatory variables have been included in the analyses and the relation between the derived factors and vascular function in preclinical subjects has been assessed. The finding of a separate inflammatory factor in both men and women suggests a distinct pathophysiological process. However, measures of obesity did not load on the inflammatory factor in either group suggesting that the level of adiposity was not a major determinant of this response as has been proposed (Yudkin et al., 1999). The inflammatory factor was however linked to the central MS by co-associations with CRP in both groups and also with IL6, SAA and fibrinogen in women. The mechanism of this relationship remains obscure but might represent an as yet undefined common aetiological factor or that the inflammatory response is involved in the genesis of the metabolic syndrome phenotype (Yudkin et al., 1999). Interestingly both FMD and DC were associated with the inflammatory factor in women, suggesting that they may be particularly susceptible to this response, whilst no association was apparent in men.

These analyses advance the comparisons of vascular function in MS and controls. and suggest that endothelial function and arterial distensibility are differentially affected by risk factors in men and women. Rather than using arbitrary criteria for the diagnosis of the metabolic syndrome, each subject is given a score determined by a range of factors identified as being part of the metabolic syndrome by the factor analysis and this can then be related to the measures of vascular function. In both men and women factor 1 was interpreted as representing the central metabolic syndrome. This factor was associated with FMD only when men and women were
considered together and was a significant and strong determinant of DC only in men. These data are consistent with previous reports in which an association between the metabolic syndrome and cardiovascular morbidity and mortality has only been found in men (Lempiainen et al., 1999).

As in previous studies the factorial analysis did not identify blood pressure as a major component of the metabolic syndrome (Lempiainen et al., 1999; Meigs et al., 1997). However, blood pressure was linked to the metabolic syndrome by shared associations with PAI1 levels and was an independent determinant of FMD and DC in both men and women.

In summary this study has demonstrated that the metabolic syndrome is associated with impaired vascular function particularly in men. The mechanisms however remain unclear but the data in matched groups suggests that glucose tolerance *per se* has little influence on early abnormalities of vascular structure or function. In contrast, recognised risk factors such as blood pressure and lipid levels are associated with impaired vascular function throughout their range. The potential for modification of these factors is well established and likely to have the greatest impact on the progression of vascular disease.
CHAPTER 5

REVERSAL OF ENDOTHELIAL DYSFUNCTION
5.1 Introduction

Although the clinical consequences of conduit artery atherosclerosis normally only become manifest in the middle aged and older generations the pathological processes that underlie its genesis begin much earlier (Berenson et al., 1992; Stary, 1989; Strong, 1995). This has lead to an increasing emphasis on the development of preventative strategies, whose aim is to retard the progression of atherosclerosis at a preclinical stage, when it is also most likely to be mutable. In view of the long natural history of atherosclerosis, in humans, the benefit of such strategies will need to be tested against surrogate markers of disease progression, rather than clinical events. Endothelial dysfunction is now recognised to be an early event in atherosclerosis and, in 'risk factor' cohorts, can be detected from as early as the first decade of life using established non-invasive techniques described in this thesis (Mullen et al., 1997). Improvement in endothelial dysfunction has been demonstrated following pharmacological (Clarkson et al., 1996a; Ting et al., 1997) and lifestyle interventions (Clarkson et al., 1999) and this might represent a beneficial effect on the progression of vascular disease. This concept is supported by the results of animal experiments in which restoration of endothelial function is associated with histologically reduced atherosclerosis (Candipan et al., 1996). In this chapter the effects of a number of interventions on brachial artery FMD in young patients with type 1 diabetes mellitus are reported.

5.1.1 Design considerations and power

The non-invasive methods of assessing vascular function described in this thesis are ideally suited for prospective studies of this nature. The rationale design of these studies will be influenced by a number of factors including the accuracy and reproducibility of the technique and biological variability in endothelial function. In addition the power of the study to detect a significant improvement will be determined by the 'effect size' which is considered to be of biological and clinical significance. Currently no long-term studies have been reported and therefore the threshold at which improvements in endothelial function might alter the clinical outcome remains unclear. An empirical approach has therefore been necessary and a change in FMD of 1.5%, which represents approximately half of one standard
deviation for a normal population, might be considered an appropriate target. Previous studies have reported the accuracy and reproducibility of the non-invasive method (Sorensen et al., 1995) and used this data to calculate power function curves based on the design of the study (parallel versus cross-over) and the number of scans performed at each visit (Figure 5.1.1). As greater variability is apparent between subjects than within subjects larger cohorts are needed to achieve sufficient power in parallel groups studies. Thus for a study with 80% power to detect a $2\%$ change in FMD following an intervention approximately 100 subjects will be required for a parallel group study and 10 to 15 subjects for a crossover study with one scan per treatment (Sorensen et al., 1995).

Figure 5.1.1 Power function curves for the relationship between hypothesised improvement in FMD and number of subjects required in a cross-over (above) and a parallel group (below) study. From Sorensen et al., (1995).
5.1.2 Who needs intervention? What intervention?

The clinical benefit of early intervention in a risk factor group is likely to be greatest in those subjects with demonstrable endothelial dysfunction at an early age and the highest absolute risk of cardiovascular disease in later life. Such benefit will be achieved at an earlier stage if this morbidity and mortality is apparent prematurely. In these groups appropriate interventions will be defined by a detailed understanding of the pathophysiology of endothelial dysfunction in relation to the particular risk factor its interaction with other risk factors and the progression of endothelial dysfunction to clinical vascular disease.

5.1.3 Type 1 diabetes mellitus – a high risk group for atherosclerosis

One such group that is at high risk for cardiovascular disease in later life are patients with type 1 diabetes mellitus (type 1 DM). In the Framingham study, the risk of developing cardiovascular disease before the age of 55 years in diabetics was twice that for non-diabetics in men and over five times the normal rate for women (Kannel and McGee, 1979). This elevated risk, and particularly the occurrence of morbidity and mortality at a young age, was also noted by Krolewski et al., (1987) when by the age of 55 years cardiovascular mortality, which affected both men and women equally, was as high as 35% (Figure 1.3.1). Autopsy studies have confirmed a relationship between early atherosclerosis and glycosylated haemoglobin as a marker of diabetes in young trauma victims (Strong et al., 1995). The risk of cardiovascular disease in type 1 DM is known to be greatest in patients who have developed microalbuminuria or frank diabetic nephropathy (Yudkin and Chaturvedi, 1999). A further adverse factor is that, at presentation, coronary atherosclerosis is often more severe and widely distributed in diabetic patients making it less amenable to intervention (Krishnaswami et al., 1996) and the long-term benefits from coronary artery bypass surgery are reduced compared to a non-diabetic population (The Bypass Angioplasty Revascularization Investigation (BARI), 1997).

The discovery of insulin by the Nobel Laureates Banting and Best in the 1920s and its widespread introduction as therapy for type 1 DM patients revolutionised their prognosis from the complications of diabetic ketoacidosis. However, insulin therapy has had relatively little impact on the incidence of cardiovascular disease in this
population, which remains the greatest cause of mortality. In the Diabetes Control and Complications Trial, of 1441 type 1 DM patients, intensive treatment with insulin improved glycaemic control and the incidence of microvascular disease (The DCCT Research Group, 1993) but did not significantly alter the outcome from macrovascular events (The DCCT Research Group, 1995). These results emphasise the need to research the pathophysiology of vascular disease in type 1 DM and to develop alternative strategies to retard atherosclerosis in this population.

5.1.3.1 Vascular dysfunction in type 1 DM

Meraji and colleagues (1987), demonstrated impaired endothelial-dependent dilatation in response to acetylcholine and histamine in aortic rings from spontaneously diabetic Wistar BB rats and confirmed the presence of a structurally abnormal endothelium by electron microscopy. These findings have subsequently been confirmed in a variety of animal models (Lindsay et al., 1997). In young type 1 DM patients, without clinical cardiovascular disease, endothelial dysfunction has been demonstrated, in both resistance vessel (Calver et al., 1992; Elliott et al., 1993; Johnstone et al., 1993) and conduit arteries (Clarkson et al., 1996a; Lekakis et al., 1997). Huszka and colleagues (1997) demonstrated significantly reduced excretion of nitrate/nitrite as a biochemical marker of NO production in type 1 DM patients. These findings however are not universal in that in other studies, of similar cohorts, no significant abnormality of endothelial function was seen (Halkin et al., 1991; Lambert et al., 1996; Smits et al., 1993). The reason for this discrepancy remains unclear, but may relate to differences in the patient characteristics, the small number of subject in many of these studies and other differences in methodology. Elliott and colleagues (1993), demonstrated an abnormality of resistance vessel function only in patients with microalbuminuria, suggesting that the stage of the disease is an important factor. In resistance vessel studies, differences in basal blood flow and the use of agents such as methacholine, which may be largely a non-NO dependent vasodilator (Chowienczyk et al., 1993), may explain some of the discrepancies. Finally a number of studies have demonstrated abnormalities of neurovascular control, in type 1 DM, and this might have an impact on, particularly, resistance vessel reactivity (Makimattila et al., 1997; Steel et al., 1993).
Four studies to date have examined endothelial function in the brachial artery. Lambert and colleagues (1996), demonstrated a small but insignificant difference (after correcting for vessel size) in FMD between normoalbuminuric type 1 DM patients and controls. However in this study an FMD of over 10% was found suggesting that the cuff was placed on the upper arm above the segment of artery being imaged. The response of arteries under these conditions may be different than when a smaller stimulus to a non-ischaemic artery is applied by placing the cuff below the target artery (Chapter 3). In a recent paper, Enderle and colleagues (1998) also found no significant abnormality of endothelial function in type 1 DM patients. However, these patients were selected on the basis of having a long duration of diabetes but not having developed any complications and therefore do not represent the general type 1 DM population. Furthermore, only 17 patients were assessed and this study might have been under-powered. Lekakis and colleagues (1997) and Clarkson and colleagues (1996a) have reported a significant abnormality of FMD in young type 1 DM patients. In the latter study, endothelial dysfunction was not related to parameters of diabetic control but to the duration of diabetes and LDL-cholesterol levels. This emphasises the continuing importance of reversible risk factors in type 1 DM. In addition to abnormalities of endothelial-dependent dilatation, elevated levels of von Willebrand factor, soluble adhesion molecules, and fibrinogen have been reported at an early stage in type 1 DM and are likely to represent generalised endothelial activation (Greaves et al., 1997; Kopp et al., 1998; Stehouwer et al., 1995). The balance of evidence supports there being an abnormality of endothelial function which occurs early in young type 1 DM subjects (Poston and Taylor, 1995).

Impaired smooth muscle response to exogenous nitrate has also been demonstrated in type 1 DM (Calver et al., 1992; Clarkson et al., 1996b). The mechanism of this abnormality is unknown but suggests a more complex and widespread abnormality than that seen in other risk factor groups. This might represent reduced sensitivity of vascular smooth muscle to exogenous nitrate or impaired metabolism of GTN to NO (Calver et al., 1992). However, the dose of GTN that has been used is supramaximal (Section 2.2.7) and the more likely explanation is that structural changes in the arterial wall limit maximal dilatation. This is supported by recent findings in healthy volunteers, in which inhibition of NO synthesis resulted in enhanced smooth muscle sensitivity to exogenous nitrovasodilators (Barba et al., 1999).
5.1.3.2 Mechanisms of vascular disease in type 1 DM

The pathogenesis of vascular injury in diabetes mellitus is likely to be complex with multiple mechanisms operating (Poston and Taylor, 1995). Hyperglycaemia and the formation of advanced glycosylation endproducts are important sources of oxygen derived free radicals (Cosentino et al., 1997; Rubanyi and Vanhoutte, 1986; Williams et al., 1998; which inactivate NO (Bucala et al., 1991; Tesfamariam and Cohen, 1992) and accelerate the peroxidation of lipoproteins (Bucala et al., 1993). Increased susceptibility of LDL to oxidation has been reported in diabetics (Cominacini et al., 1994) which may be related to poor control and duration of diabetes (Feillet et al., 1998). In addition, glycosylation or impaired metabolism of L-arginine might reduce its availability for NO synthesis (Lo et al., 1994; Wu and Meininger, 1995) and lack of essential co-factors (Hawthorne et al., 1989) or alteration in the kinetics of NO synthase (Arnal et al., 1995) might further decrease NO formation and even result in the production of superoxide anion (Wever et al., 1997; Xia et al., 1996).

5.1.3.3 Role of other risk factors

Whilst factors specific to diabetes are likely to have a major impact on the progression of cardiovascular disease epidemiological studies have demonstrated that classical risk factors continue to operate, though at a higher level (Kannel and McGee, 1979) (Figure 5.1.2). Furthermore diabetes is often associated with a clustering of risk factors including hypertension, elevated cholesterol levels and dyslipidaemia. Thus for any given level of a risk factor the attributable risk will be higher and consequently intervention is justifiable at a lower threshold.

5.1.4 Discussion

Type 1 DM is a major risk factor for premature cardiovascular disease with abnormalities of endothelial function being apparent from an early age. Preventative strategies, which target the disease at this early stage, might have a clinical benefit on cardiovascular morbidity and mortality in later life. Such strategies might include therapeutic manoeuvres that enhance NO bioavailability, reduction in concomitant risk factors and the reduction in oxidative stress. Non-invasive testing of endothelial function is ideally suited to assess the effects of such interventions on the vascular wall, over relatively short periods of time.
Figure 5.1.2 Cardiovascular risk and total cholesterol levels in diabetics and non-diabetic subjects aged 45 years. Although absolute risk is higher in the diabetic subjects classical risk factors continue to operate and a similar relationship with cholesterol exists as in non-diabetic individuals. Data derived from the PROCAM study.
5.1 Endothelial response to intravenous L-arginine in type 1 DM and smokers

5.2.1 Introduction

The pathogenesis of endothelial dysfunction in type 1 DM is complex and may be mediated by a number of factors. Experimental evidence indicates that NO synthesis might be reduced due to impaired metabolism of its substrate L-arginine or defects of endothelial nitric oxide synthase (Lo et al., 1994). In animal models of type 1 DM, oral administration of L-arginine restores abnormal endothelial function (Pieper and Peltier, 1995; Pieper and Donldinger, 1997), though this has not been a universal finding (Mayhan et al., 1996). There is little data on the impact of L-arginine on endothelial function in humans in vivo in type 1 DM.

This study examined the acute effects of intravenous L-arginine on conduit artery endothelial function in young subjects with type 1 DM and young smokers and compared the responses to those in control subjects without risk factors.

5.2.2 Specific methods

5.2.2.1 Subjects

Twenty-seven subjects were recruited for this study (9 type 1 DM patients, 9 current smokers and 9 aged matched controls). Diabetic patients were recruited from diabetic clinics if they were less than 40 years old, had had diabetes for greater than two years, and had no clinical history of vascular disease. None had clinical evidence of retinopathy or microalbuminuria (urinary albumin / creatinine ratios < 0.01). Current cigarette smokers were studied if they had a total exposure of more than two pack years (1 pack year = 20 cigarettes per day for 1 year). Findings were compared to those of 9 age and sex matched volunteers recruited from hospital staff in whom the above risk factors had been excluded. All subjects gave written informed consent and ethical approval was granted by the local ethics committee.
5.2.2.2 Study protocol

Vascular responses in the brachial artery were studied in each subject on two separate days 3 - 10 days apart, with no therapeutic interventions between. At each visit the same study protocol was followed (Figure 5.2.1). After the baseline assessment of FMD, L-arginine (Martindale Pharmaceuticals UK, 0.1 g/kg in 100 ml 0.9% saline) or 100 ml 0.9% saline was administered intravenously over 20 minutes. The order of administration was randomized in a double blind fashion, and the same operator performed all the ultrasound scans. The effect of the infusion on FMD was assessed at the end of the 20 minute infusion. After a further 10 minute rest the response to GTN (400 µg sublingually) was measured.

5.2.2.3 Assessment of endothelial function

Brachial artery FMD, in response to 4.5 minutes of distal forearm ischaemia, and dilatation to GTN were assessed non-invasively using the standard protocol and an A-mode echo-tracking device (Ingenious Systems, Netherlands). Blood flow velocity was measured at rest and at peak hyperaemia.

5.2.2.4 Biochemical measurements

At the first visit a fasting blood sample was taken for measurement of lipid levels, glycosylated haemoglobin level and plasma glucose concentration. Plasma arginine levels were also measured at baseline and immediately after the infusions. Arginine was measured by ion exchange separation and reaction with ninhydrin (Pharmacia Alpha Plus amino acid analyzer). Total plasma cholesterol was measured using the cholesterol C-system high performance CHOD-PAP method and plasma triglycerides were measured using the GPO-PAP high performance enzymatic colorimetric test (both Boehringer-Mannheim GmbH, Diagnostica). HDL-cholesterol was measured after precipitation of apoprotein B containing lipoproteins, and LDL calculated according to the Friedwald formula (Friedwald et al., 1972). Glycosylated hemoglobin (HbA1) (Rapid EP system, Helena Laboratories) and urinary albumin concentration were measured in the diabetic group.
5.2.2.5 Statistics

Descriptive data are expressed as mean ± standard deviation and statistical significance was inferred at a p value of < 0.05. Baseline FMD was calculated as the mean of the pre L-arginine and pre saline infusion FMD values, measured on the 2 separate days. Comparisons within and between groups were made by a two-way repeated measures ANOVA.

Figure 5.2.1 Study protocol. At each visit flow-mediated dilatation (FMD) was measured at baseline (FMD1) and after infusion of either saline or L-arginine (randomised double blind order). Glyceryl trinitrate (GTN) mediated dilatation was assessed at the end of each study.
5.2.3 Results

5.2.3.1 Subject characteristics

There were no significant differences in age, gender, cholesterol levels or baseline heart rate and blood pressure between the groups. The mean number of pack years smoked by the smokers’ group was $10 \pm 6.6$ (range 2 - 20). In the diabetic group, mean age at diagnosis was $15.1 \pm 8.9$ years, and duration of diabetes was $14.9 \pm 7.4$ (range 3 - 29 ) years. Glycoslyated hemoglobin levels were $10.8 \pm 2.5$ (range 8.1 - 14.4 )% in the diabetic cohort. Venous blood glucose in all diabetic subjects was between 3 and 10 mmol/l at the time of each scan. Following L-arginine, plasma arginine levels rose 25-fold from $77 \pm 43.7$ (range 47 - 212) μmol/l to $1992 \pm 1052.6$ (504 - 4017) μmol/l. There were no differences between the groups in pre and post infusion plasma L-arginine concentration.

5.2.3.2 Pre-infusion vascular study

Baseline FMD was significantly reduced in the diabetic subjects and smokers compared to the controls ($P < 0.001$) (Figure 5.2.2). There was no significant variation in baseline FMD within either group between the two visits, indicating both the reproducibility of the technique and the stable physiological status of the subjects. Mean resting vessel size, blood flow and percentage increase in blood flow during reactive hyperaemia did not differ significantly between the risk factor groups and controls.

5.2.3.3 Post infusion vascular studies

Following L-arginine or saline infusions, there were no changes in heart rate, blood pressure, resting vessel size, resting blood flow or reactive hyperaemia, indicating that the infusions had no effect on basal vascular tone. Following saline infusion there was no change in FMD in either the group. In contrast, FMD improved significantly following L-arginine infusion in the smokers, from $2.0 \pm 1.71\%$ to $3.1 \pm 2.5\%$ ($P = 0.02$), but there was no significant change FMD in the controls ($6.9 \pm 3.3$ to $6.7 \pm 3.3\%, P = 0.77$) or diabetic patients ($1.8 \pm 1.5$ to $2.2 \pm 2.1\%, P = 0.9$) (Figure 5.2.3).
In the diabetics, but not smokers, dilatation to GTN, following saline infusion, was significantly reduced by comparison with the controls (23.1 ± 7.3% vs. 13.3 ± 9.6%, $P = 0.026$) (Figure 5.2.2). There was no significant difference in response to GTN after L-arginine compared to after saline infusion in any group.

![Graph showing baseline flow-mediated (FMD) and glyceryl trinitrate (GTN-MD) dilatation](image)

**Figure 5.2.2** Baseline flow-mediated (FMD) (upper panel) and glyceryl trinitrate mediated dilatation (GTN-MD) (lower panel). FMD was significantly impaired in smokers and insulin-dependent diabetes mellitus (IDDM) subjects. In contrast GTN-MD was significantly impaired only in IDDM subjects. Data is mean ± SD.
Figure 5.2.3 Effect of L-arginine on flow-mediated dilatation in controls, smokers and patients with type 1 diabetes mellitus. Data for each subject with mean and SD in bold. After L-arginine improvement in FMD was only seen in smokers.
5.2.4 Discussion

This study shows that young subjects with different cardiovascular risk factors have a differential response to intravenous L-arginine. Endothelial function was impaired in type 1 DM subjects and current smokers compared with controls as previously described (Celermajer et al., 1993a; Clarkson et al., 1996b), however following intravenous L-arginine, endothelial function improved only in smokers, and not in subjects with type 1 DM.

Impaired flow-mediated dilatation (FMD) represents a failure of endothelial dependent homeostasis, but provides no information on the nature of the vascular injury responsible. The purposes of this study were twofold: firstly to describe the effects of L-arginine on endothelial function in conduit arteries of young subjects, and secondly to use the response to L-arginine to further characterize the vascular abnormalities associated with risk factors. The results suggest differences in the pathophysiology of endothelial dysfunction in type 1 DM and smoking and suggest a potential therapeutic approach to vascular protection in smokers.

The reasons for the failure of L-arginine to improve endothelial function in the diabetics is not clear. In animal models of diabetes, L-arginine has been shown to have a beneficial effect on endothelial function (Pieper and Peltier, 1995; Pieper and Dondlinger, 1997) though this finding is not universal (Mayhan et al., 1996). However the mechanisms which underlie these effects are not fully understood and remain controversial (MacAllister et al., 1995). An alternative explanation might be that type 1 DM patients are resistant to the effects of acute administration of L-arginine. These findings suggest that different approaches to vascular protection, based on a detailed knowledge of the pathophysiology of vascular disease, will be required in different risk factor groups. However, the findings of this study, in which the effects of transiently high circulating levels of L-arginine were examined, cannot be extrapolated directly to predict benefits that might be expected from chronic oral therapy with L-arginine which will necessitate further studies in diabetics and smokers.
5.3 The effect of atorvastatin and chronic oral L-arginine therapy on endothelial function in young type 1 diabetics with normal cholesterol levels

5.3.1 Introduction

Classical risk factor modification represents an alternative approach to reversing endothelial dysfunction in type 1 DM. Factors such as cholesterol level continue to operate in determining cardiovascular risk in diabetes (Yudkin and Chaturvedi, 1999) and its reduction is associated with reduced mortality (LIPID Study Group 1998; van der Wal et al., 1994). Consistent with this is the finding that endothelial dysfunction in young type 1 DM patients correlates with LDL cholesterol level at levels that would be considered acceptable in the non-diabetic population (Clarkson et al., 1996b).

In the previous study, no benefit was found from acute administration of L-arginine to young diabetic patients, in contrast to its effect in smokers and patients with hypercholesterolaemia (Creager et al., 1992; Drexler et al., 1991; Thorne et al., 1998). The reason for this is not clear but it is possible that chronic oral therapy might have a more pronounced benefit.

This study was designed to examine the effect of cholesterol reduction with the 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase inhibitor atorvastatin, and dietary supplements of L-arginine, two potentially synergistic interventions, on conduit artery endothelial function in young clinically well patients with type 1 DM and normal cholesterol levels.

5.3.2 Specific Methods

5.3.2.1 Subjects

Subjects aged 18 - 45 years with type-1-DM of > 2 years duration (2 – 41 years, type-1-DM defined as requiring insulin treatment from diagnosis and a history of ketoacidosis) and a LDL-cholesterol level < 4.5 mmol/l were recruited from diabetic
clinics in London. They were non-smokers (who had either never smoked or not smoked for > 2 years with a total exposure < 1 pack year), had a resting supine blood pressure < 150/90 mmHg, had no clinical evidence of large vessel atherosclerosis, and were not taking vasoactive or cholesterol lowering medication. Eleven had evidence of background diabetic retinopathy and 5 had received treatment for proliferative retinopathy. All subjects gave informed written consent and the local research ethics committee approved the protocol.

5.3.2.2 Study design

This was a randomized double blind 2 x 2 factorial study. Subjects were randomized to take either L-arginine, 7 g twice daily, in the form of a lemon flavored sugar free drink (P.M.U. South Devon Healthcare, Torquay, UK) or placebo equivalent, and atorvastatin, 40 mg once daily at night, (Parke-Davis Ltd, UK) or placebo. They were thus divided into 4 treatment groups taking either placebo only, monotherapy with either atorvastatin or L-arginine, or combination therapy (Figure 5.3.1). The dose of L-arginine was chosen on the basis of animal experiments and clinical studies in subjects with hypercholesterolaemia in whom benefit on endothelial function has been demonstrated (Clarkson et al., 1996a; Rector et al., 1996). Subjects were instructed to continue with their normal diabetic diet and insulin regime throughout the study. Endothelial function was studied non-invasively at baseline and after 6 weeks of therapy. Subjects were all studied in a fasting state and at the same time of day on both visits. No attempt was made to study subjects under conditions of euglycaemia, as this would have been logistically difficult and moreover would have introduced the potential confounding influence of a glucose and insulin infusion on endothelial function.

5.3.2.3 Assessment of endothelial function

Brachial artery endothelium dependent and independent reactivity were assessed non-invasively, using the standard protocol and an A-mode wall tracking device (Ingenious Systems, Netherlands). Brachial artery diameter was determined at rest and 55 to 65 seconds after a brief period of reactive hyperaemia induced by inflating a pneumatic tourniquet placed around the forearm to 300 mmHg and its rapid release
Figure 5.3.1 Study Protocol. Subjects were randomized to either placebo, L-arginine (7gm BD), atorvastatin (40mg OD) in a 2x2 factorial design. Vascular function was assessed non-invasively at baseline and after 6 weeks of treatment.
after 4.5 minutes. All scans were recorded onto Super VHS videotape. Hardcopy images of the brachial artery were taken and notes made of the transducer and arm position enabling precise reproduction of conditions and measurement of the same segment of artery at subsequent visits. The increase in brachial artery blood flow velocity over the first 20 seconds of reactive hyperaemia (VTIs measured every 5 seconds) was determined using pulsed wave Doppler. After a further 10-15 minutes rest to allow vessel recovery, sublingual glyceryl trinitrate (GTN) 400 µg was administered and the response to this endothelium-independent dilator assessed after 3 minutes.

On completion of each study, the stored RF data for each scan was analyzed by placement of volume sample cursors, at the near and far vessel wall interfaces. Arterial distension was tracked and end diastolic diameter determined on a beat by beat basis with a spatial resolution of 50 µm. Vessel dilatation in response to flow (flow mediated dilatation [FMD]) and GTN were expressed as the percentage increase in vessel diameter from baseline. Scans in which the image at both studies was not replicated, a satisfactory distensibility waveform was not achieved, or cursor placement was incorrect were rejected and excluded from the final analysis. Resting volumetric blood flow was calculated for each study by multiplying the velocity time integral (corrected for angle) by the heart rate and vessel cross sectional area. Although this method may lead to overestimation of blood flow, inaccuracies are consistent allowing comparison between visits and individuals.

5.3.2.4 Laboratory measurements

Subjects were characterized for the presence of microalbuminuria from two, timed, overnight urine collections (albumin/creatinine ratio > 2 mg/mmol). At each visit, venous blood was taken without use of a tourniquet, for measurement of a full blood count (Bayer (Technicon) H1 system) and for assay of biochemical parameters by reflectance spectrophotometry (Johnson & Johnson Vitros dry chemistry system, unless stated otherwise). Fructosamine (a time-averaged marker of diabetic control over the preceding 4 - 6 weeks) was determined from the rate of formation of formazan using the Dimension AR system (Roche). Arginine levels were determined following precipitation with sulfur salicylic acid by a dedicated ion exchange amino
acid analyzer (Pharmacia, UK). Total and HDL cholesterol and triglyceride levels were measured by reflectance spectrophotometry and LDL calculated using the Friedwald formula (Friedwald et al., 1972). Plasma levels of vWF and plasminogen activator inhibitor-1 (PAI1) were measured by ELISA. Fibrinogen levels were determined by the Clauss clotting method using an Amelung KC10 coagulometer.

5.3.2.6 Statistical analysis

Data are presented as mean ± standard deviation. The primary outcome measure for this study was change in FMD between the two visits. Secondary outcome measures were change in GTN mediated dilatation and change in vWF and other plasma markers of endothelial function. The effects of atorvastatin, L-arginine or combination therapy on change in FMD, GTN mediated dilatation or plasma markers of endothelial activation were assessed using analysis of covariance. Covariates for age, gender, duration of diabetes, baseline LDL-cholesterol and baseline FMD or GTN mediated dilatation were initially included as previous studies had indicated that these variables may have a significant effect on endothelial function and the response to intervention. The effects of L-arginine and atorvastatin on plasma arginine and lipoprotein subfraction cholesterol levels were compared using independent student t-tests. Univariate and multivariate analyses were used to explore relationships between FMD and GTN mediated dilatation at baseline and subject characteristics and risk factor profile. Statistical significance was inferred at a p value < 0.05.

5.3.3 Results

5.3.3.1 Baseline subject characteristics

Eighty-four subjects were randomized (53 male, 31 female). Age, gender distribution, levels of lipid subfractions, blood pressure and diabetic characteristics were well matched between the 4 groups (placebo, L-arginine, atorvastatin and combined L-arginine + atorvastatin) (Table 5.3.1). Ten subjects (12%) had evidence of microalbuminuria. Eleven had evidence of background diabetic retinopathy and 5 had received treatment for proliferative retinopathy. Seventy-seven subjects (88%) completed the study and reasons for withdrawal are outlined in Table 5.3.2.
Compliance with the study drugs was determined from the volume of L-arginine and number of tablets of atorvastatin unused on completion of the study and was 87% and 96% respectively. Atorvastatin was generally well tolerated, with no effect on measures of diabetic control. There was no incidence of muscle pains, increases in creatinine kinase or transaminase levels. Two scans from two subjects were rejected, as they were technically unsatisfactory.

5.3.3.2 Biochemical effects.

Neither L-arginine or atorvastatin therapy had any significant effect on fasting glucose concentration, insulin requirements or overall diabetic control as determined by fructosamine levels. In the subjects randomized to L-arginine, plasma arginine increased from 67 ± 15 to 159 ± 84 μmol/l (P < 0.001). L-Arginine had no significant effect on plasma levels of any other amino acid, cholesterol or triglyceride levels. In the 37 subjects treated with atorvastatin, total, LDL and HDL cholesterol and triglyceride levels decreased by a mean of 33.3 ± 9.4% (P < 0.001), 48.3 ± 10.0% (P < 0.001), 6.1 ± 21.3% (P = 0.04) and 12.1 ± 25.6% (P = 0.03) respectively (Table 5.3.3).
Table 5.3.1 Baseline subject characteristics

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Placebo</th>
<th>L-Arginine</th>
<th>Atorvastatin</th>
<th>L-Arginine + Atorvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (%male)</td>
<td>22 (77)</td>
<td>21 (72)</td>
<td>21 (53)</td>
<td>20 (55)</td>
</tr>
<tr>
<td>Age</td>
<td>34.9 ± 7.9</td>
<td>35.0 ± 6.4</td>
<td>33.7 ± 6.1</td>
<td>33.3 ± 6.8</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>16.5 ± 9.6</td>
<td>15.0 ± 9.2</td>
<td>15.1 ± 8.5</td>
<td>16.2 ± 10.2</td>
</tr>
<tr>
<td>Fructosamine (mmol/l)</td>
<td>408 ± 73</td>
<td>374 ± 75</td>
<td>427 ± 64</td>
<td>414 ± 64</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>10.8 ± 6.2</td>
<td>9.9 ± 6.1</td>
<td>10.6 ± 5.1</td>
<td>11.4 ± 5.1</td>
</tr>
<tr>
<td>Daily insulin (IU)</td>
<td>52.8 ± 17.4</td>
<td>64.2 ± 22.5</td>
<td>53.3 ± 25.6</td>
<td>50.5 ± 15.6</td>
</tr>
<tr>
<td>Arginine (µmol/l)</td>
<td>57.5 ± 15.6</td>
<td>68.9 ± 17.6</td>
<td>70.1 ± 28.6</td>
<td>65.2 ± 12.6</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.70 ± 1.07</td>
<td>4.78 ± 0.80</td>
<td>4.92 ± 1.07</td>
<td>5.08 ± 0.86</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>2.82 ± 1.0</td>
<td>2.95 ± 0.81</td>
<td>3.08 ± 0.92</td>
<td>3.02 ± 0.82</td>
</tr>
<tr>
<td>vonWillebrand factor (umol/l)</td>
<td>1.09±0.43</td>
<td>1.20±0.40</td>
<td>1.20±0.36</td>
<td>1.27±0.56</td>
</tr>
<tr>
<td>Fibrinogen (umol/l)</td>
<td>2.48±0.39</td>
<td>3.19±1.33</td>
<td>2.99±0.72</td>
<td>3.02±0.57</td>
</tr>
<tr>
<td>PAI1 (umol/l)</td>
<td>7.18±6.97</td>
<td>5.78±5.46</td>
<td>5.06±3.89</td>
<td>6.04±3.62</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.45 ± 0.33</td>
<td>1.34 ± 0.46</td>
<td>1.47 ± 0.37</td>
<td>1.62 ± 0.62</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.93 ± 0.51</td>
<td>1.09 ± 1.08</td>
<td>0.83 ± 0.44</td>
<td>0.97 ± 0.34</td>
</tr>
<tr>
<td>Resting vessel size (mm)</td>
<td>3.93 ± 0.47</td>
<td>4.05 ± 0.65</td>
<td>3.69 ± 0.70</td>
<td>3.77 ± 0.73</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>3.49 ± 2.99</td>
<td>3.33 ± 3.03</td>
<td>3.65 ± 3.20</td>
<td>3.01 ± 3.32</td>
</tr>
<tr>
<td>GTN-MD (%)</td>
<td>17.12 ± 8.49</td>
<td>16.44 ± 7.49</td>
<td>19.58 ± 8.00</td>
<td>16.07 ± 7.49</td>
</tr>
<tr>
<td>Resting blood flow (mls/min)</td>
<td>83.0 ± 48.0</td>
<td>110.6 ± 69.7</td>
<td>65.9 ± 53.0</td>
<td>86.0 ± 61.1</td>
</tr>
<tr>
<td>Hyperemic blood flow (AUC)</td>
<td>75.7 ± 26.5</td>
<td>89.3 ± 41.9</td>
<td>69.8 ± 29.0</td>
<td>78.6 ± 35.7</td>
</tr>
</tbody>
</table>

PAI1 = plasminogen activator inhibitor-1, FMD = flow mediated dilation, GTN-MD = glyceryl trinitrate mediated dilation, AUC = area under the time/flow curve for hyperemic blood flow over the first 20 seconds following release of the tourniquet.
Table 5.3.2. Withdrawals after randomization

<table>
<thead>
<tr>
<th>Study No</th>
<th>Sex</th>
<th>Age</th>
<th>Treatment group</th>
<th>Reason for withdrawal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Female</td>
<td>22</td>
<td>Atorvastatin</td>
<td>Ketoacidosis following routine meniscectomy</td>
</tr>
<tr>
<td>40</td>
<td>Female</td>
<td>33</td>
<td>L-arginine</td>
<td>Lost to follow up</td>
</tr>
<tr>
<td>42</td>
<td>Female</td>
<td>36</td>
<td>Atorvastatin + L-arginine</td>
<td>Lost to follow up</td>
</tr>
<tr>
<td>49</td>
<td>Male</td>
<td>41</td>
<td>Placebo</td>
<td>Lost to follow up</td>
</tr>
<tr>
<td>53</td>
<td>Male</td>
<td>35</td>
<td>Atorvastatin</td>
<td>Dizziness</td>
</tr>
<tr>
<td>55</td>
<td>Female</td>
<td>34</td>
<td>Atorvastatin</td>
<td>Episodes of hypoglycaemia</td>
</tr>
<tr>
<td>78</td>
<td>Female</td>
<td>38</td>
<td>Atorvastatin</td>
<td>Lost to follow up</td>
</tr>
</tbody>
</table>

5.3.3.3 Vascular Function.

Baseline vessel size, resting blood flow, reactive hyperemic blood flow, FMD and dilatation to GTN were well matched between treatment groups (Table 5.3.1). For the whole cohort, FMD and dilatation to GTN were $3.4 \pm 3.1\%$ and $17.3 \pm 7.9\%$ respectively. There was a significant correlation between baseline FMD and the area under the curve of reactive hyperemic blood flow velocity over the first 20 seconds after release of the tourniquet ($r = 0.23$, $P = 0.04$) but not with peak hyperaemic blood flow, any of the measured parameters of diabetic control, insulin levels, blood pressure, or lipoprotein cholesterol or triglyceride levels.

In subjects randomized to placebo, L-arginine, atorvastatin or combined therapy, FMD changed by $-0.33 \pm 2.11\%$, $-0.33 \pm 2.49\%$, $1.75 \pm 2.88\%$ and $0.68 \pm 2.45\%$ respectively (Figure 5.3.2). Of the prespecified baseline covariates, baseline FMD ($\beta = -0.37$, $P = 0.003$) and duration of diabetes ($\beta = -0.07$, $P = 0.03$) were significantly associated with change in FMD. After allowing for the effect of these, atorvastatin therapy was associated with a significant increase in FMD of $1.26\%$ (95%CI: 0.22 to 2.3; $P = 0.018$) (Table 5.3.4). L-Arginine was not associated with a significant change.
Table 5.3.3 Effect of atorvastatin on cholesterol and triglyceride levels

<table>
<thead>
<tr>
<th></th>
<th>Atorvastatin</th>
<th>Placebo</th>
<th>(%) change</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Baseline 6 weeks</td>
<td>Baseline 6 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41 36</td>
<td>42 41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.00 ± 0.96 3.31 ± 0.66</td>
<td>4.73 ± 0.94 4.70 ± 0.97</td>
<td>-33.3±9.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.05 ± 0.86 1.55 ± 0.48</td>
<td>2.90 ± 0.92 2.81 ± 0.92</td>
<td>-48.3 ± 10.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.54 ± 0.51 1.41 ± 0.44</td>
<td>1.39 ± 0.40 1.42 ± 0.39</td>
<td>-6.1 ± 21.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.90 ± 0.39 0.76 ± 0.29</td>
<td>1.01 ± 0.83 1.08 ± 1.01</td>
<td>-12.1 ± 25.5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

L-Arginine had no effect on any of the lipid parameters measured, therefore, data shown is only segregated by randomisation to atorvastatin.
P value (independent student t-test) refers to difference in response between the atorvastatin and placebo groups.
in FMD (-0.51%; 95%CI: -1.54 to 0.51; P = 0.32) and in subjects who received L-arginine in addition to atorvastatin there was a trend towards reduction in the benefit of atorvastatin therapy (-1.28% (95%CI: -3.35 to 0.78), P = 0.2) but this was not significantly different from the effect of atorvastatin alone (Table 5.3.4). Other variables including lipoprotein cholesterol levels, plasma glucose level, fructosamine and blood pressure were added to this model together with interactions between variables already in the model but none were found to have a significant effect. None of the treatment variables or prespecified covariates were significantly associated with change in GTN mediated dilatation (Table 5.3.5) indicating that the improvement in FMD seen in the atorvastatin subjects is likely to reflect enhanced endothelial derived NO bioavailability.

Simple correlation coefficients were used to examine the relation between improvement in FMD and the change in cholesterol levels. There was a borderline significant inverse correlation between improvement in FMD and reduction in total cholesterol (r = -0.21, P = 0.07), but no significant relation with change in HDL (P = 0.2) or LDL cholesterol (P = 0.11) or triglyceride (P = 0.16) levels.

Following atorvastatin, plasma levels of vWF decreased from 1.28 ± 0.50 to 1.10 ± 0.28 (P = 0.03 on ANCOVA), there being no significant change following L-arginine therapy. As with FMD, the effect was greatest in subjects who received atorvastatin alone with a relative diminution of the effect in subjects on combined therapy. This interaction did not, however, reach statistical significance (Figure 5.3.3) Neither intervention had any significant effect on plasma levels of PAI1, or fibrinogen.

There was a borderline significant correlation between the reduction in vWF levels and change in total cholesterol levels (r = 0.23, P = 0.06) but no relation with the improvement in FMD (r = -1.63, P = 0.2).

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Table 5.3.4 Determinants of change in flow mediated dilation.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>1</td>
<td>28.678</td>
<td>28.678</td>
<td>5.84</td>
<td>0.018</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1</td>
<td>4.942</td>
<td>4.942</td>
<td>1.01</td>
<td>0.319</td>
</tr>
<tr>
<td>Interaction</td>
<td>1</td>
<td>7.575</td>
<td>7.575</td>
<td>1.54</td>
<td>0.219</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td>1</td>
<td>33.764</td>
<td>33.764</td>
<td>6.87</td>
<td>0.011</td>
</tr>
<tr>
<td>Baseline FMD</td>
<td>1</td>
<td>73.312</td>
<td>73.312</td>
<td>14.92</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
<td>69</td>
<td>338.949</td>
<td>4.912</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>487.448</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Term</th>
<th>Effect Size</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>1.26</td>
<td>0.22 to 2.30</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>-0.51</td>
<td>-1.54 to 0.51</td>
</tr>
<tr>
<td>Interaction</td>
<td>-1.28</td>
<td>-3.35 to 0.78</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td>-0.07</td>
<td>0.02 to 0.13</td>
</tr>
<tr>
<td>Baseline FMD</td>
<td>-0.37</td>
<td>-0.56 to -0.18</td>
</tr>
<tr>
<td>Constant</td>
<td>0.37</td>
<td>-0.72 to 1.47</td>
</tr>
</tbody>
</table>

The upper table is the analysis of covariance matrix for change in flow mediated dilation (FMD) over the 6-week duration of the study and the lower table gives the regression coefficients (Effect size). Improvement in FMD was significantly associated with atorvastatin therapy even after taking into account the effect of baseline FMD and duration of diabetes. L-Arginine therapy had no significant benefit on FMD and attenuated the effect of atorvastatin when given in combination.

Abbreviations df = degrees of freedom
Table 5.3.5 Determinants of change in GTN mediated dilatation

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>1</td>
<td>34.6</td>
<td>34.6</td>
<td>0.21</td>
<td>0.648</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1</td>
<td>227.6</td>
<td>227.6</td>
<td>1.39</td>
<td>0.243</td>
</tr>
<tr>
<td>Interaction</td>
<td>1</td>
<td>127.6</td>
<td>127.6</td>
<td>0.78</td>
<td>0.381</td>
</tr>
<tr>
<td>Error</td>
<td>70</td>
<td>11483.6</td>
<td>164.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
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<table>
<thead>
<tr>
<th>Term</th>
<th>Effect Size</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>-1.35</td>
<td>-7.23 to 4.52</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>-3.50</td>
<td>-9.43 to 2.43</td>
</tr>
<tr>
<td>Interaction</td>
<td>5.22</td>
<td>-6.58 to 17.01</td>
</tr>
<tr>
<td>Constant</td>
<td>7.87</td>
<td>-1.88 to 17.62</td>
</tr>
</tbody>
</table>

Neither L-arginine or atorvastatin had any significant effect on endothelium-independent dilatation to glyceryl trinitrate.
Abbreviations: df = degrees of freedom
Figure 5.3.2 Change in flow-mediated dilatation (FMD) in the 4 study groups. FMD significantly improved in subjects randomised to atorvastatin. There was no significant change was in subjects taking placebo or L-arginine and L-arginine therapy tended to attenuate the effect of atorvastatin.
Figure 5.3.3 Change in von Willebrand factor (vWF) in the 4 study groups (mean ± 95%CI).
5.3.4 Discussion

The important role of lipids in the etiology of vascular disease in diabetes and a potential benefit from cholesterol reduction was proposed by Joslin more than 50 years ago (Joslin, 1923). Since then epidemiological studies have confirmed a link between cholesterol levels and cardiovascular morbidity and mortality (Kannel and McGee, 1979) and recent clinical trials have demonstrated improved outcome when lipid lowering therapy is commenced in diabetic patients with established atherosclerosis (LIPID Study Group, 1998; van der Wal et al., 1994). This study has extended these findings and demonstrated that endothelial dysfunction, a key factor in the initiation of atherosclerosis, can be improved in young subjects with type 1 DM and normal cholesterol levels following just 6 weeks treatment with the HMG-CoA reductase inhibitor atorvastatin. Dietary administration of L-arginine, the substrate for NO synthesis, did not have a beneficial effect on endothelial function or enhance the response to atorvastatin. The improvement in endothelial function in response to atorvastatin therapy emphasises the importance of cholesterol in the pathogenesis of diabetic vascular disease and may have a beneficial effect on the progression of large vessel atherosclerosis.

This study examined the impact of cholesterol reduction and L-arginine therapy, two therapeutic approaches with different but potentially synergistic effects on NO bioavailability, on endothelial function in young subjects with type 1 DM. Both of these interventions are known to be beneficial in non-diabetic hypercholesterolemic subjects and therefore diabetic subjects with normal cholesterol levels were purposefully selected in order to examine the effect of cholesterol reduction and L-arginine therapy on endothelial dysfunction in type 1 DM. Moreover, such subjects are likely to be representative of the general type 1 DM population.

These results indicate that atorvastatin therapy can ameliorate endothelial dysfunction associated with type 1 DM prior to the onset of clinical vascular disease. Improvement was not dependent on baseline LDL cholesterol levels indicating that, in patients with type 1 DM, cholesterol reduction may be beneficial regardless of serum cholesterol level. This response was seen using a relatively high dose of atorvastatin over a short period of time and further studies will be necessary to
determine whether lower doses would also have been effective or whether cumulative benefit would be derived from longer therapy. In these patients only a borderline significant relation between reduction in total cholesterol level and improvement in FMD or reduction in plasma vWF levels was found. The mechanism by which HMG-CoA reductase inhibitors improve endothelial function have not been fully elucidated and beneficial effects, independent of reduction in LDL cholesterol levels, have been reported (Vaughan et al., 1996).

HDL cholesterol levels were reduced following atorvastatin therapy. Paradoxically this might have contributed to the improvement in endothelial function, as in type 1 DM reduced HDL associated paraoxonase activity results in decreased clearance of oxidatively modified lipids and may render HDL proatherogenic (Mackness et al., 1991a).

In contrast to some experiments in animal models of type 1 DM (Pieper and Peltier, 1995; Pieper and Dondlinger, 1997) and previous studies in hypercholesterolaemia (Creager et al., 1992; Drexler et al., 1991), 6 weeks of oral L-arginine therapy had no benefit on endothelial function. This finding is consistent with the effects of acute administration of L-arginine described in Section 5.2. This might reflect differences in the pathogenesis of endothelial dysfunction in these risk factor groups, or an abnormality of the response mechanism that underlies improvement in endothelial function following L-arginine in type 1 DM. The mechanism, by which L-arginine improves endothelial function in hypercholesterolaemia, remains controversial and may at least in part be mediated by release of insulin (Giugliano et al., 1997). This would preclude benefit in type 1 DM patients where endogenous insulin secretion is absent. Nitric oxide synthesis or levels of nitrate or nitrite were not measured in this study and therefore it cannot be determined whether L-arginine therapy had any effect on NO production. In diabetes, increased NO synthesis in the context of enhanced oxidative stress might result in formation of the cytotoxic molecule peroxynitrite and paradoxically be detrimental to endothelial function (Wever et al., 1997; Xia et al., 1996). Indeed, a non-significant trend to reduction in FMD after L-arginine and a diminished effect of atorvastatin when both treatments were combined was noted.
Response to the endothelium independent dilator GTN was also impaired in this study, compared to normal levels previously reported (Clarkson et al., 1996b). No benefit was derived from either intervention and the mechanisms and relevance of this finding to later clinical disease remain unclear.

Cardiovascular risk in type 1 DM remains high despite improvements in the management of insulin deficiency and glycaemic control. The process of atherosclerosis, as manifest by abnormal conduit artery endothelial function, begins early in type 1 DM, even in the absence of clinical evidence of nephropathy. This study has shown that at this early stage endothelial dysfunction in young type 1 DM subjects can be ameliorated by treatment with atorvastatin. These results suggest that reduction in cholesterol levels may be indicated from an early age and at lower levels than presently recommended in the general population in order to slow the progression of atherosclerosis to clinical cardiovascular disease. The role of L-arginine therapy in diabetes remains unclear.
5.4 The effect of angiotensin converting enzyme inhibition on endothelial function in young type 1 DM subjects

5.4.1 Introduction

The renin/angiotensin system (RAS) and its inhibition might influence endothelial function through a number of different pathways (Rajagopalan and Harrison, 1996). Angiotensin-II (ATII) promotes macrophage activation and smooth muscle cell proliferation, both early events in atherogenesis (Daemen et al., 1991; Foris et al., 1983). Free radical generation, results in inactivation of NO, enhanced lipid peroxidation and cellular injury (Griendling et al., 1994; Rubanyi and Vanhoutte, 1986). Angiotensin converting enzyme (ACE) inhibitors may have beneficial effects on endothelial function via reduction in ATII levels (Rajagopalan and Harrison, 1996), and also via inhibition of bradykinin degradation (Wiener et al., 1991). ACE inhibitors have been shown to improve large vessel endothelial function in both animal models of hypercholesterolaemia (Becker et al., 1991; Chobanian et al., 1990) and clinical studies of subjects with established cardiovascular disease (Mancini et al., 1996). In type 1 DM, ACE inhibitors retard the progression of renal disease (Parving et al., 1995; Ruilope, 1995) but their effects on large vessel endothelial function have not been studied.

This study was designed to investigate the effect of the ACE inhibitor enalapril on conduit artery endothelial function in young subjects with type 1 DM to determine whether improvements of potential clinical significance could be achieved at an early stage in the natural history of arterial disease.

5.4.2 Specific methods

5.4.2.1 Subjects

Subjects with type 1 DM of > 2 years duration, aged 18 - 45 years, who were non-smokers, (had not smoked for > 2 years with a total exposure < 1 pack year) had resting supine blood pressure < 140/90 mmHg, no clinical evidence of large vessel atherosclerosis, and were not taking vasoactive medication were selected from
diabetic clinics in London. One hundred and eleven subjects were evaluated with a
detailed clinical history, physical examination and 12 lead electrocardiogram. To
characterise subjects for the presence of microvascular disease, 2 timed overnight
urine collections were made to measure urinary albumin excretion at recruitment and
again on completion of the study. After a 4 week screening phase during which all
subjects received placebo medication, 91 of the 111 subjects with stable diabetic
control (HbA1c < 14%), and satisfactory compliance with placebo were randomised.
Reasons for non-randomisation were high HbA1c in 6, poor cooperation or
withdrawal of consent in 12, and adverse experience in two subjects.

5.4.2.2 Study design

This was a randomised, double-blind parallel group study. During the first 3 weeks,
each subject was titrated from a starting dose of 5 mg of enalapril or placebo
equivalent, to a dose of 20 mg or the maximum dose tolerable. Subjects were
instructed to continue with their normal diabetic diet and insulin regime for the
duration of the study. No attempt was made to study subjects under conditions of
euglycaemia, as this would have been logistically difficult and moreover would have
introduced the potential confounding influences of a glucose and insulin infusion on
endothelial function.

5.4.2.3 Lipid measurements

At each visit blood samples were taken for full blood count, biochemistry and
HbA1c (Rapid EP system, Helena Laboratories). Fasting total cholesterol and plasma
triglycerides were measured at recruitment and on completion of the study using the
cholesterol C-system high performance CHOD-PAP and GPO-PAP high
performance enzymatic colorimetric test respectively (Boehringer Mannheim GmbH,
Diagnostica). HDL-cholesterol was measured after precipitation of apoprotein B
containing lipoproteins, and LDL-cholesterol calculated according to the Friedwald
formula (Friedwald et al., 1972). The study was approved by the local research ethics
committee and all subjects gave informed written consent.
5.4.2.4 Assessment of endothelial function

Brachial artery endothelium dependent and independent reactivity were assessed non-invasively, using the standard protocol and an A-mode wall tracking device (Ingenious Systems, Netherlands) at baseline and after 12 and 24 weeks of treatment. Subjects lay at rest for at least 10 minutes prior to the first scan and remained supine throughout the procedure. Brachial artery diameter was determined at rest and 55 to 65 seconds after a brief period of reactive hyperaemia induced by inflating a pneumatic tourniquet placed around the forearm to 300 mmHg and its rapid release after 4.5 minutes. All scans were recorded onto Super VHS videotape. Hardcopy images of the brachial artery were taken and notes made of the transducer and arm position enabling precise reproduction of conditions and measurement of the same segment of artery at subsequent visits. The peak increase in brachial artery blood flow velocity over the first 15 seconds of reactive hyperaemia was determined using pulsed wave Doppler. After a further 10 - 15 minutes rest to allow vessel recovery, sublingual glyceryl trinitrate (GTN) 400 µg was administered and the response to this endothelium-independent dilator assessed after 3 minutes. FMD and dilatation to GTN were expressed as the percentage increase in vessel diameter from baseline.

5.4.2.5 Statistical analysis

Descriptive data are expressed as mean value ± standard deviation. Withdrawn subjects were included in the analyses up to and including the time of withdrawal. Changes in the measures of vascular function within groups and between groups were assessed using paired and two sample (independent) t tests respectively. Multiple linear regression analysis was used to determine which variables appeared to be most closely associated with the FMD and GTN-induced dilatation at baseline and change in these variables over the duration of the study. Statistical significance was inferred at a p value < 0.05.

5.4.3 Results

5.4.3.1 Baseline subject characteristics

Age, levels of lipid subfractions, blood pressure, length of time since diagnosis of diabetes, total daily insulin dose and HbA1c as a measure of overall diabetic control
were comparable in both groups (Table 5.4.1). Only one subject had microalbuminuria and none had macroproteinuria.

Of the 91 subjects randomised 39 enalapril and 43 placebo subjects completed the study. Five subjects (4 enalapril and 1 placebo) withdrew because of adverse clinical experiences and 4 subjects (3 enalapril, 1 placebo) because of protocol violations or for administrative reasons. Forty-three of the 46 enalapril patients and 44 of the 45 placebo patients were titrated to the maximum dose (20 mg or placebo equivalent), though the dose was subsequently reduced in 7 of the enalapril subjects and 6 of the placebo subjects. The treatments were well tolerated and compliance determined by tablet counts was 79% for enalapril and 73% for placebo. Clinical adverse events thought to be related to the study medication were more common in the enalapril group (24% versus 11%). Six enalapril and 1 placebo subject complained of cough. Six subjects (4 enalapril, 2 placebo) had symptoms of headache or dizziness. Fifteen scans from 12 subjects (9 enalapril, 3 placebo) were withdrawn for technical reasons as described above.

There were no significant changes in diabetic control, insulin requirement, or lipid subfractions over the course of the study. Enalapril caused a mean decrease of 3.4 ± 3.0 mmHg of diastolic blood pressure (95% CI: -6.7 to -0.1, P < 0.05) but systolic blood pressure remained unchanged as did both parameters in the placebo group.

5.4.3.2 Vascular function

At baseline, resting vessel size, brachial artery blood flow, degree of reactive hyperaemia induced, FMD and dilatation to GTN were comparable in the enalapril and placebo group (Table 5.4.1). On univariate analysis there was an inverse relationship between total cholesterol levels and FMD (r = −0.22, P = 0.04) and between dilatation to GTN and total and LDL-cholesterol levels (r = −0.25, P = 0.02 and r = −0.26, P = 0.02 respectively). Response to GTN also correlated with HDL cholesterol level (r = 0.22, P = 0.05). In the multiple regression model FMD at baseline was inversely correlated with cholesterol (β = -0.77, P = 0.03) but not with age, vessel diameter, systolic blood pressure or duration of diabetes. GTN response
was related inversely to vessel diameter ($\beta = -7.57$, $P < 0.001$) and to duration of diabetes ($\beta = -0.19$, $P = 0.04$). In a separate multiple regression analysis, resting blood flow correlated with vessel diameter ($\beta = 24.3$, $P < 0.001$) and inversely with total cholesterol levels ($\beta = -8.39$, $P = 0.008$) though not with any diabetic parameters or blood pressure. This may indicate an abnormality of basal tone in distal resistance arterioles.

Enalapril had no effect on resting vessel size, brachial artery blood flow or the degree of reactive hyperaemia induced, suggesting that it did not cause significant vasodilatation in this normotensive cohort. In subjects treated with enalapril mean FMD increased from $1.6 \pm 2.4\%$ at baseline to $2.6 \pm 2.7\%$ and $2.8 \pm 2.9\%$ at 12 and 24 weeks respectively. In the placebo group, FMD also increased from $2.3 \pm 2.6\%$ to $2.6 \pm 2.19\%$ and $3.0 \pm 2.6\%$ (Figure 5.4.1). However, neither the increase in FMD within the enalapril and placebo groups reached statistical significance and there was no significant difference between responses of the enalapril and placebo group. Response to GTN did not change significantly in either group over the duration of the study (Figure 5.4.1).

Multiple regression analysis was performed to determine whether any factors in the subjects baseline profile or treatment group influenced change in FMD over the study period. Improvement in FMD was related to vessel size ($\beta = -2.83$, $P = 0.002$), duration of diabetes ($\beta = -0.15$, $P = 0.001$) baseline GTN dilatation ($\beta = -0.13$, $P = 0.02$) and systolic blood pressure ($\beta = 0.07$, $P = 0.03$) but not with subjects age, HbA1c level, LDL cholesterol level or treatment group.
Table 5.4.1. Subjects baseline characteristics and vascular reactivity.

<table>
<thead>
<tr>
<th></th>
<th>Enalapril</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects (% male)</td>
<td>45 (65.2)</td>
<td>46 (57.8)</td>
</tr>
<tr>
<td>Age (yrs)*</td>
<td>29 (18 - 44)</td>
<td>30 (19 - 44)</td>
</tr>
<tr>
<td>Duration of type 1 DM (years)</td>
<td>12.9 (7.8)</td>
<td>12.5 (8.3)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>10.2 (1.6)</td>
<td>10.3 (1.8)</td>
</tr>
<tr>
<td>Total daily insulin dose (IU)</td>
<td>57.0 (17.26)</td>
<td>51.6 (20.8)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>116.9 (10.3)</td>
<td>118.6 (8.7)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>73.7 (8.8)</td>
<td>74.6 (7.5)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.06 (0.89)</td>
<td>5.05 (1.01)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>3.08 (0.80)</td>
<td>2.95 (0.92)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.53 (0.33)</td>
<td>1.63 (0.38)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.95 (0.60)</td>
<td>0.95 (0.43)</td>
</tr>
<tr>
<td>Lipoprotein(a) (mmol/l)*</td>
<td>8.65 (0.05 - 101.80)</td>
<td>8.20 (0.05 - 103.40)</td>
</tr>
<tr>
<td>Vessel diameter (mm)</td>
<td>3.59 (0.57)</td>
<td>3.55 (0.61)</td>
</tr>
<tr>
<td>Resting blood flow (ml/min)*</td>
<td>38.5 (11.7 - 119.4)</td>
<td>33.0 (7.9 - 166.5)</td>
</tr>
<tr>
<td>Reactive hyperaemia (%)*</td>
<td>325.4 (125.1 - 653.2)</td>
<td>368.1 (125.1 - 653.2)</td>
</tr>
<tr>
<td>Flow mediated dilation (%)</td>
<td>1.60 (2.38)</td>
<td>2.31 (2.68)</td>
</tr>
<tr>
<td>GTN- mediated dilation (%)</td>
<td>17.47 (8.04)</td>
<td>18.34 (7.56)</td>
</tr>
</tbody>
</table>

Mean (SD) except in the case of * when skewed data expressed as median (range). Abbreviations: GTN = glyceryl trinitrate, HDL = high density lipoprotein, LDL = low density lipoprotein.
Figure 5.4.1 Flow mediated dilatation (top) and GTN mediated dilatation (bottom) at baseline and after 12 and 24 weeks of treatment with enalapril or placebo. Data is mean ± S.D.
5.4.4 Discussion

ACE inhibitors potentially have a number of beneficial effects on vascular structure and function, mediated either by the direct inhibition of the promitogenic and proinflammatory effects of ATII, or by enhanced bioavailability of the "antiatherogenic" molecule, nitric oxide (Lonn et al., 1994). Increased NO activity might result from attenuation of ATII mediated production of superoxide (Griendling et al., 1994; Rajagopalan et al., 1996) which can inactivate NO (Rubanyi and Vanhoutte, 1986), or via inhibition of bradykinin degradation, a potent physiological stimulus for NO release (Hornig et al., 1997; Wiemer et al., 1991). Experimental evidence suggests that ACE inhibition may reduce subendothelial accumulation of macrophages (Clozel et al., 1991; Hernandez-Presa et al., 1997) inhibit smooth muscle cell growth and enhance endothelial cell repair, factors which might be important in the early stages of atherogenesis (Daemen et al., 1991; Dzau et al., 1991; Foris et al., 1983). In hypercholesterolemic rabbits, ACE inhibition has been shown to enhance endothelial dependent dilatation and to reduce the development of atherosclerosis independently of its blood pressure lowering effects (Becker et al., 1991; Chobanian et al., 1990). In clinical trials, similar benefit on coronary endothelial function has been shown with the ACE inhibitor quinapril in non-diabetic patients with established cardiovascular disease (Mancini et al., 1996) but no benefit on atherosclerosis or the incidence of clinical vascular events has been demonstrated (Cashin-Hemphill et al., 1999).

Despite this experimental evidence of favorable effects of ACE inhibition in early atherosclerosis, in this study no benefit in conduit artery endothelial function was seen in young type 1 DM subjects after 6 months treatment with the ACE inhibitor enalapril. The inability to demonstrate an improvement after enalapril treatment compared to placebo may have a number of explanations. Firstly, the power of the study, which was designed on the difference in FMD between type 1 DM and controls subjects seen in previous studies (Clarkson et al., 1996b), may have been inadequate. There was a trend towards increase in endothelial function in both the enalapril and placebo groups. The reason for the placebo groups improvement may reflect subtle changes in subjects behaviour during the study which were outside the control of the study protocol and it remains possible that a significant result would be
achieved with a greater number of subjects or if studied over a longer period of time. The majority of subjects in this study did not have microalbuminuria which is known to increase the risk of atherosclerosis and to benefit from ACE inhibitor therapy (Krolewski et al., 1987; Parving et al., 1995; Ruilope, 1995) and it may be that the RAS has greater importance at a later stage in atherogenesis. Recent evidence suggests that endothelial expression of ACE in the conduit artery of patients may be low in the absence of atherosclerotic plaque (Diet et al., 1996) and the beneficial effects of ACE inhibition seen in animal and human studies may therefore, reflect a greater impact on more advanced atherosclerotic disease. Finally, the vasoprotective effects of ACE inhibitors may depend on their ability to inhibit tissue bound as opposed to circulating ACE (Lees et al., 1990). The potency of individual ACE inhibitors may be influenced by their pharmacological properties such as lipid solubility, affinity for ACE in vascular tissues and their clearance (Johnston et al., 1989). These findings with enalapril cannot therefore be extrapolated to apply to other ACE inhibitors. Whilst the RAS may have a role in the pathogenesis of vascular disease the complex nature of this process in type 1 DM may be resistant to individual strategies aimed at restoring vascular function.
The initial aim of this thesis was to refine and develop methods for assessing flow-mediated dilatation in vivo. The use of a fixed adjustable stereotactic clamp has facilitated the maintenance of high quality images over prolonged periods of time. Linked to this the use of acquisition software and the automated edge detection analysis to measure arterial wall diameter and determine the whole time course of arterial dilatation represents a significant improvement on earlier methods. More recent developments have resulted in a totally automatic analysis system.

In Chapter 2 and 3 the mechanisms of flow-mediated dilatation have been explored. A close dose-dependent relationship between the intensity of the flow stimulus and the subsequent dilatation has been established. The use of lower doses of GTN has been piloted and a dose-dependent relationship between GTN dose and dilatation established. This will facilitate the measurement of endothelium-independent dilatation at a more physiologically relevant range, in future studies, and its repetition over short time periods.

One of the major findings in this thesis is the important role of the duration of the flow stimulus in determining the mechanism of dilatation. Flow-mediated dilatation in response to a short episode of reactive hyperaemia was found to be dependent on synthesis of nitric oxide whilst that seen in response to a sustained flow stimulus is not affected by nitric oxide synthesis inhibition. Interestingly patients with hypercholesterolaemia had markedly impaired dilatation to reactive hyperaemia, but in contrast the dose response to a sustained flow stimulus was not different from controls. These data are relevant to the interpretation of endothelial function tests, which use flow as a stimulus, and imply that the abnormality of endothelial function in hypercholesterolaemia may be confined to the nitric oxide pathway.

In Chapter 4 the impact of a range of metabolic risk factors on endothelial function and other markers of arterial function were examined in a large population based cohort from the Whitehall II study. Both endothelial function and carotid artery distensibility were abnormal in a group of subjects with the metabolic syndrome. However, no role for glucose tolerance or insulin resistance in endothelial dysfunction or abnormalities of carotid artery distensibility were found. In contrast established cardiovascular risk factors such as blood pressure, dyslipidaemia and
obesity significantly influenced both endothelial function and carotid artery distensibility. These relationships were linear and apparent at levels of risk factor that would be considered satisfactory in the normal population.

A crucial challenge for medical practice is to develop strategies that can alter the natural history of atherosclerosis at an early premorbid stage. The assessment of endothelial function might represent one method for determining the benefit of such strategies. In Chapter 5 the effect of a number of therapeutic interventions on endothelial function have been considered. L-Arginine therapy is shown to improve endothelial function in smokers, but in contrast no benefit from either acute intravenous L-arginine or chronic oral therapy is found in young type 1 diabetic patients. These data suggest potential differences in the pathogenesis of endothelial dysfunction in these different risk factor groups.

Improvement in endothelial function is however, demonstrated in type 1 diabetic patients following treatment with the HMG-CoA reductase inhibitor atorvastatin, though a definite relationship with cholesterol reduction is not established suggesting that other mechanisms might be operating.

In a separate group of type 1 diabetic patients no benefit following angiotensin-converting enzyme inhibition with enalapril is found after 6 months chronic oral treatment.

6.1 Future directions.

Although now widely applied the measurement of flow-mediated dilatation as an assessment of endothelial function has a number of important limitations that will define future research. Firstly the mechanisms of the endothelial response to flow are not fully understood and may be heterogeneous. Elucidating the relevant pathways is pivotal in understanding the mechanisms of endothelial dysfunction in risk factor groups, interpreting the results of endothelial function tests and guiding the development of therapies targeted at retarding vascular damage at an early stage.
Specifically the nature of dilatation to sustained flow stimuli should be explored and such studies are currently underway.

Although endothelial dysfunction has been demonstrated in all the major risk factor groups, currently no long-term follow-up studies have examined the relationship between endothelial dysfunction and later cardiovascular morbidity and mortality. These studies will be important to establish the validity of endothelial function tests as a measure of early vascular disease.

Finally, benefit from interventions has been shown in individuals with endothelial dysfunction. However, it remains unknown whether such benefit will ultimately result in reduced cardiovascular morbidity and mortality. Large scale, sophisticated, interventional studies with follow-up over many years will be required to answer these important clinical questions.


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