NON-INVASIVE STUDIES OF ARTERIAL PHYSIOLOGY IN CHILDREN AND ADULTS AT RISK OF ATHEROSCLEROSIS

A Thesis submitted for the Degree of Doctor of Philosophy

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DEDICATION

To My Dad.

Although gone now for what seems an eternity, you still remain the inspiration for everything I try to achieve.

ABSTRACT

Atherosclerosis is the leading cause of death in the Western World. In clinical practice, treatment and risk factor modification usually begin only after complications of advanced disease have occurred. Pathology studies, however, have shown that atherogenesis begins much earlier and may be evident in the first decade of life. This thesis describes the development of a non-invasive method to study arterial physiology in children and adults, in order to detect early signs of vascular disease in-vivo, in presymptomatic subjects.

Endothelial dysfunction is a key early event in the initiation and progression of atherosclerosis in experimental studies, preceding formation of plaques. We have used high resolution ultrasound to study endothelial and smooth muscle function in systemic arteries in man. Arterial diameter responses to reactive hyperaemia (with increased flow causing endothelium-dependent dilatation) were compared to the responses to sublingual nitroglycerine (which acts independently of endothelial function). Phantom studies have confirmed the precision of diameter measurements (accurate to 0.1-0.2mm), and serial studies have shown reproducible results within patients between visits (coefficient of variation ≈2.0%).

Studies on over 200 control subjects without vascular risk factors showed that flow-mediated dilatation (FMD) occurs, and is inversely related to vessel size (r=-0.80, p<0.001). The effect of ageing on arterial physiology was also assessed, and FMD was impaired in normal men older than 40 years and women older than 50 years. Twenty adults with established atherosclerosis all had impaired endothelium-dependent dilatation.

Over 400 clinically well children and young adults with recognised risk factors were then studied, including children with familial hypercholesterolaemia and young adult smokers. In most, FMD was reduced compared to the relevant controls, whereas nitroglycerine-induced dilatation was preserved.

<u>Conclusions</u>. Endothelium-dependent dilatation is impaired in children and adults with risk factors for atherosclerosis, such as smoking and hypercholesterolaemia. The availability of a non-invasive method for studying arterial physiology will facilitate prospective investigation of the progression or reversibility of early vascular disease.

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LIST OF PUBLICATIONS FROM THIS WORK

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- Celermajer DS, Sorensen KE, Ryalls M, Robinson J, Thomas O, Leonard JV, Deanfield JE. Impaired endothelial function occurs in the systemic arteries of children with homozygous homocystinuria but not in their heterozygous parents. J Am Coll Cardiol 1993; in press.
- 3. Celermajer DS, Sorensen KE, Georgakopoulos D, Bull C, Thomas O, Robinson J, Deanfield JE. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation 1993; in press.
- 4. Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge J, Deanfield JE. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia, and is related to the Lp(a) level. J Clin Invest 1994; in press.

INTRODUCTION AND AIMS

A principal problem confronting preventive medicine has been the identification of asymptomatic people likely to develop atherosclerosis. In the absence of a safe and accurate screening method for visualising arterial plaques, a risk factor concept has evolved and been applied in several health promotion research programmes (MRFIT 1976, Lipid Research Clinic 1984). Many of these risk factors, such as serum total cholesterol and blood pressure, have been measured in epidemiologic surveys of children (Lauer et al 1975, Morrison et al 1977). Despite the fact that risk factor tracking occurs in adolescents and in children over 6-9 years (Clarke et al 1978, Freedman et al 1985), a substantial proportion of children with initially elevated vascular risk factors show reduced levels at follow-up. Thus the problem of identifying people in early life at high risk for adult cardiovascular disease persists. In recent years, however, non-invasive techniques for the diagnosis and assessment of atherosclerosis have improved; in particular, vascular ultrasound has the potential for safe, reliable, and cost-effective screening.

The disease process of atherosclerosis begins in childhood. The first histological signs are lipid deposits in the intima of systemic arteries (McGill 1990). Fatty streaks can be found in the aortas of children older than 3 years and in the coronary arteries by adolescence (Enos et al 1953, Stary 1989). Endothelial dysfunction is an early physiological event in atherogenesis (Healy 1990). Studies in vitro have shown that the endothelium is abnormal in the earliest stages, before plaques exist and certainly before clinical detection of disease, and that endothelial injury predisposes to thrombosis, leukocyte adhesion and proliferation of smooth muscle cells in the arterial wall (Ross 1986). An important functional consequence of endothelial dysfunction is the inability to release endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki 1980). In vivo, coronary endothelial dysfunction has been shown in response to various pharmacological and physiological stimuli, largely in symptomatic adults with established

coronary atherosclerosis (Ludmer et al 1986, Nabel et al 1990). These studies used invasive angiography, which is clearly not suitable for the investigation of either the early development of vascular damage in younger symptom-free subjects, or for serial studies of progression or reversibility.

We have therefore developed a non-invasive means of studying early changes in vascular physiology in systemic arteries. High-resolution ultrasound is used to follow changes in vessel diameter in response to increased flow and to nitroglycerine (GTN). In arteries lined by healthy endothelium, increased flow causes dilatation of the vessel (Rubanyi et al 1986, Laurent et al 1990), via release of EDRF (Pohl et al 1986). This mechanism fails with endothelial dysfunction (Young & Vatner 1987). In contrast, GTN causes vasodilatation by direct action on the smooth muscle; its effect is therefore independent of the endothelium.

This non-invasive method has the potential to detect changes in arterial physiology that precede the structural manifestations of atherosclerosis.

AIMS OF THESIS

- 1. To design a non-invasive method for testing endothelial and smooth muscle responses in the systemic arteries of children and young adults.
- 2. To validate the accuracy and reproducibility of the method.
- 3. To study arterial physiology in "normal" children and adults (those without known vascular risk factors), in order to determine the effects of ageing on endothelial function, with particular reference to differences between males and females.
- 4. To study the effects of risk factors on arterial physiology in asymptomatic subjects prone to atherosclerosis, such as young adult smokers and children with hypercholesterolaemia.
- 5. To assess the suitability of the method for studies of reversibility in subjects with endothelial dysfunction.

All studies described in this thesis were approved by the hospital's committee for ethical practice.

CHAPTER 1 - INTRODUCTION

1.1 ATHEROSCLEROSIS; A DISEASE THAT BEGINS IN CHILDHOOD

Atherosclerosis is a very old disease, having been identified even in the mummies of ancient Egypt (Ruffer 1911). The term is derived from the Greek "athero" (porridge) and "sclerosis" (hardening). In the latter half of this century, this disease and its complications have caused up to 50% of deaths in the United Kingdom. Although the clinical manifestations usually present in middle and late adulthood, the risk factors for atherogenesis are often present in childhood (Figure 1.1.1), and early histopathological changes can be found in the large systemic arteries in the first decade of life (Stary 1989) (Figure 1.1.2).

1.1.1 THE PREVALENCE OF ATHEROSCLEROSIS

Coronary heart disease is currently the leading cause of death in Britain, accounting for 26% of all fatalities, and cerebrovascular disease is the third commonest, causing 12% of deaths (British Heart Foundation 1992). The vast majority of these deaths, numbering over 250 000 each year, are due to complications of atherosclerotic plaques. Coronary deaths are commoner in men up to the age of 75 years, although thereafter the event rate is higher in women (Figure 1.1.3). The prevalence of other atheroma-related diseases, due to plaques in the renal, mesenteric and peripheral arteries, is more difficult to estimate.

Death rates from coronary and cerebral vascular disease have declined significantly over the last 30 years, mainly due to lifestyle changes, such as smoking cessation and healthier eating habits (Goldman & Cook 1984). The decrease in cardiovascular mortality may also be due to improved medical treatment of risk factors, such as hypertension, hypercholesterolaemia and diabetes. In the United States, the age-adjusted

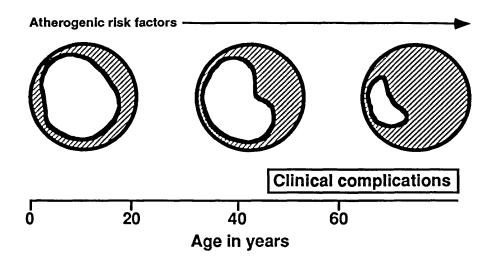


Figure 1.1.1
Although the clinical complications of atherosclerosis, such as myocardial infarction and stroke, arise from middle-age onwards, the risk factors predisposing to atherogenesis begin in childhood.

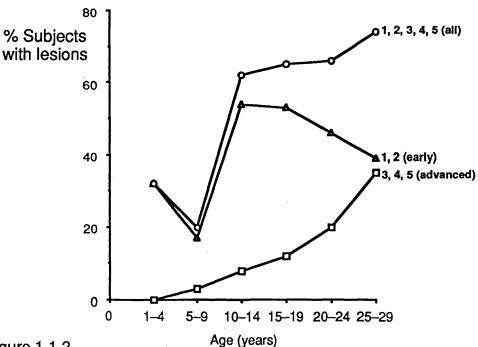


Figure 1.1.2

The prevalence of early atherosclerotic changes in the coronary vessels of children and young adults who died of non-cardiac causes (Stary 1989). Stary 1, 2 – intimal macrophages, fatty streaks. Stary 3, 4, 5 – preatheroma, fibroatheroma. By age 10 – 14 years, most children have some evidence of disease, occasionally advanced.

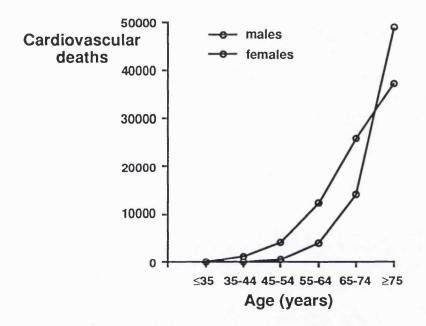


Figure 1.1.3 Cardiovascular deaths in Britain in 1991. Men have more deaths in middle-age, but women "catch up" in later life.

mortality rates from myocardial infarction and cerebrovascular accidents fell by about 50% between 1950 and 1987 (American Heart Association 1991). In the Western World, public awareness of cholesterol, nutrition and obesity have increased markedly. In contrast, in Eastern Europe and some parts of Asia, the introduction of a Western diet has caused an increase in the incidence of diabetes, hyperlipidaemia and atherosclerosis.

Other public health and medical advances have improved the outcome for patients with advanced vascular disease who have acute complications; improvements in community resuscitation programs, paramedical/ambulance care, in-hospital coronary care, arrhythmia treatment, thrombolytic therapy, balloon angioplasty and coronary surgery have all been beneficial. Despite these improvements, however, occlusive vascular disease continues to cause enormous morbidity and mortality.

1.1.2 THE LESIONS OF ATHEROSCLEROSIS

Atherosclerotic plaques may be found throughout the body, especially in medium-sized muscular (such as the coronary) and large elastic arteries (such as the aorta). In medium-sized vessels, acute complications usually involve plaque rupture or haemorrhage with subsequent occlusion of the lumen, or embolisation of atheromatous material to distal sites. Coronary, carotid and iliofemoral atherosclerosis accounts for most clinically evident disease. Aortic atheroma may result in important clinical problems because of stretching of the arterial wall, aneurysm formation and/or rupture.

The report of Enos et al (1953), in which the authors found that 77% of United States soldiers killed in action in Korea had coronary atherosclerosis, including 15% with lesions causing ≥50% stenosis of the lumen, illustrated the fact that atherogenesis begins in early life. Since then Stary (1989) has demonstrated fatty streaks and/or atheroma in 40% of young Americans killed in motor accidents, and lipid droplets in the coronaries of most children by age 13 years (Figure 1.1.2), emphasising that the pathophysiological abnormalities leading to atheroma begin in the first decades of life.

Atherosclerosis is slowly progressive. Lesions begin with insudation of lipid and macrophages into the subendothelial space. The first macroscopically visible sign of atheroma is usually the fatty streak, which is commonly found in young children and adolescents (Stary 1983). The advanced lesion, the fibrous plaque, is comprised of lipids, cells, connective tissue and necrotic debris, and usually appears in early adulthood. Plaque size and numbers increase with age (Geer 1965).

Fatty streaks usually appear first in the aorta, and then in medium-sized arteries such as the coronary (Stary 1989). Grossly, fatty streaks are yellow because of the large amounts of cholesterol and its esters deposited in the subintima. Microscopically most of this lipid is deposited in macrophages ("foam cells"), and later in smooth muscle cells. Fatty streaks occupy similar sites to those involved by advanced fibrous plaques in later life, suggesting that over time they are converted to fibroatheromatous lesions (McGill 1984). Fatty streaks do occur at other sites, but may regress. Therefore local factors such as turbulence of flow may determine which early lesions progress to advanced occlusive disease.

Advanced plaques consist of varying amounts of lipid and fibrous tissue. Fibrous lesions are usually white rather than yellow, and firmer than lipid-rich plaques. As plaques increase in size they become elevated and eventually cause intravascular flow disturbance. Microscopically such advanced plaques consist of lipid-laden macrophages and smooth muscle cells surrounded by collagen, elastin and proteoglycan. The surface of the plaque may be covered by a connective tissue cap. The composition of plaques may vary between and even within individuals. Lipid-rich plaques, such as are found in hypercholesterolaemic patients, may be associated with a propensity to ulceration and rupture (Davies & Woolf 1993).

Plaques are most prevalent in the abdominal aorta, especially near the ostia of large branches. Of the muscular arteries, coronary and carotid vessels seem to have the greatest involvement, whereas the renal, popliteal and internal mammary arteries are relatively spared. The principal clinical consequences in all these sites relate to partial or total occlusion of the lumen, with consequent distal ischaemia and/or infarction.

1.1.3 ATHEROGENESIS

1.1.3.1 Cellular Events

Understanding atherogenesis has been facilitated by the availability of animal models of disease and by improved histopathological and cell biology techniques. Rabbits (Cooke et al 1991), swine (Gerrity et al 1979) and primates (Faggiotto et al 1984) all develop atheromatous lesions similar to humans if they are given a high-cholesterol diet. Monoclonal antibodies allow determination of cell types, allowing the origin of cells that comprise atherosclerotic lesions to be identified accurately (Ross 1992). In the future, transgenic animals may also provide models of atherogenesis that allow insights into the genetic control of lipid metabolism and lesion progression.

In diet-induced atherosclerosis in animals, vessel wall abnormalities can be found after just 7-14 days of hypercholesterolaemia. Monocytes become adherent to endothelial cells, presumably because of increased expression of adhesion molecules on one or both cell types. The monocytes "roll" along the endothelial surface, and migrate to the subendothelium via intracellular spaces. Once in the vessel wall, the monocytes become macrophages, and take up modified lipoproteins (especially oxidised low density lipoproteins). These cells enlarge as they fill with lipid, accumulate underneath an (anatomically) intact endothelium, and form fatty streaks. Thereafter smooth muscle cells begin to appear in the subintimal space, and proliferate. These smooth muscle cells may also accumulate intracellular lipid. Fatty streaks may develop within 4 weeks in cholesterol-fed animals.

After several months, advanced plaques may be found, usually at arterial ostia, bifurcations or other sites of turbulent flow. The endothelial layer becomes disrupted, exposing the plaque contents to the blood. Growth factors from platelets and endothelium may be important in plaque progression from this point. An extensive fibroproliferative process may ensue, leading to increasing lesion extent and elevation. In humans, large plaques probably take decades to form (Stary 1989). Advanced lesions are then prone to fissuring, ulceration, rupture, embolisation and/or local thrombosis.

1.1.3.2 Progression of plaques

At autopsy, the major large arteries may show plaques at many stages of development, suggesting that new lesions form throughout adult life (Davies & Woolf 1993). As well as cell migration and proliferation, the processes of cell necrosis and repeated small intra-plaque bleeding may both be important in lesion progression in man. The core of extracellular lipid which characterises most plaques is probably derived from foam cell death with subsequent release of intracellular cholesterol and cholesteryl esters. This lipid core is soft and renders the plaque vulnerable to shear stress, which may in turn lead to intraplaque haemorrhage. Plaques with a lipid core comprising ≥40% of their overall volume are particularly susceptible (Richardson et al 1989). Small tears may allow blood to enter and expand the plaque. This may cause acute lumen occlusion and distal infarction, but occasionally may not cause complete luminal thrombosis. Intraplaque haemorrhage may be subclinical, and simply be followed by organisation of the thrombus and consequent lesion growth. Such episodes may be as important in lesion progression as proliferation of the plaque's cellular element.

Clinical outcome is not simply related to number, extent or size of plaques. Rupture of a relatively small, isolated lesion may lead to death if the plaque is in a major coronary or cerebral vessel, whereas many older subjects who die of non-vascular causes have extensive atherosclerosis. In general, however, disease extent and severity correlate well with the clinical vascular event rate (Salonen & Salonen 1991).

1.1.3.3 The "Response to Injury" hypothesis of atherogenesis

Most of the cellular events occurring during atherogenesis are now well characterised, however the aetiology of the process is not well known. Virchow (1858) believed that a low-grade injury to the vessel wall caused an inflammatory reaction, with subsequent insudation of leukocytes and other plasma constituents into the arterial intima. Others suggested that arterial injury leads to microthrombus formation, and that organisation of such thrombi involves cellular infiltration and thereby lesion progression (Duguid 1946). In 1973, Ross and Glomset suggested a "response to injury" hypothesis of atherogenesis, which combines many features of the two older notions of disease pathogenesis with newer cellular and molecular data. This hypothesis has since been modified as new information about endothelium, macrophages, lipid subfractions, growth factors etc. have become available, and currently incorporates most of the known events of atherogenesis (Ross & Glomset 1976, Ross 1981).

Damage to the endothelium is the key event in the response-to-injury hypothesis, in which a variety of insults may cause injury to the endothelial cells at susceptible sites in the arterial tree. Subsequently endothelial dysfunction occurs, the consequences of which promote atherogenesis. Alterations in the anti-thrombotic and permeability barrier functions of the endothelium, along with impaired local vasodilator capacity and/or increased release of vasoconstrictors, might then set up many of the cellular interactions that initiate lesion formation.

What factors may injure endothelial cells? Hypercholesterolaemia, particularly oxidised low density lipoprotein, may result in toxic endothelial damage (Cohen et al 1988). Increased monocyte adhesion, insudation and foam cell formation then results. Smoking may directly damage endothelial cells (Davis et al 1985). Hypertension may lead to physical injury to the endothelium, and hyperhomocystinaemia may cause chemical damage (Harker et al 1976). Diabetes may alter the glycation state of the endothelial

membrane. Whatever the nature of the initial injury, once endothelial damage has occurred and macrophages have subsequently accumulated in the subendothelial space, the macrophages may secrete numerous agents that further damage the endothelium, such as superoxide anions or other oxidative metabolites (Nathan et al 1980). If the cycle of endothelial injury and macrophage accumulation and stimulation is repeated, then both cells are capable of secreting potent growth factors, which may attract platelets and/or smooth muscle cells. Platelets may form local thrombi with further release of growth factors, and smooth muscle cells may proliferate within the intima. Therefore endothelial injury, which results from a variety of physical and chemical insults, may result in a sequence of events culminating in fibroproliferative lesion formation.

Therefore endothelial damage is a key early event in atherogenesis, either as a marker of early disease or more probably as an important pathogenetic mechanism. Injury to the endothelium need not result in anatomical disruption of the intima; initially the principal manifestations might simply be alterations in permeability, altered release of vasoactive metabolites and/or other consequences of abnormal endothelial physiology. For this reason, clinical research has recently focussed on in-vivo detection of endothelial dysfunction, in order to understand its causes and consequences.

1.1.4 RISK FACTORS FOR ATHEROSCLEROSIS

Large prospective epidemiology trials, such as the Framingham and MRFIT studies, have demonstrated consistent and strong associations between certain demographic and laboratory variables measured at baseline and the later risk of vascular events. As atherosclerotic burden is difficult to measure, especially in asymptomatic subjects, the concept of "risk factors" has proven useful in evaluating an individual's chance of having important vascular disease. The major associations that have been documented to date include hyperlipidaemia, cigarette smoking, hypertension, diabetes, older age and male gender (Stamler et al 1962, Kuller 1976). Of these, the factors associated with greatest risk appear to be hypercholesterolaemia, smoking and hypertension (Chapman et al 1957, Doyle et al 1957, Drake et al 1957, Kannel 1978).

1.1.4.1 Hypercholesterolaemia

Virtually every major epidemiological study has found a significant correlation between serum cholesterol level at entry and later cardiovascular event rate. The association between cholesterol and coronary disease is continuous, progressive and "dose-related", even at low cholesterol levels (MRFIT 1976). The fact that countries where hypercholesterolaemia is rare have very low incidences of occlusive atherosclerotic disease, despite high levels of smoking and hypertension, has led some investigators to suggest that hypercholesterolaemia is the single most important of the modifiable risk factors (Keys 1970). In men, almost half the population differences in coronary mortality may be accounted for by variations in serum cholesterol levels (Simons 1986). In societies where the average cholesterol level is under 4 mmol/l, the risk of atherosclerosis is greatly reduced (Farmer & Gotto 1992).

Cholesterol appears to be important from childhood onwards, as this is the time at which atherosclerosis begins. Recently a large prospective trial has confirmed the importance of cholesterol levels in young adult life. Klag et al (1993) demonstrated a strong

association between serum cholesterol levels in late teenage or early adult life and the cardiovascular event rate in middle age.

Certain lipid subfractions are particularly useful in risk stratification. Low density lipoprotein (LDL), which distributes cholesterol to peripheral tissues, is associated with atherosclerosis risk. In familial hypercholesterolaemia, where heterozygotes have a 50 per cent reduction in LDL receptors, LDL levels are high and premature atherosclerosis is exceedingly common. In contrast, high density lipoproteins (HDL), which transfer cholesterol from peripheral tissues to the liver, are protective; levels are inversely correlated with the incidence of vascular disease. Three HDL subtypes have been described. Finally lipoprotein (a) (Lp(a)), which is similar to LDL but linked to an apoprotein (a) molecule, is an independent risk factor for coronary disease. Lp(a) levels are not normally distributed, with a skew in population values towards higher levels (Genest et al 1992). The mechanism whereby Lp(a) confers independent risk is unclear.

1.1.4.2 Cigarette smoking

Despite increased public awareness of its health hazards, cigarette smoking remains the most prevalent modifiable risk factor in subjects prone to atherosclerosis; 20-30% of adults in Britain still smoke regularly (British Heart Foundation 1992). The mechanism whereby cigarette smoking causes or accelerates atherogenesis is not known, but possibilities include endothelial damage, increased platelet adherence and aggregation, increased fibrinogen levels and arterial spasm. Not only is smoking associated with an increased incidence of clinically apparent vascular events, but it has also been associated with the extent and severity of atherosclerosis, even in young adults (PDAY 1990).

Cigarette smoking has an adverse effect on other risk factors, such as lipids. Compared with non-smokers, heavy smokers have higher levels of LDL and triglycerides, and lower levels of HDL cholesterol (Mjos 1988, Tiwari et al 1989). Use of cigarettes is also associated with alterations in blood pressure; hypertension with acute smoking

(Trap-Jensen 1988) and low blood pressure in chronic smokers (Green et al 1986).

Cigarette smoke may damage endothelial cells directly (Nowak et al 1987) as well as by association with other risk factors. The components of smoke responsible are unknown. The prolongation in bleeding time found in chronic smokers is consistent with decreased endothelial production or release of prostacyclin (Meade et al 1987). The effects of smoking on platelets and clotting factors may also play a role in atherogenesis, plaque progression and/or thrombus formation at sites of haemodynamically significant lesions. Nicotine itself is a potent adrenergic agonist and may enhance vasoconstriction in coronary patients (Winniford et al 1986). Increased coronary tone may predispose to myocardial ischaemia, and smoking has been associated with increased episodes of both painful and silent ST-depression (Deanfield et al 1986). In an animal model, nicotine has also been shown to increase fibrinogen uptake by the arterial wall (Allen et al 1989); this may also contribute to atherosclerosis.

Smokers have approximately 3 times the risk of atherosclerosis compared to non-smokers. There is a positive correlation between relative risk of cardiac events and number of cigarettes smoked (2.1 for the lightest and 4.0 for the heaviest smokers) (Kaufman et al 1983). The risk does not seem to relate to the quantity of tar, nicotine or carbon monoxide in each cigarette.

Attention has recently been drawn to "passive" smoking as a risk factor for vascular disease. In MRFIT, husbands of smoking women had an increased prevalence of coronary disease compared to controls (Svendsen et al 1987). Therefore even exposure to low doses of exhaled cigarette smoke may cause arterial wall damage.

1.1.4.3 Hypertension

One of the major reasons cited for the recent fall in cerebrovascular and coronary event rates is the widespread improvement in diagnosis and treatment of hypertension. The availability of well-tolerated, long-acting anti-hypertensive drugs has facilitated blood pressure control, often without the need for specialist referral. Hypertension has been shown to be a major risk factor for both men and women, regardless of age or gender (Koren et al 1991). The mechanism of damage is probably related to increased shear stress on the arterial walls, particularly at sites of turbulent flow, such as bifurcations. In addition, elevated blood pressure frequently coexists with hyperlipidaemia (Kannel 1978, Williams et al 1988); this combination of risk factors may be particularly important in atherogenesis.

Treatment of hypertension has now been shown to reduce cerebral and coronary event rates. In a prospective randomised trial, metoprolol reduced atherosclerotic death rates in hypertensive smokers, including coronary mortality, despite adverse effects of this therapeutic agent on lipid levels, glucose tolerance and insulin resistance (Wikstrand et al 1988). It is perhaps for these reasons that the beneficial effects on stroke mortality are more marked than for coronary disease (Kaplan 1991). Angiotensin-converting enzyme inhibitors, which lower blood pressure without many of the detrimental metabolic effects, may prove to be more effective in lowering coronary event rates in hypertensive patients.

1.1.4.4 Diabetes Mellitus

Both insulin-dependent and non-insulin dependent diabetics have a marked increase in atherosclerosis and its complications (Garcia et al 1976). Age adjusted death rates for diabetic men and women are over twice those seen in non-diabetics, with the excess mortality largely being due to vascular disease (Kleinman et al 1988). Diabetics also tend to have a higher incidence of hyperlipidaemia and hypertension; this metabolic "Syndrome X" may be related to hyperinsulinaemia and insulin resistance (Stolar 1988). Myocardial ischaemia is often silent in diabetic patients, perhaps related to coexisting sensory neuropathy. The mechanism whereby diabetes promotes atherogenesis is uncertain, but high glucose, insulin and lipid levels may alter both endothelial and

platelet physiology. In addition insulin is a growth factor, and may stimulate smooth muscle cell proliferation.

1.1.4.5 Physical activity

Although exercise improves lipid levels, blood pressure, insulin sensitivity and subjective well-being, its role in preventing coronary death is still controversial. Paffenbarger et al have found that sudden cardiac death is less common in patients who exercise regularly after myocardial infarction (1977). Exercise, however, may be associated with an increased risk of ventricular arrhythmia, particularly in unfit subjects. Recent studies, in general, have shown a trend towards a beneficial effect of exercise on vascular mortality rates (Farmer & Gotto 1992).

1.1.4.6 Family history

There is a familial aggregation of cases of atherosclerosis. This related, in part, to the genetic influences controlling hypercholesterolaemia, hypertension and diabetes, as well as environmental influences such as cigarette smoking. In some cases, genetic and environmental influences may be difficult to distinguish (Feinlieb 1983). Whatever the mechanism, children born in families with a high prevalence of risk factors are more likely to develop vascular disease (Sinaiko & Wells 1990). Despite the fact that many familial cases can be attributed to known risk factors, a positive family history per se probably confers independent risk (Jorde & Williams 1988). This is almost certainly attributable to genetic factors that have not yet been identified. For example, the gene encoding the angiotensin converting enzyme has been shown to be independently related to risk of myocardial infarction, in otherwise healthy subjects (Cambien et al 1992).

1.1.4.7 Gender

There is a marked gender difference in vascular event rates, despite the fact that the process of atherosclerosis does not appear to differ between men and women, and that the major risk factors of cholesterol, hypertension and smoking affect both sexes. Atheroma is much less common in young and middle aged women than men, especially between aged 35 and 55 years. The "protection" of women from vascular disease is much less evident after the menopause, when coronary death rates begin to converge (Matthews et al 1989). Oestrogens appear to exert a beneficial effect on both the lipid profile (Jacobs & Loeffler 1992) and the arterial endothelium itself (Williams et al 1990). Male gender is therefore an independent risk factor for atherosclerosis.

1.1.4.8 Other risk factors

Certain other factors have been identified as possible independent risk predictors. These include personality type (Ragland & Brand 1988), hypercalcaemia (Weinstein & Heiden 1989), hypercoagulability (Meade et al 1986a), and hyperuricaemia (Brand et al 1985).

Risk factors have therefore been linked with both the presence of established atherosclerosis and its clinical complications. Whether these same factors can be linked to the early presymptomatic stages of atherogenesis will depend on the availability of methods to identify subjects with abnormal arterial structure or function, and to correlate results of such tests with individuals' risk factor profiles.

1.2 ENDOTHELIUM; NORMAL FUNCTIONS AND CONSEQUENCES OF INJURY

INTRODUCTION

For many decades the endothelium, the cell layer which lines the blood vessels, was viewed simply as a semipermeable barrier between blood and interstitium, facilitating the exchange of water and small molecules. However over the last 20 years, a series of experiments have demonstrated that the endothelium has an enormous range of vital homeostatic functions. Instead of serving as an inert barrier, the endothelium is a widely distributed organ of considerable biological potential, that not only extends throughout the body in the convenient form of an anti-thrombogenic vascular lining, but also participates in metabolic, synthetic and regulatory pathways at different vascular sites and in individual organs. As the complexity of normal endothelial function has been elucidated, it has become apparent that endothelial cell dysfunction is implicated in several important disease processes, such as atherosclerosis and hypertension.

The endothelium lies between the lumen and the vascular smooth muscle. Therefore it is in a position to control blood fluidity and/or composition by secreting substances into the lumen, and to influence vascular tone, reactivity and growth. It is able to "sense" changes in haemodynamic forces, or blood-borne signals, by membrane receptor mechanisms, and respond to physical and/or chemical stimuli by synthesis and/or release of a variety of vasoactive and thromboregulatory molecules, and growth factors. Substances released by the endothelium include prostacyclin, endothelium-derived relaxing factor (EDRF), endothelins, endothelial cell growth factor(s), interleukins, plasminogen inhibitors and von Willebrand factor.

Given that normal endothelial function plays a central role in vascular homeostasis, it follows that endothelial dysfunction probably contributes to disease states characterised

by vasospasm, vasoconstriction, excessive thromobosis and/or abnormal vascular proliferation. In terms of the pathogenesis of vascular disease, loss of EDRF secretion may be a particularly important consequence of endothelial injury, as EDRF is not only a potent vasodilator, but also an inhibitor of both platelet aggregation and vascular smooth muscle proliferation.

1.2.1 NORMAL ENDOTHELIAL FUNCTION

The vascular endothelial cells fulfil an extraordinary variety of homeostatic and metabolic functions. Although only one cell layer thick, the role of the endothelium includes maintenance of blood fluidity, vascular tone and permeability, as well as regulating inflammatory responses and transducing a variety of blood-borne signals (Table 1.3.1). In addition to these "universal" functions, the endothelium may have organ-specific roles which are differentiated for various parts of the body, such as gas exchange in the lungs, control of myocardial function in the heart or phagocytosis in the liver and spleen. More than 50 different types of endothelial cell have now been described.

Table 1.2.1. Some functions of normal vascular endothelium.

Maintenance of thromboresistance

Maintenance of selective permeability

Regulation of vascular tone

Regulation of vascular growth

Regulation of inflammatory reactions

Synthesis and secretion of peptides

Integration and transduction of blood-borne signals

(Adapted from Petty and Pearson 1989)

Studies of endothelial structure and function have been accomplished by a variety of techniques, including ultrastructural studies (Palade 1953), in vitro experiments for endothelial cell isolation and culture (Gimbrone 19761, Jaffe 1984), physiological studies in animals (Renkin 1977) and most recently clinical studies in man (Ludmer 1986, Celermajer 1992). This knowledge has facilitated the development of certain treatment strategies based on administration of endothelial products, such as prostacyclin and nitric oxide, or their antagonists.

1.2.1.1 Thromboresistance

Endothelium has anticoagulant, antiplatelet and fibrinolytic properties. Endothelial cells are the major site for 2 anticoagulant reactions involving thrombin. The first is a receptor-mediated thrombin inactivating reaction (Machovic 1986); this may be accelerated by the presence of heparin. The other involves a thrombin-thrombomodulin interaction on the endothelial surface, which enhances activation of the circulating anticoagulant protein C (Esmon 1987). This anticoagulant activity may be further increased by endothelium-derived protein S, which is a cofactor for protein C (Stern et al 1986).

Platelet adhesion to endothelial cells is markedly inhibited by the endothelium-derived arachidonic acid metabolite, prostacyclin. The same stimuli that activate platelets, such as thrombin and adenosine di- and triphosphate (ADP, ATP), also act to release prostacyclin from the endothelium, which allows the endothelium to limit the extent of platelet plug formation (Jaffe 1987). The interactions between platelets and endothelium regulate both platelet function, coagulation cascades and local vascular tone (Pearson & Gordon 1985).

In addition, endothelial cells may secrete tissue plasminogen activator (t-PA) (Hekman & Loskutoff 1987), a power thrombolytic agent which is now in frequent clinical use for

treatment of coronary thrombotic occlusion. Plasminogen activator release is stimulated in-vivo by noradrenaline, vasopressin or stasis within the vessel lumen. Thrombin may also stimulate t-PA release, providing a further endothelium-mediated safeguard against uncontrolled coagulation.

1.2.1.2 Maintenance of selective permeability

Endothelial cells and their basement membrane provide a barrier to the extravasation of fluid, proteins, cells and other blood products. The composition of the basement membrane is closely regulated by the endothelium, and may include collagen, elastin, proteoglycans, fibronectin and a variety of other proteins. Endothelial cells both synthesise these products and determine the overall composition of the subintimal layer (Sage 1984, Madri et al 1988). This may vary considerably between organs, allowing high permeability in some areas (eg the glomerulus, exocrine glands) but low permeability in others (such as in the cerebral vessels). Endothelial cells probably also release the collagenases that remodel basement membranes and permit new vessel growth (Gross et al 1982).

Normally small molecules may move through the endothelium and large molecules between cells. Movement of fluid is controlled by a balance of hydrostatic and osmotic forces. In certain pathological states, however, endothelial permeability may be markedly increased, resulting in inflammatory oedema. Uncontrolled endothelial permeability is also probably involved in the pathogenesis of septic shock and the adult respiratory distress syndrome.

1.2.1.3 Regulation of vascular tone

The central role of endothelium in controlling vascular tone has only been appreciated since the discovery of the potent vasodilators prostacyclin and nitric oxide (Moncada et al 1977, Furchgott & Zawadzki 1980). Endothelium controls underlying smooth muscle

tone in response to a variety of physiological stimuli. This involves a number of luminal membrane receptors, complex intracellular pathways and synthesis and release of a variety of relaxing and constricting substances. In addition to making its own vasoactive mediators, endothelial cells may transduce signals from or even inactivate circulating vasodilators and constrictors, such as thrombin, bradykinin, ADP and ATP (Ryan & Ryan 1984).

Endothelium-derived vasoactive factors

Endothelium-derived relaxing factor (EDRF). The existence of EDRF was first postulated by Furchgott and Zawadzki in 1980, when they noticed that rabbit aortic rings relaxed to acetylcholine only in the presence of an intact endothelium. Since then it has been shown that EDRF is a humoral agent which is released continuously in the basal state, and after stimulation by increased flow or by certain pharmacological agents, in animals and in man.

Identification. After the initial finding in 1980 of a non-prostanoid vasodilator secreted by endothelium, it was several years before its chemical identity was discovered. The short half-life and instability of EDRF is due to rapid metabolism to nitrite and nitrate, and this made its isolation and identification difficult (Griffith et al 1984, Rubanyi et al 1985a). Martin et al found that EDRF binds directly to and is inactivated by haemoglobin, especially oxyhaemoglobin (1985). This suggests that EDRF can only have local actions (Edwards et al 1986, Evans et al 1989).

In 1988, both Furchgott et al and Ignarro et al suggested that EDRF was nitric oxide (NO), a gas. Both EDRF and NO were labile, with short half-lives and activated soluble guanylate cyclase in smooth muscle cells, leading to increased cyclic-GMP levels, reduced intracellular calcium and thereby vasorelaxation. The potent vasodilator effects of both EDRF and NO were inhibited by haemoglobin and free radicals, and potentiated by superoxide dismutase. Palmer et al (1988) then demonstrated that EDRF and NO

possessed identical biological and chemical characteristics, using an in vitro bioassay technique. The amount of NO released by the endothelium in response to bradykinin could account for all the smooth muscle relaxant activity of the released EDRF.

Since then other investigators have suggested that EDRF is a nitrosothiol precursor of NO, which is then released after EDRF breakdown (Myers et al 1990). In either case, the biological effects of EDRF are mediated ultimately by nitric oxide.

Synthesis. EDRF is synthesised from L-arginine by an enzyme, nitric oxide synthase (NOS) (Palmer et al 1988). The reaction is stereospecific, and L-arginine is converted to NO and L-citrulline. The generation of NO from L-arginine can also be specifically blocked by arginine analogues, such as N^G-monomethyl-L-arginine (L-nMMA) which has recently proven to be a useful tool in clinical research, allowing investigation of the biological distribution and role of EDRF/NO. NO synthase has at least 2 isoforms; one constitutive, which is responsible for basal EDRF release and that stimulated by most physical and pharmacological agents (Bredt & Snyder 1990), and the other is inducible, and may produce large amounts of EDRF after activation by certain cytokines (for example, in septic shock).

Release. EDRF maintains low arterial tone at rest. There is evidence for basal release of EDRF in animals (Amezcua et al 1989) and in humans (Vallance et al 1989). In addition EDRF release is stimulated by increased flow (leading to increased shear stress on the endothelium) and by bradykinin, thrombin, acetylcholine and a variety of other circulating agents, which increase EDRF release via activation of specific endothelial cell membrane receptors.

Mode of vasodilator activity. EDRF interacts with the iron atom of heme in guanylate cyclase, causing its activation, and thereby increasing intracellular c-GMP levels (Arnold et al 1977, Griffith et al 1985). In smooth muscle cells, this results in a reduction of intracellular calcium and thereby relaxation (Collins et al 1986). The same pathway is

involved in the mechanism of action of exogenous nitrovasodilators, such as sodium nitroprusside and glyceryl trinitrate.

Endothelium-derived hyperpolarising factor. Stimulation of the endothelium by acetylcholine may produce hyperpolarisation of the underlying smooth muscle and thereby vasorelaxation. This is not mediated by NO, but by another endothelium-derived factor, which acts by increased K⁺ conductance. The resulting vasodilatation is not inhibited by L-nMMA, the specific antagonist of EDRF (Feletou & Vanhoutte 1988, Taylor & Weston 1988). In contrast ouabain, a Na⁺/K⁺ ATPase inhibitor, does not affect EDRF-induced relaxation, but prevents the action of endothelium-derived hyperpolarising factor. The physiological role of this factor is uncertain.

Endothelins (endothelium-derived contracting factors). In 1985, Rubanyi & Vanhoutte showed that hypoxia caused release of a constricting factor by endothelial cells, which was not blocked by cyclo-oxygenase inhibitors. In 1988 Yanagisawa isolated, purified and sequenced this factor, a 21 amino acid peptide, and named it endothelin. This peptide is closely related to the snake venom sarafatoxin, and is the most potent vasoconstrictor yet identified. There are 3 isoforms of endothelin, but only one (ET-1) has been shown to be released from endothelial cells (Masaki 1989). Endothelin receptors have been found in the brain, heart, kidney, intestine and vessel wall (Koseki et al 1989). Like EDRF, ET-1 is synthesised de novo in the endothelial cells, and released in response to a variety of stimuli, such as adrenaline and hypoxia (Boulanger & Luscher 1990).

ET-1 has a short half-life (Anggard et al 1989), suggesting that it too is mainly a locally active vasoregulator. Its physiological role is not known, however it is present in healthy subjects in low concentrations (Davenport et al 1990). Elevated endothelin levels have been found in hypertension, coronary disease and heart failure; in these conditions, a pathological role has been postulated but not proven (Warrens et al 1990, Cody 1992).

Prostacyclin. Another major endothelium-derived vasodilator is prostacyclin, which is derived from arachidonic acid via the enzyme cyclo-oxygenase (Moncada et al 1977). Prostacyclin also has anti-thrombotic and anti-platelet activity. Its release may be stimulated by bradykinin and adenine nucleotides. Like EDRF it is chemically unstable, with a short half-life (FitzGerald et al 1981), however unlike EDRF it acts via stimulation of adenylate cyclase and increase in intracellular levels of cyclic-AMP. It is a potent vasodilator, and is active in both the pulmonary and systemic circulations.

1.2.1.4 Endothelium-derived growth factors

Endothelial cells may stimulate smooth muscle cell proliferation, probably via release of a growth factor (Di Corleto & Bowen-Pope 1983). In addition, endothelium-derived growth factors may stimulate proliferation of other endothelial cells (Gajdusek & Schwarz 1982). In contrast, some oligosaccharides secreted by endothelial cells powerfully inhibit smooth muscle cell growth and proliferation (Castellot et al 1981); therefore endothelial dysfunction may be associated with disinhibition of smooth muscle proliferation, which may be important in atherogenesis.

1.2.1.5 Interactions of endothelial cells with leukocytes

In normal humans a large proportion of neutrophil leukocytes are temporarily adherent to the endothelium ("marginated"). In the early stages of inflammation, these adherent neutrophils migrate into the tissue, release chemotactic and other mediators and thereby initiate the inflammatory response. Cytokines may increase expression of neutrophil adhesion molecules on the endothelial surface (Mantovani & Dejana 1987). In contrast, monocytes are not generally adherent to normal endothelial cells, however endothelial dysfunction may be associated with increased expression of intracellular adhesion molecules such as ICAM-1. This facilitates monocyte adhesion and migration, and is important in atherogenesis (see above).

In addition, normal endothelium also plays an important role in immunological regulation, metabolism of circulating amines, lipoprotein metabolism and integration and transduction of blood-borne signals. Interestingly, coronary microvascular endothelium may regulate myocardial contractility (Treasure et al 1990, Ricou et al 1992). Details of these and other functions are beyond the scope of this introduction.

1.2,2 CONSEQUENCES OF ENDOTHELIAL INJURY

A variety of insults may damage endothelial structure and/or function. These include physical injuries, such as abrasion or increased shear stress (for example, with hypertension), biochemical injury, such as toxin-mediated damage (for example, by the highly reactive amino acid homocystine, or by cytokines) and immune-mediated damage. Injury severe enough to cause denudation of endothelium has catastrophic consequences due to the loss of the normal barrier function of this cell layer. More usually these insults cause alterations in endothelial physiology, with impairment or loss of certain cell functions; for example, hypercholesterolaemia may impair EDRF release but not endothelial permeability, or certain cytokines may impair endothelial barrier functions but not vasoregulator substance metabolism.

Endothelial injury is involved in a wide variety of disease states, either as a consequence or as a cause of the disease process (Table 1.2.2). Particularly in atherosclerosis, endothelial injury appears to be a key early event, and the resulting dysfunction may have a variety of consequences that promote atherogenesis. In systemic and pulmonary vascular disease endothelial dysfunction is an early event (Panza et al 1990, Celermajer et al 1993a); whether it proves to be the primary aetiology or an association of elevated pressure is not yet known.

Table 1.2.2. Diseases related to endothelial cell dysfunction

Atherosclerosis Tumour angiogenesis

Thrombosis Graft rejection

Hypertension Haemolytic uraemic syndrome

Pulmonary vascular disease Kawasaki syndrome

Septic shock Thrombotic thrombocytopaenic

Vasculitis purpura

Inflammation Idiopathic thrombocytopaenic

Diabetic vasculopathy purpura

Endothelial injury and atherogenesis

Endothelial injury is now regarded as the initial event in atherogenesis (Ross 1986, Clarkson 1987). In even normocholesterolaemic animals, physical damage to the endothelium can lead to atherosclerotic-lesion formation (Moore 1973). Hypertension has been shown experimentally to disrupt endothelial integrity (Reidy & Schwartz 1982). Hyperhomocystinaemia, which causes chemical endothelial injury, is associated with premature atherosclerosis and thrombosis (De Groot et al 1983). The fact that many of these insults, which are associated with clinical progression of vascular disease, are clearly related to endothelial injury, has added weight to the "response to injury" hypothesis of Ross & Glomset (1973) (see above).

The consequences of endothelial damage that promote fatty streak and plaque formation include increased adherence of leukocytes (Steinberg 1987), increased permeability to macrophages and lipoproteins, which then accumulate in the vessel wall, increased platelet adherence, and increased smooth muscle migration and proliferation (Henderson

1991). The latter may be mediated by loss of endothelium-derived heparin-like oligosaccharides, which normally inhibit smooth muscle cells (Castellot et al 1981), or by allowing platelet adhesion and thereby increased local concentration of platelet derived growth factor, which is also an important smooth muscle mitogen (Stiles 1983).

Endothelial dysfunction is also accompanied by loss of EDRF secretion. As EDRF is a local vasodilator, and also inhibits platelet adherence and aggregation, smooth muscle proliferation and endothelial-cell leucocyte interactions, reduced EDRF activity may contribute to the initiation and progression of atherogenesis. Dietary supplementation with L-arginine, the EDRF precursor, prevents atherogenesis in hypercholesterolaemic rabbits (Cooke et al 1992). This exciting observation strongly supports the concept that endothelial dysfunction is an important and potentially treatable early event in the atherogenic process.

Finally the endothelium also plays an important role in subjects with advanced atherosclerotic disease. At the site of a large plaque, endothelial dysfunction is associated with dynamic plaque constriction, in response to acetylcholine (Ludmer et al 1986) or even exercise (Gordon et al 1989). In addition endothelial dysfunction may promote local thrombus formation at the site of a plaque; thrombosis is usually the pathological event underlying clinical catastrophes such as myocardial or cerebral infarction.

Therefore endothelial dysfunction is probably important across the entire spectrum of atherosclerotic disease; as a key initiating event, in the development of the cellular interactions resulting in lesion progression, and in the behaviour of advanced plaques and their propensity to cause arterial occlusion.

1.3 MEASURING ATHEROSCLEROSIS; INVASIVE AND NON-INVASIVE TESTS

1.3.1 OVERVIEW

Measurement of atherosclerosis is important in 3 distinct clinical/research settings. Firstly in patients with complications of occlusive vascular disease, such as angina pectoris or transient cerebral ischaemia, diagnosis and management decisions may be based on the identification of atherosclerotic lesions. Secondly in trials of antiatherogenic interventions for symptomatic patients, very precise measurement of disease extent and severity are required to allow valid comparison of the pre- and post-treatment extent of atheroma. Finally measuring atherosclerosis is important in asymptomatic patients (for example, screening high risk groups). As atherosclerosis has a long presymptomatic phase early in its natural history, techniques for identifying the presence, extent and severity of disease have been sought. Clearly invasive methods of measurements are less suitable for investigation of asymptomatic subjects being studied at an early stage in the course of their vascular disease.

The major techniques that are used currently to diagnose and quantify atherosclerosis fall into two categories; invasive and non-invasive. Only invasive methods are available currently for detailed imaging of the coronary arteries, whereas both invasive and non-invasive methods are suitable for imaging the extracranial carotid and iliofemoral arteries. Of the invasive methods, angiography is the most widely practised. Limitations of this technique include visualisation of the lumen rather than the vessel wall and plaque, the tendency to underestimate plaque size and the inability to detect early disease. More recently intravascular ultrasound has been developed, and provides images of the vessel wall of proximal large arteries.

The main non-invasive method in clinical use is external vascular ultrasound, which has been used to study atherosclerosis since the 1970's, in order to detect and approximate the severity of stenoses in the peripheral arteries. B-mode images can be obtained quickly and painlessly in the majority of patients. Its disadvantages include considerable variability in disease quantification, both between observers and in studies of the same patient on different days. B-mode can be used to study early disease (quantification of the intima-media thickness of the vessel wall) or late disease (visualisation of plaques). The availability of Doppler flow velocity measurement has enhanced the ability of ultrasound to diagnose the haemodynamic significance of a given stenosis. Other ultrasound techniques (pulsed Doppler, A-mode scanning) have been developed to study arterial compliance and elasticity, which may be altered early in the atherogenic process.

Finally magnetic resonance imaging (MRI) has developed to the point where high quality pictures of coronary and peripheral arteries may be obtained non-invasively. The equipment, specialised gating and spin sequences and appropriate processing software are not widely available, however MRI may prove to be an accurate method of identifying the presence or absence of important flow-disturbing plaques in proximal large arteries.

Therefore there is, as yet, no perfect technique for the identification of early atherosclerosis or the quantification of late disease. Despite this, the techniques above are widely used and are currently being refined, to provide accurate and reproducible measurements of plaque burden within the arterial system.

1.3.2 INVASIVE MEASUREMENT OF ATHEROSCLEROSIS

1.3.2.1 Angiography

The commonest angiograms performed in clinical practice are coronary studies, because of the prevalence of coronary syndromes in the community and because non-invasive methods are not available for delineating coronary anatomy (in contrast to the carotid and

femoral circulations). The major advantages of angiography are the obvious direct relevance of coronary arterial anatomy to angina and infarction, and the ability to visualise proximal and distal vessels. It is widely applicable and quick. The major disadvantages are its invasive nature, the measurable procedure-related risk, the cost and the inability to image the arterial walls. Furthermore the angiographic severity of disease may not correlate well with the degree of flow reduction or the presence of distal ischaemia.

Prior to 1958, coronary arteries could only be visualised during ascending aortography. In the late 1950's Sones et al (1958) pioneered selective coronary angiography, and the images obtained delineated the site, extent and severity of coronary atheroma. This revolutionised the care of patients with angina, heart failure and infarction, and paved the way for successful coronary artery surgery. Initially investigators focussed on disease severity as an indicator for treatment (Ricketts & Abrams 1962, Sones & Shirley 1962), however many found that subjective interpretation of lesion severity was extremely variable. Intra-, interobserver error and poor reproducibility of visual estimation of coronary stenosis (Brown et al 1982) prompted attempts to improve reliability, initially by averaging the measurements made by 2 or 3 independent experienced observers, or by taking readings performed by a panel of experts (Rouen et al 1977). Calipers and calibrated grids represented the first attempts at systematic quantification of lesion stenosis, and in the last decade a variety of computer-assisted methods for quantifying disease extent and severity have become available (Brown et al 1986). Although such precise measurements are not necessary in routine clinical practice, these highly reproducible systems are essential for interpretation of angiograms obtained for disease Computer packages incorporating validated edge-detection regression studies. algorithms, digitised pictures and videodensitometry are now widely available, and allow accurate determination of percent stenosis (diameter and area), and precise measurement of lumen diameters.

Quantification of lesion severity is therefore important both for individual patient management and for interpreting clinical trials of anti-atherogenic interventions. The major weakness of angiography, however, is that the images only delineate the intravascular lumen, but provide relatively little information about the anatomy of the arterial wall. Plaques appear as "negative" areas, or filling defects, where the contours of the contrast image is not smooth. This means that estimates of plaque size may be inexact, and depend on the quality of the contrast injection and the angles of imaging. Furthermore plaques not only cause luminal narrowing, but are also associated with atrophy of the adjacent vessel wall. Eccentric lesions may shift the arterial wall outwards rather than just protruding into the lumen (Crawford & Levene 1953). Therefore small plaques may be angiographically invisible, as the outwardly shifted lesion may still lie outside the original line of the arterial wall. Large plaques are almost always associated with compensatory dilatation of the vessel wall to preserve the lumen, and this capacity to maintain lumen size is not overcome until the area of the plaque exceeds 40% of the artery's original cross-sectional area (Glagov et al 1987). This implies that angiography almost always results in an underestimation of lesion severity and size.

Furthermore visual or computer-assisted assessment of stenosis severity involves comparison of an "involved" segment with an adjacent "reference" diameter that is presumed normal. For example, coronary arteries in transplant recipients may be diffusely severely narrowed, but appear "normal" angiographically because the arteries are uniformly small, but still smooth. If the reference segment of an artery is itself narrow, this will also lead to an underestimate of stenosis severity. On the other hand, reference segments may be ectatic, leading to overestimation of lesion severity. Absolute lumen dimensions are therefore probably more reproducible than estimates of percent stenosis (Ellis et al 1986), and in this setting automated edge detection has also been useful. In general, computer assisted estimations of disease severity are more reproducible than visual assessments, for both mild and severe disease (Detre et al 1982). A review of the techniques, reproducibility and reliability of computerised methods for

measuring absolute lumen diameter and percent stenosis has recently been published (Crouse & Thompson 1993).

Another potentially confounding factor in coronary angiography, particularly in the comparison of serial films from the same patient, is that coronary arterial tone may vary between visits. Relative states of relaxation or spasm, which may depend on temperature, autonomic nervous system activation, circulating catecholamine levels, endothelial function etc may alter the diameter of "normal" segments or at the sites of diffuse or focal atherosclerotic disease. Treatments that improve endothelial function, for example, may be associated with less dynamic stenosis of plaques (Ludmer et al 1986), and thus the lumen may appear larger on an angiogram, even if the plaque has not changed in size. This phenomenon, by potentially decreasing reproducibility of serial angiographic assessments, may be important in interpretation of disease reversibility studies using coronary angiography to measure the study end-points.

Angiography therefore delineates the site of stenoses and the extent of disease, albeit imprecisely. Despite the above limitations, there is a strong correlation between the severity of coronary disease as estimated by angiography and the severity of symptoms (Proudfit et al 1966). Luminal stenosis of ≥50% has frequently been used as a threshold for intervention (ie the presence of "significant" disease), although recently increased importance is being attached to disease extent as well as severity, to left ventricular function and to tests of the extent of myocardium "at risk" in the presence of coronary stenoses (White et al 1984). Some of the supplementary techniques have been developed to allow the assessment of the extent and severity of end-organ ischaemia distal to a stenosis include isotope scans and positron emission tomography.

Angiography is also used in clinical practice for the diagnosis of atheroma severity and extent in the carotid, aorto-iliac, renal, mesenteric and other vascular beds. The principles of major advantages and limitations are similar to those described above for coronary studies. Angiography, as a modality that delineates lumen stenosis rather than

arterial wall anatomy, is most useful for studies of clinical outcome in symptomatic patients, particularly in arteries that are inaccessible to ultrasound, such as coronary or renal vessels.

1.3.2.2 Intravascular ultrasound

Imaging. Autopsy studies in cholesterol-fed animals and in humans with atherosclerosis first showed that there was often a significant discrepancy between arterial lumen appearances and the extent of atheromatous change in the vascular wall. This was later confirmed by surgical observations at the time of coronary grafting. In 1987, McPherson et al provided the first in-vivo pictures of the coronary arterial wall by performing epicardial echocardiography using high frequency ultrasound. They were able to estimate lumen diameter, wall thickness and composition in both normal and diseased vessels, and their findings confirmed that arteries that appeared smooth on an angiogram often had substantial diffuse atherosclerosis. Since then, similar findings have been confirmed by using high frequency, high resolution intravascular ultrasound transducers.

The combination of high resolution ultrasound imaging technology and low profile catheters that could safely be introduced into arteries has the potential to greatly improve the validity of coronary imaging. A miniaturised transducer is mounted on the tip of a catheter, and the probe is advanced in a retrograde fashion to the coronary artery of interest. Cross sectional images are obtained as the ultrasound transducer rotates at the tip of the catheter, and software is now available to allow three-dimensional image reconstruction by "stacking" the two-dimensional images obtained during controlled withdrawal of the catheter. The vessel lumen, the intima, media and adventitia all have differing acoustic properties, and the reflected sound from the tissue boundaries allows accurate localisation of most arterial wall structures. Connective tissue and calcium have particularly high acoustic impedance, and this usually permits clear identification of the lumen/intima interface (Fields & Dunn 1973), the adventitia, the anatomy of collagen-

rich plaques and any areas of calcification. On ultrasound, blood, lipid and collagen have different characteristics, and therefore lesion composition may be assessed, as well as arterial wall thickness, plaque size and extent of disease. The development of flexible, small diameter catheters has allowed study of the major peripheral and coronary arteries.

Since its inception in the last decade, intracoronary ultrasound has been applied in both clinical and research settings. It has been especially useful in visualising arterial wall anatomy before and after various mechanical interventions at the site of the plaques, such as balloon angioplasty or atherectomy. Certain lesions can be identified prospectively as being "high risk" for dissection or rupture, and some lesions may be identified that are "resistant" to angioplasty (Coy et al 1991). This information may spare some patients from unnecessary risk. The effect of interventions on plaque size, arterial wall integrity and wall composition can be accurately assessed (Tanaglia et al 1992). Appearances obtained after intervention may correlate with late restenosis, and help physicians understand risk factors for this important clinical problem.

Another area in which intracoronary ultrasound has proven useful is in the assessment of the coronary arteries of heart transplant recipients. In these patients, atherosclerosis is invariably silent (as the heart is denervated), and disease may be diffuse and therefore difficult to diagnose by standard angiography. In one study of 60 transplanted patients, intracoronary ultrasound delineated the presence of atherosclerosis in every subject, even though 42 had had "normal" coronary angiograms (St Goar et al 1992).

Intravascular ultrasound has also been used to study the pathogenesis of stable and unstable angina pectoris (Mintz et al 1992), to evaluate coronary stents (Schymer et al 1992), to assess disease in saphenous vein grafts (Nase-Huppmeier et al 1992) and to study chronic arterial occlusion. It has also been used extensively to study peripheral arterial disease, particularly iliofemoral atherosclerosis.

One other research application suggested for this technique is the use of intra-arterial ultrasound as the end-point in atheroma regression trials, rather than angiography (Nishimura et al 1992, Walker et al 1992). This may result in better understanding of anti-atherogenic strategies and their effects on the arterial wall. Rigorous evaluation of the technique's validity and reproducibility will be required, however, before intracoronary ultrasound can be used in this way. In comparison with conventional angiography, this technique still does not provide images of the distal coronary vessels, and it is also more time-consuming to perform a full evaluation of all the coronary branches. Therefore angiograms may still be more accurate for defining disease extent, whereas intravascular ultrasound for delineating individual lesion severity.

Doppler. Intracoronary Doppler ultrasound has also been developed for in-vivo use in humans. The principle of Doppler ultrasound, that there is a frequency shift in sound depending on the speed and direction of the moving target, has recently been applied to external cardiovascular ultrasound, and now permits velocity mapping and colour flow patterns to be obtained. Intra-arterial Doppler measurements are theoretically attractive, as flow velocity is inversely related to lumen diameter, and therefore blood accelerates at the site of a stenosis. Development of miniaturised Doppler ultrasound crystals mounted on flexible catheters has therefore facilitated intra-arterial Doppler measurements, where the haemodynamic significance of luminal stenoses may be assessed (Harley & Cole 1974). In 1977, Cole and Hartley utilised this method for quantifying flow in human coronary arteries; simultaneous measurement of flow velocity and luminal cross-sectional area allows calculation of absolute flow. Subsequently this method has been used to estimate lumen stenosis (Wilson et al 1985, Sibley et al 1986). As contrast injection may itself dilate an artery, ultrasound estimation of stenoses may be more accurate.

The other major application of intracoronary Doppler has been to allow the estimation of coronary flow reserve (Johnson et al 1989, Nabel et al 1990). Coronary flow may be calculated at baseline and after maximal vasodilatation has been produced (for example, by papaverine infusion). The measurement of coronary flow reserve has facilitated

research into the control of normal and diseased coronary resistance vessels (Drexler et al 1991, Egashira et al 1993).

Therefore intravascular ultrasound (imaging and Doppler) may not only provide better quantification of plaque size and stenosis severity, but may also allow plaque tissue characterisation. Its main disadvantages, however, are its invasive nature (therefore it is unsuitable for screening or presymtomatic diagnosis), and the inability to visualise distal small arteries, which are especially important for management surgical decision-making in coronary patients.

1.3.3 NON-INVASIVE METHODS

Clinical manifestations of coronary atherosclerosis occur late in the natural history of the disease, as they result from obstruction of an arterial lumen. Detection of the disease process at an earlier stage is an important goal, but is seldom achieved. Fluoroscopy for coronary wall calcification is insensitive, ultrafast computed tomography to detect calcific microdeposists is sensitive but of uncertain clinical value, and magnetic resonance imaging is only just now allowing angiographic visualisation of the lumen of coronary arteries but not of vessel walls. Many other non-invasive techniques have been used to investigate atherosclerosis over the last 2 decades, including carotid phonangiography, electronic stethoscopes and plethysmographic studies, such as oculoplethysmography to detect severe carotid stenoses. The most enduring method, however, has been ultrasonography, as it is sensitive, specific, painless and quick to perform.

Ultrasound allows accurate study of vessel structure (for example, wall thickness) and function (for example, compliance), which may be altered early in the disease process. Although these methods are not applicable to coronary arteries directly, ultrasound is usually applied to the visualisation of other arteries as "surrogate" vessels, given that atheroma is a diffuse disease. Techniques studying carotid wall thickness (Salonen &

Salonen 1991), femoral atheroma (Olsson 1991), aortic compliance (Dart 1991) and brachial artery elasticity (Hirai 1989) have been developed and proposed as useful non-invasive methods of studying the atherosclerotic process. This section provides an overview of the most commonly used non-invasive methods for assessing atherosclerotic disease.

1.3.3.1 Ultrasound and atherosclerosis

<u>Ultrasound imaging</u>. Ultrasound provides information about the vessel wall anatomy by imaging techniques (B-mode), and about flow disturbances by Doppler velocimetry. Ultrasound images rely on the principle that the sound waves transmitted through the tissues are reflected from interfaces between adjacent structures that have different acoustic impedances. The appearance of the reflected waves depends on the density of the tissues, and the software processing of the returning signal. In the first ultrasound systems, only one line could be interrogated at a time (M-mode), but subsequently a series of adjacent lines could be interrogated simultaneously, and the reflected signals formatted into a two-dimensional image (B-mode). The higher the frequency of the transmitted pulse, the shorter the wavelength and therefore the better the resolution. Unfortunately high frequency ultrasound has poor depth penetration, and therefore is only suitable for imaging fairly superficial structures.

The initial application of medical ultrasound was in obstetrics. In the 1980's, ultrasound images of almost every type of organ and tissue were obtained, including cerebral, cardiac, hepatobiliary and soft tissue applications. Ultrasound transducers became miniaturised and can now be used externally or with a variety of probes designed for intracavitary use. Due to its safety and ease of application, it has become one of the most widespread diagnostic imaging techniques.

Vascular ultrasound was first applied to the study of abdominal aortic disease (Bernstein et al 1976). B-mode images allow good visualisation of arterial walls and pulsatility, and

also of venous structures. Gradual improvements in resolution and the development of "real-time" imaging have facilitated study of most major vessels. Those that have a relatively superficial course, such as the common carotid, brachial and superficial femoral arteries, can be imaged easily and with great detail available. Deeper vessels, like the coronaries or aorta, can be visualised, but usually only with poor resolution.

Doppler techniques. In 1971, Hokanson introduced the technique of Doppler imaging. The Doppler effect was first used in conjunction with ultrasound to detect movement of blood within arteries. This simple application is still used clinically in patients with impalpable peripheral arterial pulses. This technique was improved by transmitting the ultrasound in short bursts, allowing the depth of the returning signal to be calculated if the speed of sound through the tissues was known (the time gate principle of "pulsed" Doppler). Continuous wave Doppler does not allow selection of a sample depth, but can detect much higher flow velocities than pulsed Doppler (because of a higher repetition frequency); therefore both techniques provide important and complementary information. Finally the returning Doppler signal can be colour-coded for the direction and velocity of flow, and colour-flow mapping has further facilitated interpretation of complex flow patterns in a given sample volume. Colour signals are particularly useful in allowing rapid localisation of arteries and veins, even in the presence of a poor quality grey-scale image.

The Doppler signal changes in arteries where a significant stenosis is present. The frequency increases in proportion to the severity of the stenosis, and spectral broadening may also be noted, particularly in the presence of early disease (Breslau et al 1982, Roederer & Strandness 1984b).

Duplex ultrasound is the combination of B-mode imaging and Doppler techniques. Many investigators have documented a high degree of accuracy in detecting stenoses causing 75-96% obstruction of the lumen (Roederer et al 1984a, Boggousslavsky et al 1986, Moneta et al 1989). The technique may be inaccurate in the presence of early disease,

where a lesion may lie outside the plane of a given two-dimensional image, and before detectable flow disturbance has occurred. Also in very severe disease, especially total occlusion, duplex ultrasound may be inaccurate. As images and Doppler signals are now of high quality and easy to obtain with commercially available equipment, duplex scanning is widely used in clinical practice to obtain non-invasive structural and haemodynamic information from many arterial sites.

<u>Ultrasound of plaques and assessment of risk</u>. Stenosis severity is indicative of the risk of clinical events in the carotid circulation. Prospective natural history studies have shown that relatively minor stenosis (<80%) have an annual risk of producing a stroke of 1-3%, whereas the risk is 4-12% per year in the presence of a stenosis occluding greater than 80% of the lumen (Moneta et al 1987, Caracci et al 1989). The risk is greater still if the end-diastolic flow at the stenosis is greater than 200 cm/s, suggesting severe obstruction to anterograde flow in systole (Moneta et al 1989).

In addition to plaque severity, the composition of a plaque may be important in the risk of disease progression and clinical events; soft, lipid-rich lesions are more vulnerable to rupture or to distal embolisation than hard, fibrous plaques (Davies & Woolf 1993). Attempts have been made, therefore, to provide information about tissue characteristion using ultrasound, and thereby to identify subjects at highest risk. Ultrasonographically homogeneous plaques are relatively benign, whereas most symptomatic patients have heterogeneous plaques, an appearance which correlates well with intraplaque haemorrhage and/or ulceration (Reilly et al 1983). Lipid-rich plaques are much more lucent than fibrous lesions, and Johnson et al (1985) found that almost all patients with lucent ("soft") plaques that were causing over 75% stenosis developed symptoms over 5 years follow-up. Gray-Weale et al (1988) have classified lesions according to their ultrasound appearance, and related lesion type to the risk of subsequent clinical events.

In current practice intraplaque haemorrhage can be reliably detected, with high sensitivity and specificity, plaque composition can be characterised, and percent lumen stenosis can be measured approximately. These ultrasound features can be used to determine treatment strategies in the clinical setting.

The major limitation of B-mode imaging for the quantification of plaque size is poor reproducibility. B-mode provides a two-dimensional picture, but plaques are not only three-dimensional but almost always eccentrically placed within the artery. Depending on the location of the scanning plane, images of plaque size and composition may vary considerably. Consecutive parallel transverse scans may be obtained and reconstructed (Blankenhorn et al 1983), however images of the lateral walls are poor in transverse sections. Calcification may produce shadowing and make vessel wall details even more difficult to quantify. Furthermore the patient's position relative to the transducer and variable machine parameters such as frequency, dynamic range and processing curves render precise quantification of plaques difficult to achieve. Consequently most studies of B-mode imaging of plaques show poor reliability for measurement of lesion size or percent stenosis (Comerota et al 1981, Ratliff et al 1985).

In contrast to plaque quantification, B-mode images of the thickness of the arterial wall are easily obtained, reproducible and may be important in the detection of early structural changes associated with atherosclerosis.

1.3.3.2 Intima-media thickness (IMT)

Given that ultrasound measurement of plaque size and extent may not be accurate and reproducible, investigators have sought a "surrogate" marker of the atherosclerotic process. Measurement of the thickness of the arterial wall in a vessel prone to atheroma may provide such a marker, and therefore the IMT of the common carotid, which is easily obtained and measured, has been extensively investigated.

IMT is the distance between the lumen/intima interface (the "i" line on an ultrasound image of the vessel wall) and the media/adventitia interface (the "m" line). Common

carotid IMT can be measured in almost all subjects, and is currently being studied in many clinical trials and epidemiological studies worldwide. IMT increases with age and is thicker in subjects with atherosclerotic disease at distant sites (Belcaro et al 1991). The measurement is not of a plaque itself, but of a segment of arterial wall that becomes thicker in people with risk factors for and in the presence of occlusive vascular disease elsewhere in the body; that is, IMT is simply a marker for the atherosclerotic process. In some cases, increasing IMT is due to medial hypertrophy in response to hypertension or as a result of fibromuscular hyperplasia; in these subjects, increasing IMT may not be related, therefore, to worsening atherosclerosis.

Measurement of IMT can now be made accurately and reproducibly in the common carotid, bifurcation and internal carotid arteries (Wendelhag et al 1991). Common carotid IMT is greater in hypercholesterolaemic than in normal adults (Wendelhag et al 1992), and also correlates well with carotid and femoral atherosclerosis (Belcaro et al 1991). Salonen & Salonen (1993) have recently shown that thickened IMT is related to later ischaemic event rates. They measured common carotid IMT in 1257 men aged 42-60 years, and showed that IMT≥1.0mm was associated with an increased risk of subsequent myocardial infarction (MI) compared to a normal value (IMT<1.0mm) (relative risk 3.0, 95% confidence interval 1.4-6.5). Furthermore the maximal IMT, when analysed as a continuous variable, was also associated significantly with the risk of MI; for each 0.1mm increase in IMT, risk of MI increased by 11% (95% confidence interval 6-16%). These data establish the predictive value of carotid IMT measurement (in middle aged men only), in confirms the close relation between carotid and coronary atherosclerosis reported from autopsy studies.

Intima-media thickness and a variety of non-invaively derived arterial wall thickness "scores" are being used in prospective, long term epidemiological studies. In the Atherosclerosis Risk In the Community (ARIC 1989) study, increased IMT was positively correlated with total serum cholesterol, LDL cholesterol and systolic blood pressure. Salonen & Salonen have not only correlated IMT with age, cholesterol,

smoking and other risk factors in middle-aged men, but also shown that carotid IMT was predictive of the subsequent risk of myocardial infarction (1993), as above. Similarly Belcaro et al (1991), who described a grading system for arterial wall thickening ("ultrasonic biospy"), have shown that high grade lesions correlate with risk of developing clinical disease in asymptomatic subjects followed up for 3 years. Carotid ultrasound may therefore reflect the total body "atherosclerotic burden".

The results of larger multicentre epidemiological studies using IMT as a proxy measure of coronary disease are awaited before recommendations about widespread screening or the use of IMT in clinical trials of disease progression or reversibility can be made.

1.3.3.3 Arterial wall elastic properties

As the histologic structure of vessels change with normal ageing or with the development of disease processes, so too their dynamic properties may alter (Kohn 1977). Information on the properties of the arterial wall has long been sought because these factors are altered in various cardiovascular diseases, such as hypertension (which alters wall thickness) and atherosclerosis (which reduces compliance). Typically the measurements required to assess the functional status of the arterial wall are diameter and thickness (for the geometry), and compliance, distensibility and the elastic modulus (for the mechanical properties).

Non-invasive methods have been developed to estimate vessel diameter and wall thickness, other than B-mode images. These include "A-mode" scanning, which uses an ultrasonic pulse echo-tracking device, and a pulsed-Doppler technique, to measure lumen diameter as the width of a column of moving blood. The A-mode device relies on high frequency 10mHz ultrasound, a stereotactic arm for precise positioning, a sampling frequency of 100mHz and sophisticated processing of each radio-frequency line which detects the blood-vessel wall interface. This method is reproducible (Mooser et al 1988), but is cumbersome, technically demanding, and relies on precise transducer position

without the use of a two-dimensional image to guide probe placement. The pulsed Doppler technique detects the width of a moving column of blood, and so measures lumen diameter but not the vessel wall directly. Limitations include suboptimal resolution of ≈ 0.4 mm, and the effect that non-laminar flow might exert on diameter measurements.

The pulse echo-tracking devices may also be used to assess arterial compliance, distensibility and elastic modulus (Meister et al 1992). Using this and similar techniques, non-linear elastic properties of arteries have been found to change with ageing (van Merode et al 1989), chronic hypertension (Safar et al 1981) and atherosclerosis severity (Hirai et al 1989). These techniques are not in routine clinical use, and long-term trials are awaited to assess their predictive value for disease progression or vascular events.

1.3.3.4 Magnetic resonance imaging

Magnetic resonance imagine (MRI) is based on imaging of the proton, the positively charged spinning nucleus of hydrogen, which is abundant in tissues containing water, proteins and lipids. Using a powerful magnetic field to align these nuclei and one or more radiofrequency pluses to perturb them, followed by measurement of their relaxation patterns, high quality tissue images can be obtained. Flow rate profoundly alters the MR characteristics of blood, and so flow can be "imaged" using special pulse sequences. This principle has recently been applied to allow the development of MRI "angiograms" (Edelman & Warach 1993).

MRI is non-invasive and safe. It has not yet provided high resolution pictures of the vessel wall nor allowed the visualisation of atheromatous plaque. However MRI angiography now provides excellent images of the carotid (Masaryk et al 1991) and iliofemoral arteries (Owen et al 1992), including the proximal large vessels, and medium- sized arteries such as the circle of Willis and the anterior tibial vessels. In the coronary circulation, Manning et al (1993) have developed an ultrafast echo gradient

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sequence which, when combined with breath-holding and ECG gating, provides excellent pictures of the proximal coronary vessels, with high sensitivity and specificity when compared with conventional angiography. MRI coronary angiography is able to identify the presence of substantial stenoses correctly in most subjects, although details of distal vessel anatomy are currently unavailable. Furthermore, subjects must have a regular cardiac rhythm, and be able to breath-hold for ≥ 15 seconds for each picture.

MRI angiography has the same limitations as conventional angiography, in that it provides images of the lumen rather than the vessel wall. However MRI angiography may be combined with anatomical and functional MRI, as well as MRI perfusion imaging, to provide a comprehensive cardiac and coronary evaluation, by non-invasive means.

MRI is therefore an exciting non-invasive technique for evaluating atherosclerosis and its complications. Compared to ultrasound, however, it remains more costly and less available. As images of the vessel wall are not obtainable by MRI, this technique is as yet not applicable for the detection of the earlier stages of atherogenesis, in childhood or young adult life.

1.4 MEASURING ENDOTHELIAL FUNCTION; IN-VITRO AND IN-VIVO STUDIES

Endothelial function is important in controlling local vascular tone and in the interaction between the vessel wall and platelets and leucocytes. The ability of intact endothelium to release vasoactive substances in response to a variety of pharmacological and physiological stimuli has been used as the basis for a variety of in-vitro and in-vivo tests of endothelial physiology.

1.4.1 IN-VITRO STUDIES

Most in-vitro studies of endothelial control of vascular tone have been performed on isolated strips or rings of arteries mounted on strain gauges in organ chambers. Typically arteries are dissected free, cleared of excess fat and connective tissue and cut into rings. Endothelium can be removed by gentle rubbing on the luminal side. Rings with and without endothelium are then mounted on wires attached to a force transducer, and maintained in oxygenated physiological buffer solution at 37°C. The rings are then preincubated with indomethacin (to inhibit synthesis of protaglandins) and preconstricted, usually with phenylephrine, to achieve a stable contractile state.

This preparation can then be exposed to endothelium-dependent vasodilators such as acetylcholine and ADP, to test endothelial function, and exogenous nitrates such as nitroprusside, to test smooth muscle relaxation. This experimental design has been used to study endothelial function in systemic and pulmonary arteries from a variety of animals (Furchgott & Zawadzki 1980, Chand & Altura 1981, Miller et al 1986), and in pulmonary arteries from excised human tissue (Dinh-Xuan et al 1991).

1.4.2 IN-VIVO STUDIES

All techniques used for in-vivo study of endothelial function in human arteries have been invasive to some extent. Methods to study large (conduit) artery physiology are catheter-

based, and rely on measuring small changes in arterial diameter by quantitative angiography, in response to a variety of infused vasoactive agents. Studies of endothelial and smooth muscle function in small (resistance) arteries rely on infusion of pharmacological agents followed by a measurement of change in flow velocity or absolute flow. Due to their invasive nature, these studies are mainly applicable to patients with symptoms of established vascular disease.

1.4.2.1 Large artery studies

The technique for studying endothelial function in conduit arteries in man was first described in the coronary circulation (Ludmer et al 1986), and modifications of this catheter-based method have since been used to investigate vascular responses in other systemic arteries, such as the femoral artery (Liao et al 1991) and recently in the pulmonary arteries (Celermajer et al 1993a).

Cardioactive medications are usually stopped at least 12 hours before catheterisation. After completion of the diagnostic tests (such as angiography), heparin is given, and a large guiding catheter is placed in the target artery. Through this a smaller infusion catheter is introduced into the lumen of the artery; this catheter is used for the administration of vasoactive agents, while pressure is monitored and contrast agent is injected through the larger guiding catheter. The infusion catheter may have a Doppler ultrasound crystal mounted at its distal end to allow simultaneous measurement of flow velocity in the artery (Figures 1.4.1, 1.4.2).

Serial intra-arterial infusions are then administered, including control solutions, endothelium-dependent dilators (usually acetylcholine or substance P) and endothelium-independent dilators (such as nitroglycerine or nitroprusside). Throughout each infusion, ECG, heart rate and arterial pressure are recorded continuously. At the end of each infusion, an angiographic picture is taken, for later quantitative measurement of arterial diameter, and flow velocity may be measured. Stability of the position of each catheter

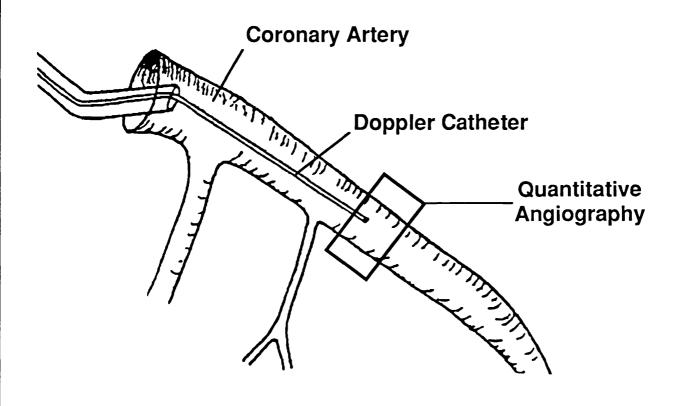


Figure 1.4.1
Schematic drawing of placement of guiding and Doppler flow velocity catheters for invivo invasive assessment of coronary endothelial function.

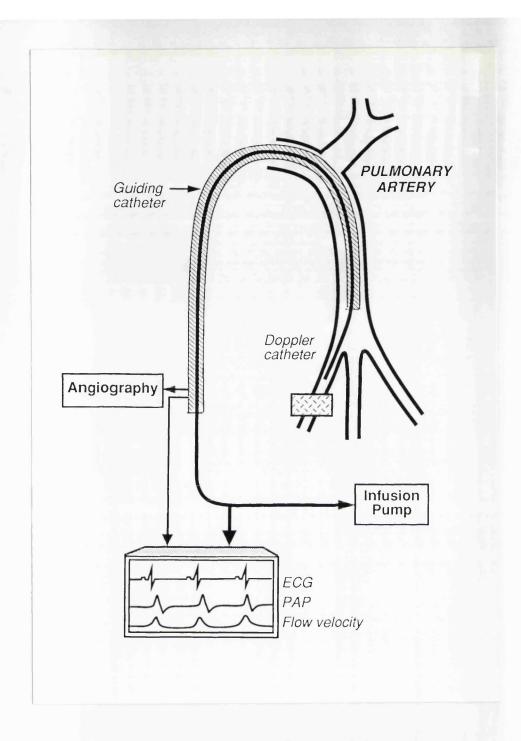


Figure 1.4.2

The experimental setup for investigation of in-vivo pulmonary endothelial function (PAP - pulmonary artery pressure, stippled box indicates the segment analysed by quantitative angiography).

is confirmed by fluoroscopy.

Using this technique, important insights into the role of endothelium in the pathogenesis of atherosclerotic disease have been obtained. Normal endothelium mediates vasodilatation, but at the site of atherosclerotic plaques, dysfunctional endothelium may mediate paradoxical vasoconstriction in response to acetylcholine (Ludmer et al 1986). This observation underscores the importance of dynamic plaque constriction as an active mechanism in ischaemia, in addition to ischaemia which may result from fixed stenoses limiting tissue perfusion at times of increased metabolic demand. Similar experiments have linked endothelial dysfunction to failure of vasodilatation or constriction of arteries in response to exercise (Gordon et al 1989), increased flow (Nabel et al 1990), sympathetic stimulation (Nabel et al 1988) and mental stress (Yeung et al 1991). A similar shift towards constrictor responses has been observed in angiographically smooth arteries in patients who have overt disease in other coronary vessels (Zeiher et al 1991), and in subjects with known risk factors for atheroma (Vita et al 1990). Increasing age is also associated with impaired endothelium-dependent responses (Yasue et al 1990). These latter observations in angiographically smooth vessels emphasise the importance of endothelial dysfunction in the early stages of atherosclerosis (Zeiher et al 1991).

1.4.2.2 "Small Vessel" Studies

Endothelium plays a critical role in the control of vasomotor tone in resistance as well as large conduit arteries, and this too can be tested in-vivo, albeit by an invasive technique involving arterial cannulation. Endothelial function has been studied in the coronary resistance vessels in animals (Sellke et al 1990) and in man (Drexler et al 1991a). Recently investigations of endothelial function of the forearm resistance vessels have been described (Panza et al 1990); as the protocol is less invasive, involving only brachial artery cannulation, it can be applied to asymptomatic subjects with risk factors for (but no clinical evidence of) vascular disease. Although resistance vessels do not develop atheroma, endothelial dysfunction has been demonstrated in these arteries in

subjects prone to atherosclerosis.

In these experiments, subjects have a cannula inserted into the brachial artery under local anaesthesia, for determination of blood pressure and infusion of vasoactive substances. Infusions include control solutions, endothelium-dependent dilators (such as methacholine) and endothelium-independent dilators (such as nitroprusside), often in incremental doses. Forearm blood flow is measured by venous occlusion plethysmography, using calibrated strain gauges (Creager et al 1990). Forearm vascular resistance can then be calculated from pressure and flow data.

Using this technique, it has been found that endothelium-dependent dilatation is impaired in the small vessels in the forearm and coronary circulations in hypercholesterolaemic humans (Creager et al 1990, Drexler et al 1991b), and in the forearm in hypertensive subjects (Panza et al 1990). Whether endothelial dysfunction is related causally to hypertension is still unclear, and no "dose-response" relationship between blood pressure level and degree of vascular physiological abnormality has yet been established.

Due to their invasive nature, all of the above in-vivo studies of endothelial function in man have enrolled relatively few patients (less than 40 each, often fewer than 20 subjects). Furthermore endothelial function in children, in whom atherogenesis may well have commenced, has not yet been studied. Therefore a non-invasive, widely applicable test of arterial physiology is required, to facilitate cross-sectional and serial study of endothelial and smooth muscle function in asymptomatic children and adults at risk of atherosclerosis.

CHAPTER 2 - THE NON-INVASIVE METHOD

2.1 DEVELOPMENT OF GENERAL METHODS

2.1.1 RATIONALE

In current practice, diagnosis and treatment of atherosclerotic vascular disease are usually instituted late in the natural history, at a stage when plaques are well established and clinical complications have occurred. As the process of atherogenesis begins in childhood (Stary 1989), research has been directed towards detection of early vessel wall changes in asymptomatic subjects at increased risk, by non-invasive means.

Studies of arterial structure have failed to reveal important changes in high risk children and young adults. As physiological abnormalities may precede anatomical changes, a test of arterial reactivity rather than structure might detect early disease. Given that endothelial dysfunction is an important early event in animal models of atherogenesis and may play a role in initiation and progression of atherogenesis in man, a non-invasive method of testing endothelial function might prove useful for understanding the disease process and identifying "at risk" subjects in early life. In-vivo tests of endothelial function do exist, based on arterial catheterisation and the response of arteries to infused vasodilator substances, followed by quantitative angiography. These techniques, however, are clearly inappropriate for either study of asymptomatic subjects, or for serial investigation of disease progression or reversibility.

2.1.2 DESIDERATA

A non-invasive test of arterial physiology should be:

(i) Widely applicable

- (ii) Well tolerated
- (iii) Accurate
- (iv) Reproducible and
- (v) Should distinguish between subjects with and without disease.

The following sections outline a non-invasive method for testing arterial physiology that fulfils the above criteria. The method is derived from the principles of the invasive coronary endothelial studies outlined above; that is, accurate measurement of arterial diameter in response to endothelium-dependent and independent stimuli. In the invasive method, measurement is by quantitative analysis of angiograms, the endothelial stimulus is a pharmacological agent such as acetylcholine or substance P and the smooth muscle stimulus (endothelium-independent) is intra-arterial GTN. In the non-invasive test, the measurements are made using high resolution vascular ultrasound, the endothelial stimulus is increased arterial flow (reactive hyperaemia) and the smooth muscle stimulus is sublingual GTN spray.

2.1.3 DESCRIPTION OF THE PROTOCOL

The diameter of the target artery (superficial femoral or brachial) is measured from 2-dimensional ultrasound images, using a 7.0mHz linear array transducer (L7384) and a 128XP/10 system (Acuson, Mountain View, California) (figure 2.1.1). In all studies, scans are taken at rest, during reactive hyperaemia, again at rest and after sublingual GTN (figure 2.1.2).

Cardioactive medications are stopped \geq 24 hours prior to the test. The subject lies at rest for \geq 10 minutes before the first scan. The target artery (either the superficial femoral artery just distal to the bifurcation of the common femoral, or the brachial artery 2-15 centimetres above the elbow) is scanned in longitudinal section. The centre of the artery is identified when the clearest picture of the anterior and posterior intimal layers is obtained. The transmit (focus) zone is set to the depth of the near wall, in view of the

Vascular Study **Dynamap Ultrasound ECG** Scanner 7.0 mHz **Linear Array Transducer Pneumatic Tourniquet**

Figure 2.1.1
Diagram of a subject undergoing non-invasive study. The transducer (7.0mHz linear array) is placed over the brachial or superficial femoral artery, which is scanned in longitudinal section. A pneumatic tourniquet is placed distal to the target artery. The blood pressure may be taken in the left arm, and the ECG is monitored continuously.

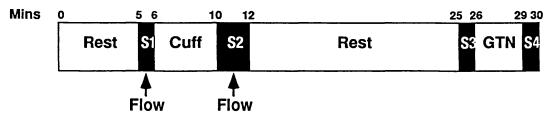


Figure 2.1.2
Protocol of the non-invasive study. The study takes 30 minutes.
Cuff – period of cuff inflation, Flow – Doppler-derived flow measurement made, GTN – glyceryl trinitrate, S – scan.

greater difficulty of evaluating the near compared to far wall "m" line (the interface between media and adventitia) (Pignoli et al 1986, Nolsoe et al 1990). Transmit power is - 9dB, log compression 40dB and overall gain 3dB. Individual depth and gain settings are set to optimise images of the lumen/arterial wall interface, images are magnified using a resolution box function (leading to a television line width of approximately 0.065mm), and machine operating parameters are not changed during any study. The processing curves chosen are pre-processing 2, persistence A and post-processing 5, to enhance the vessel wall/lumen interface (high contrast, crisp borders). No studies are performed on arteries that have been previously catheterised. Only studies in which the near and far wall m-lines are clearly seen during each scan are later analysed.

When a satisfactory transducer position is found, the skin is marked, and the limb remains in the same position throughout the study. Care is taken to apply the transducer without undue pressure. A resting scan is recorded and blood flow velocity is measured in the artery, using a pulsed Doppler signal at a 70° angle to the vessel, with the range gate (1.5mm) in the centre of the artery. Increased flow is then induced by inflation of a pneumatic tourniquet to a pressure of 300 mmHg for 4.5 minutes followed by release. A second scan is taken for 30 seconds before and 90 seconds after cuff deflation, including a repeat Doppler flow velocity recording for the first 15 seconds after the cuff is released. Thereafter 10 minutes is allowed for vessel recovery, after which a further resting scan is taken. Sublingual GTN spray (400 mcg) is then administered, and 3-4 minutes later the last scan is performed. The electrocardiogram is monitored continuously. In the first 40 patients studied, blood pressure was recorded in the left arm at 2 minute intervals.

<u>Data Analysis</u>. Vessel diameter is measured by 2 observers, who are unaware of the condition of the subject and the stage of the experiment. The arterial diameter is measured at a fixed distance from an anatomical marker, such as a bifurcation, using ultrasonic calipers. Measurements are taken from the anterior to the posterior "m" line (the interface between the median and aventitia) at end-diastole, incident with the R-wave

on the electrocardiogram (ECG). For the reactive hyperaemia scan, diameter measurements are taken 45-60 seconds after cuff deflation. Four cardiac cycles are analysed for each scan and the measurements averaged. The vessel diameter was measured in each of the scans (that is, at rest after reactive hyperaemia, after a further 10 minutes rest and 3-4 minutes after GTN), and were then expressed as a percentage relative to the first control scan (100%). Flow is calculated by multiplying the velocity-time integral of the Doppler flow signal for one cardiac cycle by the heart rate by the vessel cross-sectional area (π x radius²). Reactive hyperaemia is calculated as the maximum flow recorded in the first 15 seconds after cuff deflation divided by the flow during the resting (baseline) scan.

<u>Interobserver variability</u>. Throughout this thesis, all scans from all patients were analysed by at least 2 observers. This was determined early in the study, because small inaccuracies in diameter measurement might have important impact on the calculation of percent dilatation in response to various stimuli. Taking the average of 4 scans per condition for each of 2 observers was considered the best strategy to minimise measurement error.

We calculated the between-observer coefficient of variation as part of 3 studies that appear below; for absolute measurement of "phantom artery" diameter (section 2.2), for study of our first 50 normal subjects (section 3.1) and for the study of 200 subjects who were current, former or life-long non-smokers (sections 4.3, 5.3). For the in-vitro phantom study, the between-observer error was neglible (<1%). For the two in-vivo studies, the interobserver coefficient of variation for measurement of FMD was 1.3-1.4%. The range of differences of measurement of FMD between observers was 0-7%, mean $1.6\pm1.3\%$.

2.1.4 CHOICE OF ARTERY

External ultrasound imaging with high frequency linear array transducers provides

detailed pictures of vessel wall anatomy, but its application is limited to relatively superficial arteries. Images of deeper arteries, such as the coronary or renal vessels, requires lower frequency ultrasound, which has better depth penetration but lower resolution. Therefore we evaluated the use of several superficial systemic arteries, to assess their suitability for the non-invasive method. The ideal vessel is not too superficial, as compression by the application of the transducer might occur (e.g. radial or dorsalis pedis artery), not too deep, so that adequate resolution could still be obtained (excluding coronary arteries), and a vessel that could be scanned with the subject in a comfortable position (excluding the popliteal artery).

The arteries considered were therefore the brachial, superficial femoral and carotid. Excellent images of the arterial wall could be obtained for each of these vessels. We then evaluated methods for inducing a condition of increased flow, in order to test endothelium-dependent dilatation. For the limb vessels, distal circulatory arrest by use of a pneumatic tourniquet followed by reactive hyperaemia was well tolerated and reliably provided flow increase of 200-800%. Images during reactive hyperaemia were easy to acquire. In contrast, increased flow in the carotid was more difficult to achieve. We found that hyperventilation followed by mask breathing of 6% CO₂ in air produced a flow increase of 60-100% in the internal carotid, however this was poorly tolerated, especially by children, and made scan acquisition difficult.

Therefore the best arteries to study using this technique are the brachial and superficial femoral, as they are large systemic arteries in which reactive hyperaemia can be induced easily. As atherosclerosis is a diffuse process, which often follows a parallel course in the coronary, carotid and femoral vessels, our initial studies were carried out on the superficial femoral artery. When we found an inverse relationship between flow-mediated dilatation and resting vessel size in normal subjects (see section 3.1), we confined our study to arteries with diameter ≤ 6.0 mm; that is, superficial femoral arteries in children and brachial arteries in adults.

2.1.5 CHOICE OF CUFF PRESSURE AND INFLATION TIME

Other investigators studying reactive hyperaemia have used suprasystolic inflation pressures, and cuff times of 2-10 minutes (Anderson & Mark 1989, Sinoway et al 1989, Laurent et al 1990). In our pilot studies, we found that cuff pressures over 300mmHg and inflation times greater than 6 minutes resulted in significant discomfort, especially in children. Cuff pressures under 200mmHg do not reliably produce circulatory arrest, and inflation times less than 3 minutes do not produce maximum hyperaemic responses, as assessed by change in Doppler flows. Therefore we have used cuff inflation pressures of 250-300mmHg and inflation times of 4-5 minutes. This has proven tolerable and effective for both children and adult subjects.

2.1.6 FLOW-MEDIATED DILATATION

Increasing blood flow through large arteries produces an increase in vessel diameter, both in animals (Hintze et al 1984) and in man (Anderson & Mark 1989, Sinoway et al 1989, Laurent et al 1990). This phenomenon of flow-mediated dilatation (FMD) is now known to be endothelium-dependent, as a normal arterial response is critically dependent on the presence of a functionally intact endothelium (Smiesko et al 1985, Pohl et al 1986). The fact that FMD is endothelium-dependent forms the basis for non-invasive testing of endothelial function.

Control of vessel diameter by changes in blood flow was first proposed in 1933 (Schretzenmayr), who observed dilatation of the canine femoral artery in response to increases in femoral blood flow. Originally the mechanism of this vasodilatation was thought to be due to a retrograde wave of dilatation starting in the arterioles and spreading to the large proximal arteries; so called "ascending dilatation" (Macho et al 1981). This was soon disproven, however, after experiments in which cutting the femoral artery distal to an AV fistula failed to abolish FMD (Ingebrigtsen & Leraand 1970, Lie et al 1970).

It was hypothesised therefore that a local mechanism controls arterial responses to flow. Endothelial cells are ideally situated to "transduce" the signal of increased flow/shear stress into a vessel wall response via modulating the production and release of vasoactive substances. Endothelial cells in culture are known to react to changes in mechanical forces by altering metabolism (Franke et al 1984, Van Grondelle et al 1984). Recently Smiesko et al (1985) and Pohl et al (1986) have shown that FMD occurs in normal arteries and is abolished completely by techniques used to injure the endothelium (mechanical or pharmacological). Furthermore Rubanyi et al (1986) have demonstrated that FMD is related to the release of EDRF, and Gruetter et al (1981) have shown abolition of FMD by the concomitant administration of the EDRF inhibitor, methylene blue. Both absolute flow increase and pulsatility are powerful stimuli of endotheliumdependent FMD. The mechanism whereby endothelial cells sense an increase in shear stress is not well characterised, but is probably mediated via activation of a K+ channel in the endothelial cell membrane (Rubanyi et al 1990). Vascular relaxation may also occur by direct transmission of endothelial cell hyperpolarisation to the underlying smooth muscle via gap junctions (Olesen et al 1988).

In our experiment, flow increase is produced by distal circulatory arrest (using a pneumatic tourniquet) and subsequent reactive hyperaemia. We have considered three alternative mechanisms whereby reactive hyperaemia may cause vasodilatation, other than by increased flow causing endothelium-dependent dilatation.

Firstly, tourniquet inflation and deflation may alter artery diameter by disturbing cardiovascular reflexes. We studied 40 subjects, and monitored blood pressure in the left arm and heart rate throughout our experimental protocol. Neither cuff inflation nor release altered these haemodynamic parameters (Figure 2.1.3). Similarly Anderson & Mark (1989) reported no change in heart rate or blood pressure after forearm cuff inflation to suprasystolic pressure for 3-10 minutes in 29 healthy men and women.

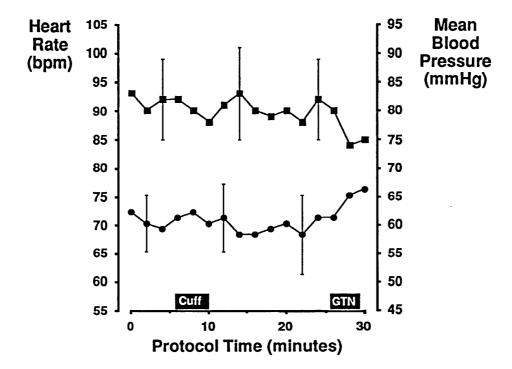


Figure 2.1.3
Blood pressure (■) and heart rate (●) do not change during the experimental protocol.

Secondly, we considered the possibility that systemic circulation of ischaemic metabolites from the distal forearm might cause vasodilatation immediately after cuff release. We studied 10 normal subjects (5 male, 5 female) by inflating the cuff on their left arm (suprasystolic pressure, 4-5 minutes) and measuring the right brachial artery diameter, and compared observed vasodilatation to the results obtained from the same subjects studied by the standard method (ipsilateral reactive hyperaemia) (Table 2.1.1).

Table 2.1.1. Response of right brachial artery to reactive hyperaemia in the contra- and ipsilateral arm.

Subject	Left cuff	Right cuff
1	0.4	10.5
2	0.9	11.1
3	0.7	9.1
4	0	5.1
5	-1.4	8.2
6	-1.8	11.2
7	1.6	6.9
8	-1.4	10.9
9	-0.2	12.8
10	1.1	7.3
Mean	0±0.8%	9.3±1.5% (p<0.0001)

Therefore dilatation was not observed when reactive hyperaemia was performed in the contralateral arm. Similar results have been reported by Lie et al (1970) and Anderson & Mark (1989).

Finally it is possible that intra-arterial distending pressure might change during this

protocol, and thus alter vessel diameter; that is, distending pressure may increase during cuff inflation and decrease immediately thereafter. However the resting pressure gradient across the brachial artery is small (approximately 4mmHg) (Heistad et al 1968). Melkumyants et al (1964) have studied feline arteries, and found that any intra-arterial pressure decrease after cuff release is offset by the increase in flow, resulting in no change in distending pressure. Anderson & Mark (1989) have measured intra-arterial pressure in man during and after distal vessel occlusion, and confirmed that there are no consistent changes in distending pressure.

Therefore flow mediated dilatation is an endothelium-dependent phenomenon. In our experiment, distal cuff inflation and release do not mediate diameter changes in the proximal artery by either a reflex, local pressure change or circulating ischaemic metabolite mechanism, and this method has now been accepted as a valid test of endothelial function by several major peer-reviewed journals. Theoretically, performing the experiment whilst infusing an EDRF antagonist (such as L-nMMA) into a proximal part of the target artery might provide definitive proof that this technique directly tests endothelium-dependent dilatation, and such experiments are currently underway.

2.1.7 GTN-INDUCED DILATATION

We selected sublingual nitroglycerine (or glyceryl trinitrate, GTN) as the endothelium-independent vasodilator in our protocol, as it acts directly on smooth muscle, has a rapid onset of action and short half-life, is easy to administer and has no serious side effects.

Nitrovasodilators have been used clinically for over 100 years in the treatment of angina pectoris, and more recently for hypertension, heart failure and complications after cardiac catheterisation. Until the last decade, little was known about their mechanism of action. In 1977, two groups found that organic nitrates induced a dose-dependent increase in levels of cGMP in smooth muscle (Schultz et al 1977, Katsuki et al 1977). Subsequently it was shown that all nitrovasodilators activate soluble guanylate cyclase

(Murad et al 1978). The consequent increase in cGMP levels induces a sequence of protein phosphorylation that leads to smooth muscle relaxation either by inhibiting calcium entry, promoting calcium exit or interfering with the actin/myosin interaction (Moncada et al 1991) (Figure 2.1.4).

GTN is the oldest and most frequently prescribed compound in the nitrovasodilator class. As it acts directly on smooth muscle, its relaxant action is independent of whether the endothelium is intact or not. GTN acts on arteries and veins, reduces coronary spasm in susceptible individuals, dilates coronary arteries at the site of eccentric stenoses (and thus improves flow to ischaemic areas) and may improve collateral flow (Mickelson et al 1990).

GTN given sublingually or via the buccal mucosa is absorbed rapidly, and exerts a peak effect in 2-10 minutes. Its duration of action is only 15-30 minutes. As a result it is usually used for acute relief of angina, or prophylactically prior to short-term activities that might precipitate an attack. As GTN is volatile it must be kept in a stoppered vial. Adverse reactions are rare, other than transient headache.

Oral nitrates are also effective, but have a slower onset of action, and low bioavailability due to significant first-pass metabolism by the liver. Physiological tolerance may occur with chronic usage. Cutaneous GTN ointment produces long-lasting vasodilatation (4-6 hours), but takes 60-90 minutes to act. Intravascular GTN is quick-acting, but inappropriate for use in a non-invasive study.

GTN given sublingually is a safe and effective vasodilator with a rapid onset and short duration of action, which produces arterial relaxation by a mechanism that does not depend on intact endothelial function. Therefore its use is ideal as a non-invasive marker of the capacity of arterial smooth muscle relaxation.

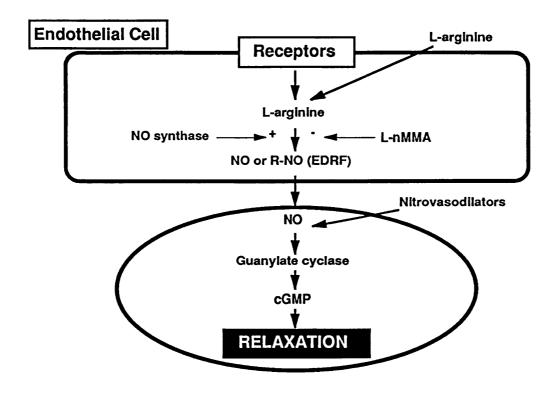


Figure 2.1.4
Mechanism of action of nitrovasodilators, such as GTN. These drugs produce smooth muscle relaxation by direct activation of guanylate cyclase and consequent increase in cyclic-GMP production.

2.1.8 TIMING OF MEASUREMENTS

- (i) <u>During reactive hyperaemia</u>. In order to determine the optimal time to measure vessel diameter and flow velocity after cuff deflation, we studied 10 normal subjects, and measured brachial artery diameter at rest and every 10 seconds after cuff release for 2 minutes. We then studied 8 further subjects, and measured flow every 5 seconds after cuff release for 30 seconds. As can be seen from Figure 2.1.5, the best time to measure flow mediated dilatation is 45-60 seconds after cuff deflation. Reactive hyperaemia is maximal much earlier, 5-15 seconds after cuff release. This is similar to findings in other studies (Sinoway et al 1989, Laurent et al 1990).
- (ii) After sublingual GTN. We studied 6 subjects to determine the time course of GTN-induced dilatation of the right brachial artery. Maximum response could always be observed 3-4 minutes after administration of the spray. Dilatation could first be observed 60 seconds after GTN, and the artery returned to baseline diameter after 30 minutes in 5/6 subjects; in one, GTN-induced dilatation was still present 1 hour after administration.

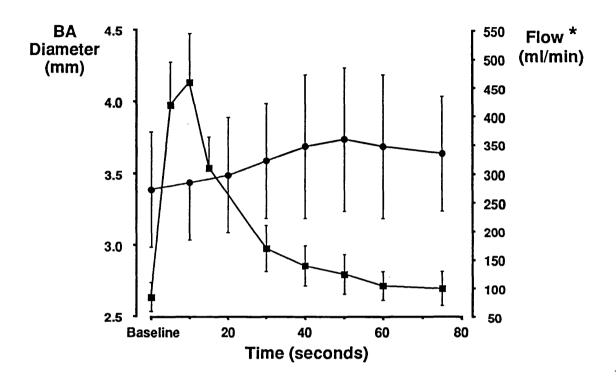


Figure 2.1.5 Changes in brachial artery diameter (\bullet) and flow (\blacksquare) during reactive hyperaemia. Diameter is maximal 45 – 60 seconds after cuff release, hyperaemic flow is maximal much earlier (5 – 15 seconds).

* Derived from vessel size, flow velocity and heart rate

2.2 PHANTOM STUDIES AND ULTRASOUND CONSIDERATIONS

INTRODUCTION

Developments in B-mode ultrasound technology have facilitated non-invasive study of arterial structure and function at an early stage of the atherogenic process. The features of early vascular damage that may be detected accurately by ultrasound include increased arterial wall thickness (Salonen & Salonen 1991), decreased compliance (Van Merode et al 1989) and impaired endothelial function (Celermajer et al 1992). The latter two indices of arterial physiology are calculated from relative changes in arterial diameter, either within the cardiac cycle, or before and after reactive hyperaemia. These methods depend more on accurate measurement of diameter change than of absolute diameter itself.

The best resolution available from ultrasound is obtained with high frequency transducers; the cost of improved resolution, however, is decreased tissue penetration. For the evaluation of superficial arteries with high frequency ultrasound, the theoretical axial resolution can be derived as follows.

The wavelength W of the ultrasonic pulse is given by W=c/f, where c is the speed of sound in biological tissue (approximate 1540 metres per second) and f is the transducer frequency. The pulse length 1p is given by $1p=n \times W$, where n is the number of cycles that are used to produce the ultrasonic pulse (usually 2-3 in most commercial machines). The minimum thickness d of a resolvable structure is d=1p/2. Combining these equations, $d=n \times c/2f$. In a typical case with n=3, $c=1.5 \times 10^2 \text{ msec}^{-1}$ and f=7 mHz, then d=0.3 mm. Therefore the best theoretical resolution (where n=1) of 7 mHz ultrasound is 0.1 mm, but in practice, with n>1, the usual theoretic resolution is approximately 0.3 mm. This is in accordance with experimental results from several laboratories (Pignoli et al 1986, Wendelhag et al 1991). This lower limit of resolution is important in considering the accuracy of measuring the distance between two points that are extremely close together, such as in assessment of intima-media thickness.

In our experiment, however, the issue is not one that is limited by the axial resolution of a system, as we are not measuring tiny distances; rather, we are measuring differences between larger diameters (for example, the change of diameter of an artery from 4.0 to 4.4 mm). That is, we need to know the precision of a measurement of distance between 2 points that are greater than one wavelength apart. This is a function of echo pattern and television line width, rather than axial resolution, and has not previously been investigated.

We therefore performed a phantom experiment using the same equipment and machine operating parameters that we have used for in-vivo studies of arterial physiology, in order to assess whether small diameter changes could be detected accurately using B-mode ultrasound.

2.2.1 SPECIFIC METHODS

The phantom. A phantom with 10 cylinders ("arteries") was constructed (Danish Phantom Design, Jyllinge, Denmark). The cylinders measured 2.8, 3.0, 3.2, 3.4, 3.6, 4.0, 4.1, 4.2, 4.3 and 4.4 mm respectively, chosen as the most frequent range of brachial artery diameters observed in-vivo. This provided 7 pairs of "arteries" with a diameter difference of 0.2 mm, and 4 pairs with a difference of 0.1 mm. The phantom was made up of agar moulded around steel cylinders of known diameter. Al₂O₃ and SiC were added to the background agar to obtain a liver equivalent backscatter, with an attenuation of 0.5 dB/MHz x cm and a sound velocity of 1540 m/s. The cylinders had the following specifications: attenuation < 0.05 dB/Mhz x cm, backscatter <-20 dB to background and sound velocity 1490 m/s. The "arteries" were arranged in random order with the anterior aspect of each "artery" being approximately 15 mm from the surface of the phantom (Figure 2.2.1). Magnetic resonance imaging was used to confirm the diameters of "arteries".

PHANTOM STUDY



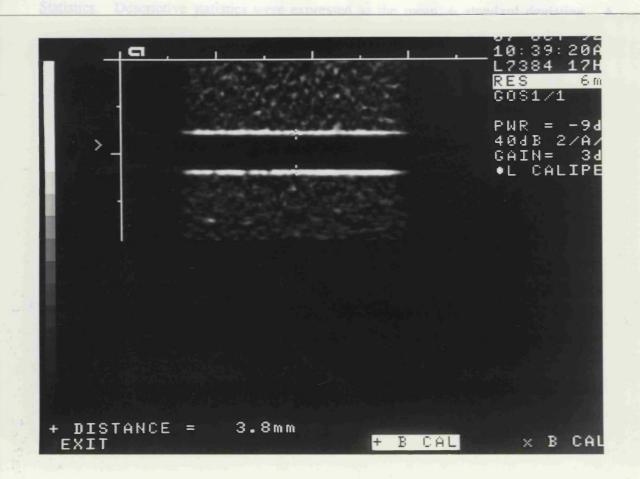
- "Arteries" 2.5 5.0mm diameter
- 7 pairs 0.2mm different,
 4 pairs 0.1mm different
- Ultrasound settings as for in vivo studies
- 2 operators, 6 sets of scans, 4 observers, average of 3 observations for each scan

Figure 2.2.1
Schematic diagram of the "phantom", containing 10 "arteries" of slightly different diameters.

<u>Ultrasound scanning and measurements</u>. Images were obtained using a commercially available Acuson 128XP/10 system and L7384 7.0 MHz linear array transducer (Acuson, Mountain View, California, U.S.A.). The machine operating parameters were identical to those used for the in-vivo studies of brachial artery anatomy and function: transmit power of -9dB, log compression 40dB, preprocessing curve 2 ("crisp borders"), persistence A, postprocessing curve 5 ("high contrast") and overall gain 3dB. These parameters were chosen to maximise the contrast at the lumen/vessel wall interface. The transmit (focus) zone was placed at the level of the phantom "artery", which was always scanned in longitudinal section. System gain settings were also set to optimise images of the lumen/wall interface, and images were magnified using a resolution box function (leading to a television line width of approximately 0.065 mm). Machine operating parameters were not altered throughout the experiment.

Two experienced sonographers scanned each of the 10 phantom "arteries" in longitudinal section, on 3 separate occasions each. All scans were recorded on super-VHS videotape. Vessel diameter was then measured by 4 independent observers, who were unaware of the operator identity and the "artery" size. "Arteries" were presented to each observer in a random order. For each scan of each phantom "artery", 3 measurements were taken and averaged, and the result rounded to the nearest 0.1 mm.

All measurements were made directly from the tape by the built-in software and calipers accurate to 0.1 mm. Measurements were taken from the trailing edge of the near wall interface to the leading edge of the far wall interface, near the center of the "artery" (Figure 2.2.2). This is a similar measurement technique to that used for in-vivo assessment of arterial physiology, where the leading edge of the anterior wall adventitial zone is unclear and often irregular. This was expected to introduce a systematic underestimation of the true "artery" diameter by at least the thickness of the anterior interface between the 2 agar layers.



differences was 0.04 me. Differences between the overall mean error in estimation of diameter

Figure 2.2.2

A phantom "artery" with calipers positioned for measurement. The diameter measurement is made from the trailing edge of the near wall to the leading edge of the far wall.

measuring "artery" diameters were within operator, between operators, within observer

<u>Statistics</u>. Descriptive statistics were expressed as the mean ± standard deviation. A nested analysis of variance estimated components of variation attributable to between-"arteries", between-observers, between-operators, within-observers, within operators, and residual interaction. The 3 measurements made by each observer on each scan were averaged and rounded to the nearest 0.1 mm to give 240 observations. Recorded differences between "arteries" known to be 0.1 mm and 0.2 mm were tabulated. The average bias between observed and true diameter was calculated.

2.2.2 RESULTS

Measurements of absolute diameters. For all 10 "arteries", the absolute diameter measurement was consistently 0.6 mm below the true "arterial" diameter. The anterior wall "thickness" using the settings described measured 0.4 mm and when we measured from from leading edge to leading edge the difference between the measured and the true diameter was 0.2 mm (Figure 2.2.3). By reducing the gain settings to -18 dB an additional reduction in the discrepancy between true and measured "artery" diameter to 0.1 mm was obtained. Short axis measurement of phantom "arteries" (that is, transverse sections) did not reduce the error further.

Measurements of diameter differences. The overall mean error in estimation of diameter differences was 0.04 mm. Differences between pairs of "arteries" whose diameters were 0.1 mm apart were correctly estimated in 50 of 96 (52%) cases. No measurement in vessel diameter difference of 0.1 mm was more than 0.1 mm in error. Differences between pairs of "arteries" whose diameters were 0.2 mm apart were correctly estimated in 112 of 168 (67%) cases. No measurement in vessel diameter difference of 0.2 mm was more than 0.1 mm in error (Figure 2.2.4).

<u>Interoperator and interobserver variability</u>. The possible sources of variability in measuring "artery" diameters were within operator, between operators, within observer, between observers and between "arteries". Of the total variance, only 1% was random

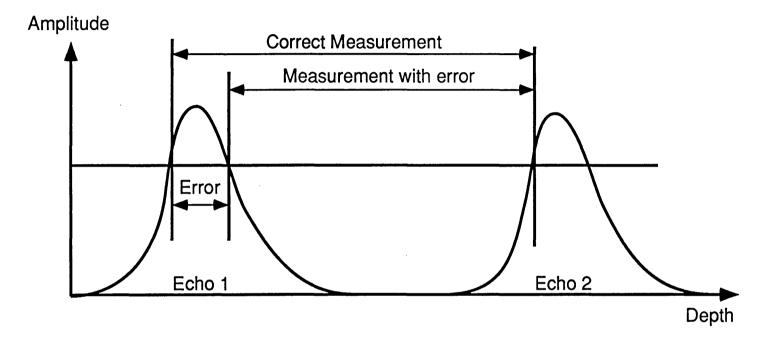


Figure 2.2.3
This measurement technique (Figure 2.2.2) introduces a systematic underestimation of artery diameter by at least the thickness of the near wall, as shown.

measurement error, the difference between the individual "arteries" accounting for 99% (Table 2.2.1).

<u>Table 2.2.1</u>. Sources of variation in measuring phantom "artery" diameter.

Sources of Variation	Variance	Percent of total
Between-observer	0.00005	0
Between-operator	0.00009	0
Within-operator	0.00004	0
Within-observer	0.00157	1
Interaction	0.00047	0
Between "arteries"	0.30673	99

2.2.3 DISCUSSION

Measurements of small changes in vessel diameter are required for calculations of vascular distensibility and cross-sectional compliance, and for non-invasive assessment of endothelial function in systemic arteries. Studies of arterial physiology have been used to examine subjects with risk factors for atherosclerosis, to characterise arterial changes with increasing age and blood pressure, and may be useful to assess the effect of drugs and/or risk factor modification on the progression and reversibility of the early stages of atherosclerosis. Many authors have cited an interobserver variability for diameter measurement as 2-3% (Salonen & Salonen 1991, Wendelhag et al 1992), and used these data to support the precision of these measurements. However interobserver variability is not a measure of precision. Recent work has also addressed the issue of axial resolution of vascular ultrasound systems, and shown a practical resolution of 0.3-0.4 mm (Pignoli et al 1986), because commercially available ultrasound machines most often use cycles

with 3 pulses. However in our experiment the issue is not so much axial resolution as the precise measurement of distance between 2 lines that are greater than one or 2 wavelengths apart. Beam-plot and phantom experiments performed by the manufacturer under ideal conditions have demonstrated that changes in axial distance from the transducer of 0.1 mm can be visualised and made accurately (Acuson, private communication). Our data show that using commercially available equipment, machine settings appropriate to clinical use and consistent caliper positioning on the near and far wall, changes in diameter measurement of 0.1-0.2 mm can be detected accurately, independent of operator and observer.

Although other investigators have demonstrated that measurements of trailing edge and near wall images may be inaccurate in the measurement of precise arterial diameter, both are neccessary for measurement of internal diameter of an artery. In our experiment this approach introduced a systematic measurement error of 0.6mm, constant for all 10 "arteries". Measuring from leading edge to leading edge eliminated most but not all of this error. By significant but clinically inappropriate reduction in the gain settings to -18 dB, the minimum required to visualise the "arterial walls", a further reduction in error to 0.1 mm was achieved. Although the ultrasound system used theoretically performs best at approximately 25 mm depth, correcting the distance from transducer to phantom "arteries" did not reduce the measurement error further. The final difference between measured and real diameter may be explained by a "slice thickness effect" (Figure 2.2.5). However measurements made from leading edge to leading edge in transverse rather than longitudinal sections did not overcome this minimum error.

Given that changes in arterial diameter of 0.1-0.2 mm may be measured accurately, this is equivalent to a 2.5-5% change in diameter of a 4 mm brachial artery or a 2-4% change in a 5 mm internal carotid artery. This "minimum error" should be considered in all studies of vascular physiology performed using high resolution B-mode ultrasound, although the error should be random rather than systematic.

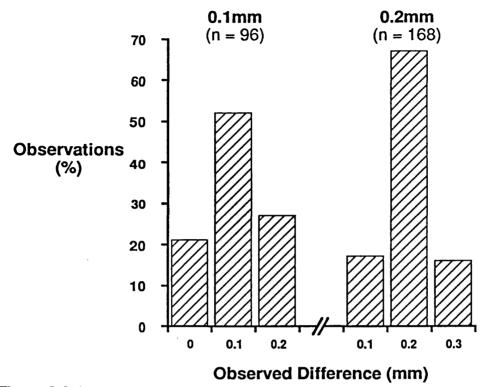


Figure 2.2.4
Diameter differences of 0.1 – 0.2mm can be determined accurately in most cases. No averaged measurement of diameter difference was >0.1mm in error, for any observer.

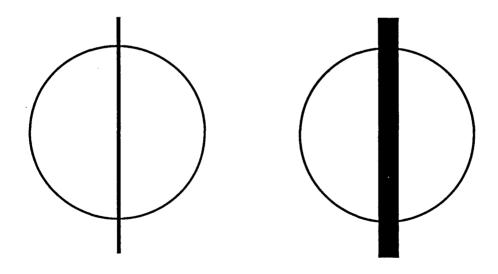


Figure 2.2.5
Ultrasound slices are not infinitely thin. If longitudinal sections are taken, vessel diameter may be underestimated, as images are not just obtained from the centre (or widest point).

The results of phantom experiments are not neccessarily applicable to clinical studies, as the "arteries" are smooth and non-pulsatile. However they demonstrate the best performance obtainable if the in-vivo images are of high quality. In brachial artery studies in over 100 subjects, we found an interobserver variability of approximately 2% (Celermajer et al 1992), only slightly higher than in this phantom study, suggesting that good quality images can be obtained in-vivo on most occasions.

The overall variance in this phantom study was very small, and almost exclusively (99%) due to the differences in "artery" diameter, whereas the intraoperator, interoperator, intraobserver and interobserver variability were all negligible. This is consistent with variability studies measuring arterial wall thickness (Salonen & Salonen 1991).

B-mode ultrasound measurements of diameter changes can therefore be used to study changes in arterial physiology that accompany atherosclerosis early in its development. As external ultrasound is non-invasive, painless, poses no risks and is easily repeatable, it is also ideal for prospective monitoring. This depends not only on technical precision of measurements, but also on the biological variability of the same subject studied on different occasions. The issue of scan reproducibility is addressed in the next section.

2.3 REPRODUCIBILITY STUDIES

INTRODUCTION

One of the important issues in evaluating a new method for investigating arterial physiology is whether similar results are obtained from the same subject, studied on different occasions; that is, the within-subject variability. If this is high, it suggests that either technical factors limit the method's accuracy, or that there is true biological variability in the arterial responses. If the natural variability is low, this validates the method's suitability for comparison of different groups of patients and controls.

Endothelium-dependent arterial responses to a variety of pharmacologic and physiologic stimuli have been studied, usually using invasive catheter-based techniques (Nabel et al 1990, Zeiher et al 1991, Mugge et al 1993). These are not suitable for serial evaluation of arterial physiology, either to permit study of within-patient variability, or for response to risk factor modification or other potentially anti-atherogenic strategies, especially in asymptomatic subjects. The non-invasive method of testing endothelial function in the systemic arteries of children and young adults at risk of atherosclerosis, described in Section 2.1, facilitates serial study. We therefore investigated 40 adults on 4 occasions each, to determine the natural variability in arterial responses over time.

2.3.1 SPECIFIC METHODS

<u>Subjects</u>. Forty adults (20 males, 20 females) aged 22-51 years were recruited from amongst hospital staff. All were asymptomatic, normotensive and non-diabetic. Eight were current smokers throughout the study, 8 were former smokers and 3 had total cholesterol level >6.0 mmol/1. None were taking regular medications. Each patient had 4 studies to assess responses in the right brachial artery, incorporating intervals between visits of 1-2 days, 1-2 weeks, and 2-4 months. The ultrasound operating parameters were similar to those described in section 2.1, and those used for the phantom studies

described above (Figure 2.3.1). The subjects were not necessarily scanned at the same time of each day, and were not instructed to avoid food, drink or cigarette smoking before any study. In each case the operator was unaware of the previous study results in that subject. All subjects gave informed consent.

Data analysis. Arterial diameters were measured directly from super-VHS videotape recordings by two independent observers who were unaware of the scan sequence and the identity of the subject. For every scan, vessel diameters were measured from the anterior to the posterior interface between media and adventitia (the "m-line") at a fixed distance from an anatomical marker, such as a vein or a fascial plane. The mean diameter was calculated from 4 cardiac cycles incident with the R wave on the ECG.

<u>Statistics</u>. Descriptive statistics are expressed as the mean \pm standard deviation. For each study of each patient (total of 160 studies), flow-mediated dilatation (FMD) was calculated for each of the 2 observers, as the arterial diameter during the hyperaemia scan divided by the average diameter of the 2 resting scans. Diameter changes were expressed as the percentage change relative to the average baseline scan (100%). For each study, FMD was calculated as the average of the values obtained by the 2 observers. Interobserver variation was summarised by calculating the mean and standard deviation (SD) of the absolute difference between observers for FMD at each of the 160 studies. A four-level nested analysis of variance was then carried out to determine the relative magnitude of the different sources of variation: between patients, between days (within weeks), between weeks (within months) and between months (within patients). Coefficients of variation were obtained from the between scan variation divided by the overall mean (taking into account the variations between days, between weeks and between months). As a further indication of within patient variability, the mean or "true" FMD was calculated, and subtracted from each of the 4 values obtained from the 4 visits for each subject.

2.3.2 RESULTS

Subjects. All 160 ultrasound studies were of sufficient quality to be analysed (Figure 2.3.1). The interobserver variability for measurement of FMD was $1.2\pm0.4\%$, and was $\leq 2\%$ in 146 of the 160 (91%) studies. Interobserver variability was $1.8\pm0.4\%$ for measurements of GTN-induced dilatation.

Sources of variation. The sources of variation in FMD (between subjects, within subjects between months, between weeks and between days) are shown in Table 2.3.1. The largest sources of variation were between patients and between days within patients. No significant additional variability was found between studies separated by weeks or up to 4 months.

Within-patient variability. The overall coefficient of variation for FMD for all subjects was 1.8 % (1.6% for women, 1.9% for men, p=0.10). The difference between FMD in each of the 160 studies and the mean or "true" FMD for each of the 40 subjects is shown in Figure 2.3.2. In 34 of the 40 subjects (85%), all measurements of FMD were within 2.5 % of the mean FMD for that subject (Figure 2.3.2). Only 5 % of measurements in any subjects were more than 4 % from the mean for that individual. The 2 most "consistent" subjects had FMD measured as 10, 10, 10 and 11 % and 4, 4, 4 and 4 %. The 2 most variable subjects had FMD measured as 9, 10, 11 and 0 % and 1, 1, 7 and 8 %; both of these were men aged 30-40 years.

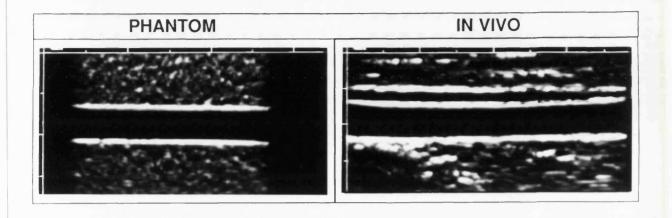


Figure 2.3.1

Ultrasound scans of a phantom and an in-vivo artery (from a young adult male) taken using the same machine operating parameters.

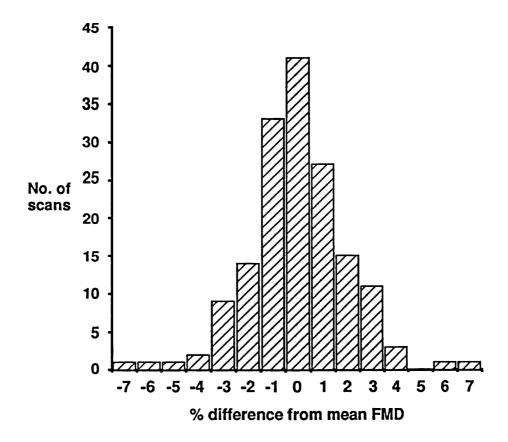


Figure 2.3.2
Plot of the difference of each study from the mean flow-mediated dilatation (FMD) for each subject, showing that the observed FMD was within 4% of the mean FMD in 95% of subjects.

<u>Table 2.3.1.</u> Sources of variation in measuring flow-mediated dilatation in the brachial artery of 40 adults, studied on 4 occasions each.

Sources of Variation	Variance	Percent of total
Between-patients	11.1	72
Between-months	1.3	8
Between-weeks	0.1	1
Between-days	2.8	19

2.3.3 DISCUSSION

In Section 2.2, we found that high resolution ultrasound allowed accurate measurement of small differences in vessel diameter, in a phantom experiment. Phantom studies demonstrate the "best case" for measurement accuracy, as the "arteries" are smooth and non-pulsatile. The current study shows that in the clinical setting, where measurement of arterial diameter may be more difficult because of minor irregularities of the vessel wall/ lumen interface, this difficulty can be overcome by careful selection of the arterial segment to be scanned and measured. None of the 160 scans acquired as part of this investigation were unsuitable for analysis. In this experiment, we found a very low coefficient of variation for in-vivo measurement of FMD. We studied 40 young adult subjects with smooth arteries, and selected ultrasound parameters to maximise the contrast between lumen and vessel wall ("crisp borders"). The experiment was carried out under highly controlled conditions, by an experienced vascular technician and experienced ultrasound observers. Each measurement of arterial diameter represented the average of 8 values (4 measurements by each of 2 observers). These factors might act to reduce variability significantly. Therefore under optimum conditions designed to minimise measurement error, we have found that within patient variability of FMD is small, over a period of up to 4 months.

These findings are consistent with pilot data we acquired from 21 different subjects, who were studied 2 or 3 times; the coefficient of variation was 2.3 % (Celermajer et al 1992). Interestingly, data are lacking to demonstrate whether endothelial responses to pharmacological stimuli (such as acetylcholine) are similarly reproducible, when measured by quantitative angiography, or whether endothelial function tests in subjects with established atherosclerosis will give consistent results on different occasions.

In only 2 of our subjects was there large variation over the 4 scans (7-10 % between the lowest and highest values for FMD); this could either have been be due to biological variability or to technical factors. The results in the remaining subjects were highly reproducible, despite the fact that no attempt was made to standardise such potentially important biological influences as time of day or relationship to meals. It is noteworthy that variability in females was no greater than in males, suggesting that the oestrus cycle has little influence on this measure of endothelial function.

Variability within patients was mainly from day to day, with little additional variation introduced by longer intervals between studies. This suggests that the gap between studies before and after interventions is not critical as long as the day to day variability is taken into account (up to a period of months) (see Section 5.2).

Endothelial function in systemic arteries can therefore be studied non-invasively in man, making it possible to detect early abnormalities accurately and reproducibly. The low natural variation in endothelium-dependent dilatation suggests that it is feasible to test whether this early manifestation of arterial damage can be detected reliably, both in "normal" subjects and those with known risk factors for vascular disease.

CHAPTER 3 - STUDIES ON NORMAL SUBJECTS

3.1 THE IMPORTANCE OF VESSEL SIZE

Using the non-invasive method outlined in Chapter 2, we initially performed a "pilot study" of 50 subjects, in order to assess the feasibility, applicability, tolerability, safety and value of this technique.

3.1.1 SPECIFIC METHODS

These 50 subjects, recruited from hospital staff and their families, were aged 8-57 years. All were life-long non-smokers, normotensive, non-diabetic, had no family history of premature vascular disease and had plasma total cholesterol ≤5.5mmol/l (range 3.0-5.5mmol/l). There were 16 children (8-16 years) and 34 adults. The arteries studied were the superficial femoral (in all the children and 14 of the adults) and the brachial artery (in 20 adults). The superficial femoral vessel had been chosen because of its accessibility to ultrasound and because it is a frequent site of atherosclerotic disease. When flow-mediated dilatation was not observed in some adult femoral vessels, the brachial artery was studied in order to explore the relationship between FMD and vessel size. Examples of a brachial study from a normal subject are shown in Figures 3.1.1, 3.1.2.

The mean vessel diameter and percent dilatation for each subject were obtained by taking the average of measurements made by the 2 observers. The relation between percent flow-mediated dilatation and vessel diameter was then assessed by linear regression, for vessel size alone and then with adjustment for age, cholesterol and type of artery. The procedure was repeated for percent GTN-induced dilatation. Descriptive statistics are expressed as mean \pm standard deviation. Statistical significance was taken as p<0.05.

CONTROL

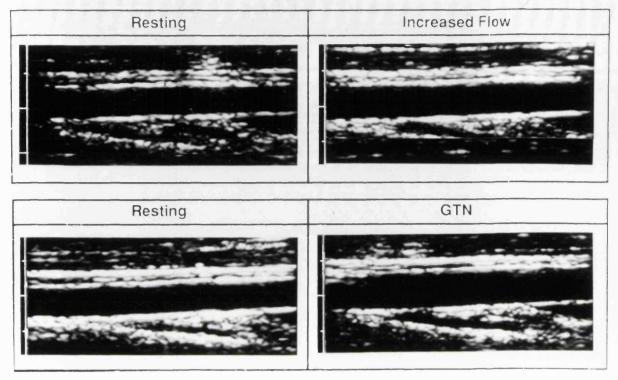


Figure 3.1.1

An example of 4 scans from the brachial artery of a control subject, showing both flow-mediated and GTN-induced dilatation. The artery has the same diameter in both of the resting scans.

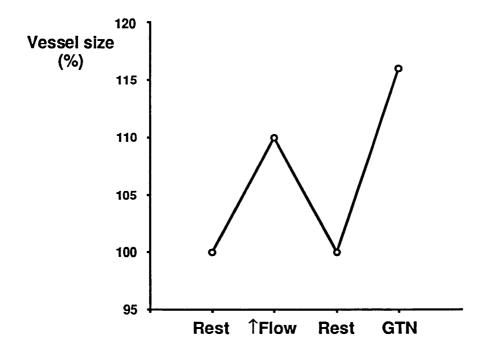


Figure 3.1.2
Graphic representation of figure 3.1.1, with percentage flow-mediated dilatation expressed relative to the resting vessel size.

3.1.2 RESULTS

Of the 50 studies, performed by an experienced ultrasound technician, 49 were considered suitable for analysis; the hyperaemia scan from one adult whose brachial artery was studied was uninterpretable. Three of the 50 subjects declined sublingual GTN.

In 49 control subjects reactive hyperaemia was $440\pm132\%$ in the superficial femoral artery (n=30) and $628\pm192\%$ in the brachial artery (n=19). The arteries dilated 1-18% in response to this increase in flow (Figures 3.1.1, 3.1.2). Dilatation was correlated inversely with resting vessel diameter (r=-0.81, p<0.001; Figure 3.1.3). On multivariate analysis, flow mediated dilatation (FMD) was still closely correlated with vessel diameter (p<0.0001), but not with subject age, cholesterol concentration or target artery. Vessels of 6.0 mm or less dilated $10\pm2\%$ (range 2-18%). Vessel dilatation to GTN was also inversely correlated with resting vessel diameter (r=-0.80, p<0.0001), and findings on multivariate analysis were similar to those for flow-mediated dilatation.

3.1.3 DISCUSSION

This pilot study confirmed that the technique is feasible, widely applicable to children and adults and well tolerated. The relationship between vessel dilatation in response to increase in flow or GTN and resting vessel size, which had not been described previously, allowed us to define an optimal range of vessel size for future studies.

For arteries of diameter <6.0 mm, FMD is about 10% in control subjects; however for arteries ≥ 6.0 mm, FMD is small, even in healthy people. Given that our hypothesis is that FMD might be abnormal in subjects at high risk of atherosclerosis, the method is obviously best applied to the study of smaller arteries in adults (such as the brachial or internal carotid) and larger arteries in children (such as the superficial femoral). A lower limit of suitable resting arterial diameter of 2.0 mm was chosen, as below this vessel size

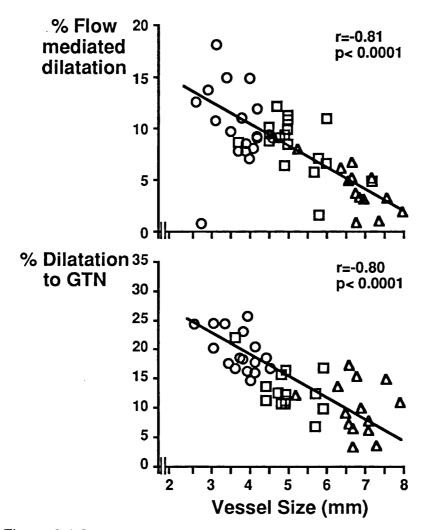


Figure 3.1.3 The relation between resting arterial diameter and flow-mediated and GTN-induced dilatation in the systemic arteries of control subjects.

∆= superficial femoral artery in adults.

□= superficial femoral artery in children.

O= brachial artery in adults.

the effect of a measurement error of as little as 0.1mm could lead to an error of $\geq 5\%$ in the estimation of percent dilatation. Therefore an optimal range of artery size of 2.0-6.0 mm was set for future experiments. Interestingly, invasive studies on adult coronary arteries have demonstrated that FMD is 10-12% for vessels of 3-4 mm diameter in control subjects (Nabel et al 1990), which is consistent with our observations (Figure 3.1.1).

Arteries respond to increased flow by dilating, and the resulting increase in cross-sectional area allows more blood to be conducted to the metabolising tissues. Larger arteries clearly need a smaller increase in diameter to achieve the same increase in cross-sectional area, compared to smaller vessels (cross-sectional area is related to the square of the diameter). This may be the explanation for our findings. Alternate explanations may relate to the geometry of radially-arranged smooth muscle and its relaxation, in small compared to large arteries, or that shear stress for a given increase in flow is much greater in a small compared to a large artery (inversely proportional to the fourth power of the radius), and therefore small arteries may have a greater dilator response.

Whatever the mechanism, the importance of vessel size in determining response to dilator stimuli has 2 major implications for planning experimental studies; the definition of an optimal range of vessel size, and the importance of ensuring that vessel diameter is matched in control and high risk groups of subjects.

3.2 NORMAL CHILDREN; FEMORAL STUDIES

In order to study the earliest physiological manifestations of atherogenesis, we aimed to study children in the first decade of life and up to the early teenage years, who might be at high risk of atherosclerosis. Two conditions that may present in childhood and are associated with premature atherosclerosis are familial hypercholesterolaemia (section 4.2) and homozygous homocystinuria (section 4.4). An age and sex-matched control group of children was required to allow comparison of vascular responses between healthy and high-risk subjects. We therefore studied the superficial femoral arteries of a group of children with no known vascular risk factors, to define a normal range of values for FMD and GTN-induced dilatation of the superficial femoral artery.

Thirty-two healthy children were recruited from amongst the families and friends of hospital staff, including 16 subjects reported in section 3.1. There were 16 boys and 16 girls, aged 5-15 (11.2 ± 2.4) years. All were non-smokers, normotensive, non-diabetic and had no family history of premature vascular disease. Total plasma cholesterol was measured in all; mean 4.2 ± 0.7 mmol/l, range 3.0-5.3.

These children all underwent a femoral arterial study, as described above. Their vessel size was 4.9 ± 0.6 (range 3.6-6.1) mm, baseline flow was 285 ± 95 (105-520) mls/min and hyperaemia values were 395 ± 105 (235-660)%. FMD was 7.0 ± 3.7 (-2-+12)% and GTN induced dilatation was 11.3 ± 3.7 (3-22)% (Figures 3.2.1, 3.2.2). On multivariate analysis, both FMD and GTN-induced dilatation were inversely related to vessel size (p ≤ 0.02 for both), but not with subject age, sex or cholesterol.

These data provide a normal range for arterial physiological responses in the femoral vessels of healthy children.

Impaired FMD (<3%) was found in 5 subjects. Technical factors may have accounted for the low FMD values in some of these subjects; if such studies were regarded as

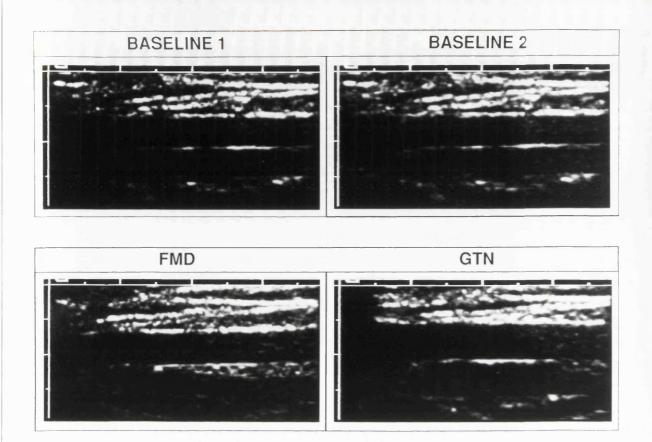


Figure 3.2.1
Scans from the superficial femoral artery of a control child, showing flow-mediated and GTN-induced arterial dilatation.

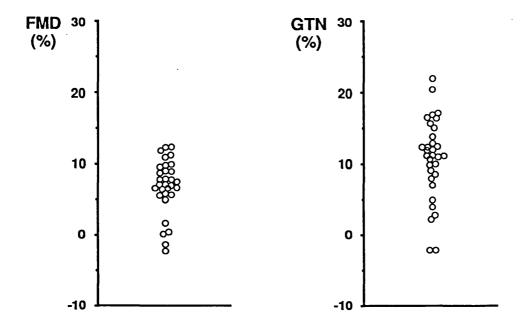


Figure 3.2.2 Flow-mediated (FMD) and GTN-induced dilatation in 32 control children.

important prognostic indicators for individual patients, it would clearly be important to repeat the scans on these patients. For practical reasons, rigorous reproducibility studies on femoral responses in children, such as those reported for adult brachial studies in section 2.3, have not been undertaken. Alternatively, given that the atherogenic process begins in childhood (Stary 1989) and that occlusive plaques may be found in even normocholesterolaemic older teenagers (Enos et al 1953), these children may already have abnormal arteries. Long term follow-up would be required to investigate this possibility.

3.3 THE POOLE COHORT OF NORMAL TEENAGERS

INTRODUCTION

In order to interpret the results of arterial studies in teenagers prone to atherosclerosis, we required a large group of "normal" adolescents to provide control data. We had previously established an optimal range of vessel size for study (2.0-6.0mm), and investigated a group of healthy younger children (aged 6-15 years), to define the normal range of flow-dependent and GTN-induced responses in the superficial femoral arteries. We had also found that most older teenagers (≥ 15 years) had superficial femoral artery diameter > 6.0mm, but brachial artery sizes within the optimal range. We therefore sought a group of older children without vascular risk factors to study brachial artery responses.

In 1991, Sporik et al reported the cholesterol values in a cohort of normal children from Poole in Dorset, initially recruited in 1976-7 (at birth) for a prospective study on atopy. No attention had been paid to the presence of familial coronary risk factors in this selection, which had been made antenatally at the maternity outpatient clinic of Poole General Hospital. The cohort comprised 31 boys and 37 girls, all but one of whom were white. No attempt was made to modify the children's diet during the study. The social class of the families determined by the paternal employment when the child was aged 5 was: I-13%, II-36%, III-16%, IV-27%, V-6% and 2% unclassified. This cohort represented the remaining children available for study at age 11 from an original cohort of 95, and had been seen annually for the first five years of life and at the age of 11 when all except one girl were still prepubertal.

In 1992, with the collaboration of Dr J J Cogswell, we contacted and studied 48 of these teenagers, now aged 15-16 years. The aims were to provide control data for brachial artery responses of teenagers without vascular risk factors, and to determine whether their vascular reactivity correlated with age, sex, vessel size, total cholesterol or lipid

subfraction levels.

3.3.1 SPECIFIC METHODS

Brachial artery studies were performed in the standard way, in a temperature-controlled room, at Poole General Hospital. Measurement of plasma lipids and lipoproteins in the 48 children was performed on EDTA plasma after a 14 hour overnight fast. Plasma triglyceride was measured using the GPO-PAP high performance enzymatic colourimetric test (Boehringer-Mannheim GmbH, Diagnostica). High density lipoprotein cholesterol was measured after precipitation of apoprotein B containing lipoproteins and LDL-cholesterol was calculated according to the Friedwald formula (1972). Lipoprotein (a) (Lp(a)) was measured using an ELISA method (Immuno GmbH).

Results are described as mean \pm standard deviation. The relationship between flow-mediated dilatation and age, sex, vessel size, total cholesterol and lipid subfractions was explored by univariate and multivariate regression analysis. These analyses were confined to the 45 non-smokers.

3.3.2 RESULTS

There were 19 boys and 29 girls, all aged 15-19 years at the time of study. Three were regular smokers (2-10 cigarettes/day for 1-4 years, 2 girls and 1 boy). Two had a family history of coronary disease in a first-degree relative aged <55 years, and 2 others had a father being treated for hypercholesterolaemia (neither had xanthomata). Their total cholesterol level was 4.4 ± 0.8 (3.0-6.6) mmol/l (Figure 3.3.1). Triglycerides were 0.6 ± 0.3 mmol/l, HDL 1.3 ± 0.3 mmol/l, LDL 2.8 ± 0.8 mmol/l and LP(a) 18.9 ± 20.8 (1-75) mg/dl.

Flow mediated dilatation was observed in all subjects (2-15, $10\pm2.8\%$), as was GTN-induced dilatation (9-35, $22\pm6.7\%$) (Figure 3.3.2). On univariate analysis, FMD was

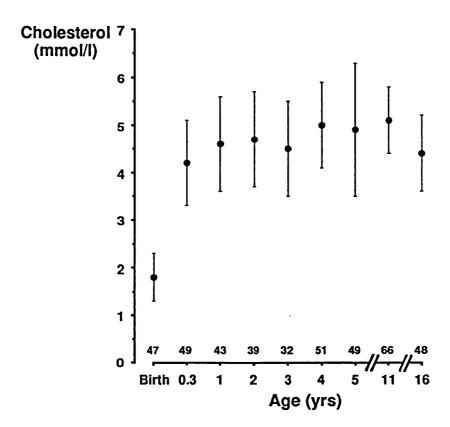


Figure 3.3.1 Serial total cholesterol levels in the Poole cohort, from birth to 16 years (mean, standard deviation).

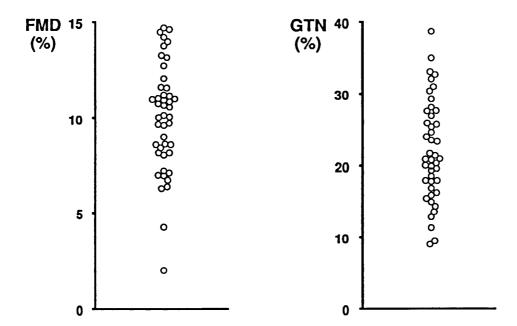


Figure 3.3.2 Flow-mediated (FMD) and GTN-induced dilatation in the 45 non-smoking teenagers from Poole.

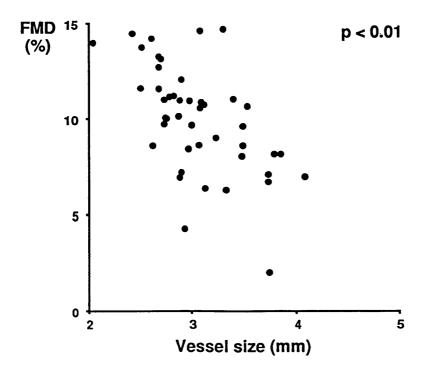


Figure 3.3.3
The relationship between flow-mediated dilatation (FMD) and vessel size in the Poole cohort.

inversely correlated with vessel size (r=-0.58, p<0.001), but not correlated with total cholesterol or any lipid subfraction (Figure 3.3.4). Results of multivariate analysis are in Table 3.3.1. GTN was also inversely correlated with vessel size (r=-0.62, p<0.001), but not with any other variable, on uni- or multivariate analysis.

Table 3.3.1. Multivariate regression analysis for determinants of FMD in 45 non-smoking teenagers from Poole

<u>Variable</u>	Partial regression coefficient	p-value
Sex	+0.24	0.20
Vessel size	-0.38	0.03
Cholesterol	+0.24	0.24
Triglycerides	-0.10	0.59
Lp(a)	-0.15	0.42

3.3.3 DISCUSSION

The serial cholesterol levels from these teenagers show similar "tracking" of values to studies from the United States (Lauer et al 1975) and Australia (Godfrey et al 1972). Cholesterol values rise towards puberty, fall thereafter and slowly climb to pubertal values in late adolescence (Tamir et al 1981). In this cohort, the average cholesterol values are slightly higher than the normal values published in American series, although this group contains a preponderance of girls and an excess of higher social classes (both of these factors are associated with increased cholesterol levels). Nevertheless 23% had total cholesterol ≥5.2mmol/l, the current "upper limit of normal" in the British Hyperlipidaemia Association guidelines (Cobbe & Shepherd 1993).

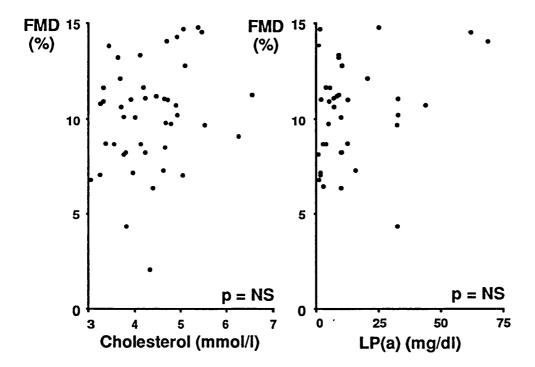


Figure 3.3.4
The relationship between FMD and total cholesterol (left panel) and Lp(a) (right panel).

Lp(a) levels were available in 44 children, and showed the expected "skewed" distribution of values, rather than a normal distribution. This has been described in adult subjects (Jenner et al 1993). Lp(a) has recently been shown to be an independent risk factor for coronary artery disease (Genest et al 1992, Scanu et al 1992). In this group, 9/44 (20%) had Lp(a) value >30mg/dl; the significance of this in otherwise well children with normal total cholesterol is uncertain.

Both flow-mediated and GTN-induced dilatation were observed in all the children with a normal risk factor profile. There was a wide range of values, most of which could be accounted for by the range of vessel sizes encountered. Two children had FMD < 5% in the absence of recognised risk factors; both had total cholesterol level < 6.0mmol/l, one had an elevated Lp(a) level (33mg/dl) and in the other the Lp(a) value was unavailable. Long-term follow-up will be required to determine if these 2 teenagers will be at increased risk of later atherosclerosis. Overall, FMD was not related to total cholesterol level, Lp(a) or any other lipid subfractions. We did not measure certain other variables that may be related to endothelial function, such as angiotensin-converting enzyme phenotype (Cambien et al 1992). On the basis of pathology studies atherosclerosis in youth (Stary 1989, PDAY 1990), it is likely that many of this "normal" group have early arterial disease; some of the determinants of these early processes may still be unidentified (Farmer & Gotto 1992).

The results of these vascular studies provide control data for two types of "at risk" subject; those of similar age with known risk factors, such as cigarette smoking or diabetes, and adult subjects who also have no identified predisposing factors for atherosclerosis, other than older age. In the next section, we have studied the effects of ageing *per se* on endothelial function in man.

3.4 THE EFFECTS OF AGEING: SEX DIFFERENCES IN THE PATTERN OF AGE-RELATED ENDOTHELIAL DYSFUNCTION IN MEN AND WOMEN PARALLELS THE RISK OF CARDIOVASCULAR DISEASE

INTRODUCTION

Life expectancy is greater for women than men, in large part due to their lower incidence of cardiovascular death in middle age (British Heart Foundation 1992). Male gender remains a cardiovascular risk factor, even after controlling for other influences such as cigarette smoking and hypertension (Castelli 1984, Lipid Research Clinics 1984). Many investigators have suggested that the basis for the protection of young and middle-aged women from atherosclerosis is their relative excess of oestrogenic hormones (Colditz et al 1987, Witteman et al 1989, Haarbo et al 1991). We therefore aimed to assess whether ageing is associated with progressive endothelial dysfunction, whether the pattern of any age-related decline in vascular health is different in men and women, and if any sex difference is consistent with known changes in hormonal status.

Not only is endothelial dysfunction an important early event in experimental models of atherosclerosis (Ross 1986), but it may also contribute to morbidity and mortality by disturbing arterial physiology in adults with established coronary stenoses (Ludmer et al 1986, Nabel et al 1990). Previous studies have shown that increasing age is associated with coronary endothelial dysfunction, but have only examined small numbers of subjects, most of whom had multiple vascular risk factors and were undergoing catheterisation for suspected coronary disease (Vita et al 1990, Yasue et al 1990). Using the non-invasive method, we have examined endothelial function and its relationship to age in a large number of healthy men and women, who did not have these potentially confounding influences. Our observations suggest that there is a difference in the pattern of age-related vascular injury in men and women, with a steep decline in endothelial

function occurring in women after the menopause. This supports the concept of a hormonal influence on vascular disease and risk.

3.4.1 SPECIFIC METHODS

<u>Subjects</u>. We studied 238 subjects aged 15-72 (38 ± 17) years, including 103 men and 135 women. Subjects were recruited from hospital staff, families, friends and community volunteers over a 2 year period, and were only included in this study if they were asymptomatic, lifelong non-smokers and had no family history of premature vascular disease. None were known to have hypertension, diabetes or hypercholesterolaemia. None were taking regular medications. Thirty-six of the women had not had a menstrual period for more than one year; their average age at menopause was 51 ± 1 years. None of these post-menopausal women were taking hormone replacement therapy.

<u>Study design</u>. Total plasma cholesterol and resting supine blood pressure were measured in every subject. All then had a non-invasive ultrasound study of the right brachial artery to test endothelial and smooth muscle function, as described above. Seven of the subjects declined sublingual GTN.

<u>Data analysis</u>. All scans were recorded on super VHS videotape for later analysis. Vessel diameter was measured by 2 observers, unaware of the clinical details and stage of the experiment, as described above. The mean vessel diameter and percent dilatation for each patient were taken as the average of the measurements of the 2 observers.

<u>Statistics</u>. The distributions of FMD, GTN, age, cholesterol, vessel size and hyperaemia were summarised by sample means and standard deviations, while pairwise relationships were expressed by Pearson correlation coefficients. Treating FMD and GTN as response variables and age, cholesterol and vessel size as independent variables, their simultaneous relationship was investigated by calculating partial correlation coefficients

with each of the three independent variables in turn, while adjusting for the other two. Men and women were then analysed separately. For each individual i, FMD and GTN were adjusted for vessel size by substracting $b(s_i - S)$, where b is the appropriate regression coefficient on vessel size, s_i is the individual's vessel size, and S is the mean vessel size. These adjusted FMD and GTN values were then plotted against age.

For GTN, the regression coefficient b arose from a simple linear regression on vessel size. For FMD a multiple regression was carried out with a 'vessel size' term which provided the coefficient b, plus two 'age' terms with coefficients depending on whether the individual's age was above or below a specified changepoint c. max. This 'most likely' changepoint produced the minimum residual standard deviation $\sigma_{c.max}$ over a range of possible changepoints from 30 to 65 years. To summarise the support for each possible changepoint c, the "profile likelihood" $\sigma_{c.max}^2/\sigma_c^2$ was plotted, which has maximum 1 at c = c.max, as derived from a likelihood-ratio test procedure (Hinkley 1991). In order to compare the two-straight line model with a smooth curve, a quadratic function in age was fitted and the residual standard deviation calculated. Men and women were compared with respect to the gradient of adjusted FMD with age, both before and after their estimated changepoint, using a Z-test based on their estimated standard errors. The significance of the difference between changepoints was obtained by comparing the most likely changepoint for both sexes using a standard t-test.

3.4.2 RESULTS

The age distribution of the subjects and their vascular responses to increased flow and to GTN are shown in Figures 3.4.1 and 3.4.2. Scans from all 238 subjects were of sufficient quality for analysis, and no plaques were observed in any subject. Total plasma cholesterol was 4.9 ± 1.0 (range 2.6-7.7)mmol/l. No subjects were hypertensive (blood pressure <160/90 mmHg in all). In response to an increase in flow of $487\pm172\%$ after cuff deflation, there was an increase in brachial artery diameter of 7.9 ± 3.9 (range -1 to 18)%. In response to GTN, arterial diameter increased by

 19.4 ± 5.8 (range 4 to 38)%.

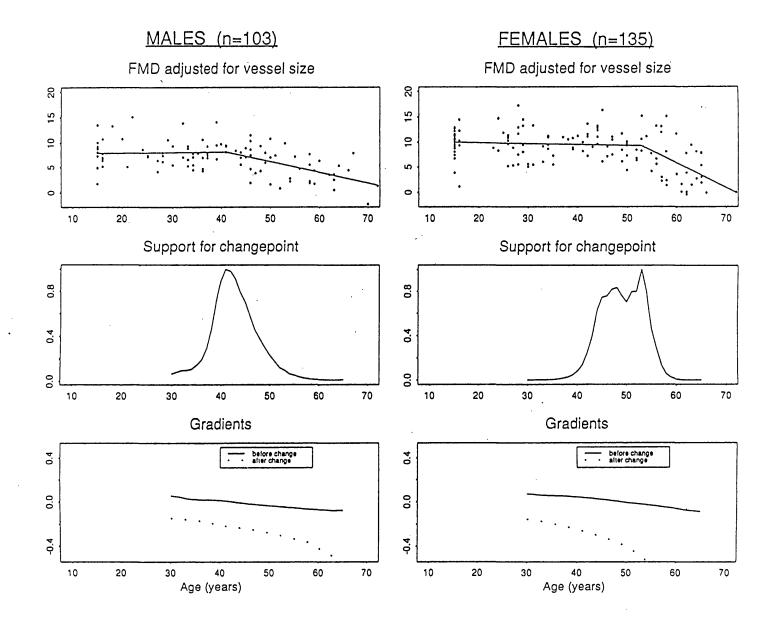
On multivariate analysis of all subjects, flow-mediated dilatation was independently related to age (r=-0.38, p<0.0001) and vessel size (r=-0.38, p<0.0001), but not to the degree of reactive hyperaemia (p=0.21) nor to the cholesterol level (p=0.27). In contrast, while the GTN response was also related to vessel size (r=-0.44, p<0.0001), there was no relationship with age (p=0.18) (Table 3.4.1).

Table 3.4.1 - Determinants of flow-mediated (FMD) and GTN-induced dilatation in 238 subjects with no known risk factors for vascular disease.*

	<u>FMD</u>		GTN		
<u>Variable</u>	Partial regression	p-value	Partial regression	p-value	
	coefficient		coefficient		
Vessel size	-0.38	<0.001	-0.44	<0.001	
Age	-0.36	<0.001	-0.09	0.18	
Cholesterol	0.07	0.27	-0.05	0.40	

^{*}On multivariate analysis

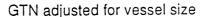
<u>Sex difference</u>. In men, FMD (adjusted for vessel size) did not decline up until the end of the fourth decade. Change-point analysis suggested that an age-related decline occurred subsequently, from age 41 years, after which FMD fell by 0.21% per year. In women, vessel-size adjusted FMD was preserved to age 53 (p=0.04 compared to men), after which it declined significantly faster than in men (0.49% per year, p=0.002). As there was a very narrow range in the age at menopause in this study, separate analysis of years since menopause versus FMD in women yielded a similar slope of decline. In contrast, there was no age-related decline in GTN-induced dilatation in either sex.

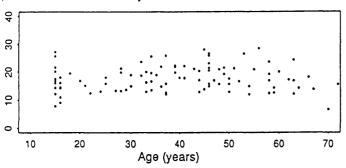


MALES (n=102)

FEMALES (n=129)

GTN adjusted for vessel size





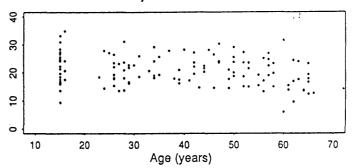


Figure 3.4.1 (Previous page)

Ageing and flow-mediated dilatation (FMD) in men (left) and women (right). The top two panels show the individual data points for percent FMD adjusted for vessel size. The centre panels show the likelihood of a changepoint existing at any given age; this is highest for men at age 41 years and women at 53 years. The lowest panels show the gradients before and after the changepoints; in women, there is a steeper decline after their changepoint.

Figure 3.4.2 (This page)

Ageing and glyceryl trinitrate (GTN) induced dilatation for men (left) and women (right). The response to GTN is preserved with increasing age, in both sexes. Seven of the 238 subjects had declined GTN.

3.4.2 DISCUSSION

This study has shown that ageing is associated with progressive loss of flow-mediated dilatation in the systemic arteries of healthy subjects with no risk factors for vascular disease. As responses to GTN are preserved into old age, this suggests that ageing leads to endothelial dysfunction. The effects of age on arterial physiology, however, are different in men and women. In men, endothelial responses decline gradually after the fourth decade, whereas in women, vascular physiology remains healthy for a further 10 years. Subsequently the rate of decline is faster in women, so that endothelial dysfunction is present in almost all subjects by 65 years of age. This pattern of vascular damage parallels the known difference between the sexes in cardiovascular morbidity and mortality in middle and old age (British Heart Foundation 1992), where men are at much higher risk from age 40-50 years, but women "catch up" in later life.

The process of atherosclerosis does not appear to differ between men and women, and the risk factors known to predispose to vascular disease affect both sexes equally (Farmer & Gotto 1992). We have previously shown that this non-invasive method can detect flow-mediated dilatation in healthy subjects (Chapter 2), and have also found that risk factors such as hypercholesterolaemia and cigarette smoking are associated with abnormal endothelial physiology in both male and female children and young adults (see Chapter 4). Ageing is associated with an increasing prevalence of diabetes, hypertension and hypercholesterolaemia, and in the previous studies of both Vita et al (1990) and Yasue et al (1990), in which coronary endothelial dysfunction was found in most subjects over 30 years of age, risk factors may have had an important confounding effect.

In this study, by excluding all subjects who had ever smoked, or who had known hyperlipidaemia, hypertension, diabetes or a positive family history, we have investigated the effects of ageing *per se* on vasomotor responses, and have compared endothelial function in male and female subjects. Previous studies enrolled too few patients to examine any gender differences in the effects of ageing on endothelial

function. The method we use to study endothelial and smooth muscle function is non-invasive and well tolerated, allowing investigation of a large number of subjects with neither risk factors nor symptoms.

Ageing is associated with a number of anatomical and haemodynamic changes in the vascular system of all subjects, including collagen degeneration, loss of elastin, increased intima-media thickness of the arteries and reduced vascular compliance (Smulyan et al 1983, Rosenthal 1987). Despite these age-related changes, the arterial response to GTN was preserved in our study. This is consistent with other studies that have shown that pharmacological doses of exogenous nitrovasodilators remain effective in older adults (Feldman et al 1981, Parker et al 1986), and that these drugs are still therapeutically effective in geriatric patients (Mickelson et al 1990). In contrast, we observed an age-related decline in endothelial function in both men and women. Previous investigators have shown that the dilator capacity of both large and small coronary vessels in response to acetylcholine, an endothelium-dependent vasodilator, also decreases with increasing age in subjects with chest pain but angiographically smooth coronary arteries (Vita et al 1990, Yasue et al 1990, Egashira et al 1993).

The mechanism whereby ageing is associated with impaired endothelial function is not known. Possibilities include age-related decrease in release of endothelium-derived relaxing factor(s), or their increased catabolism in the vessel wall, and/ or increased release of constricting factors (Egashira et al 1993). In vitro ageing is associated with increased monocyte-endothelial cell adherence, which may be due to age-related increases in peroxidative stress (Molenaar et al 1989). Our data suggest that women may be protected from these age-related changes for a decade longer than men.

This sex difference in the pattern of age-related decline in endothelial function is best explained by the influence of hormonal factors. Oestrogens may protect women from atherogenesis (Bush et al 1987, Haarbo et al 1991). After the menopause, decreased oestrogen levels are associated with a more atherogenic lipid profile (Gambrell & Teran

1991, Jacobs & Loeffler 1992), however the effects of oestrogen on plasma lipids can explain only a small part of the beneficial effects on the risk of vascular disease (Williams 1993). Oestrogens are vasoactive hormones and have direct effects on the arterial wall (Magness & Rosenfeld 1989, Ganger et al 1991). In experimental animals, surgically-induced menopause is associated with a loss of endothelium-dependent coronary vasorelaxation (Williams et al 1990), and in post-menopausal animals, oestrogen replacement may restore normal endothelial function (Haarbo et al 1991). Intravenous oestrogen acutely improves endothelium-dependent relaxation in post-menopausal women (Williams et al 1992). In our study, the age at which endothelial dysfunction was seen in women was around the time of the menopause. These data therefore suggest, but do not prove, that oestrogens may protect against endothelial injury.

Cardiovascular disease is the commonest cause of death in the United Kingdom, and the known sex difference in life expectancy is largely due to the increased risk of coronary and carotid atherosclerotic complications in middle-aged males. Our data suggest that ageing per se, irrespective of exposure to known risk factors, is associated with earlier physiological abnormalities in the systemic arteries of men compared to women. This is consistent with observations that both old age and male gender are independent risk factors for cardiovascular mortality, and that oestrogens may protect women from the age-related decline in vascular health. The association between menopause and the subsequent change in endothelial function that we have observed is provocative, but prospective studies will be required to see whether hormone replacement therapy prevents or retards the age-related changes in arterial physiology in post-menopausal women. All such women are potentially candidates for hormone replacement therapy, not just for well-being and prevention of osteoporosis but also for protection of the arterial wall and reduction of vascular risk. The availability of a non-invasive method to assess vascular health will allow investigation of the appropriate type and timing of such hormonal therapy, both in individuals and in clinical trials.

CHAPTER 4 - STUDIES ON HIGH RISK SUBJECTS

4.1 ADULTS WITH ESTABLISHED ATHEROSCLEROSIS

INTRODUCTION

During the early stages of development of the non-invasive technique, we performed "pilot" studies on 3 types of subject; those with no known risk factors for vascular disease, those who were clinically well but prone to atherosclerosis because of the presence of one or more risk factors, and a small group of subjects with known coronary atherosclerosis.

Other investigators had previously shown that subjects with established coronary plaques had endothelial dysfunction in the coronary circulation (Ludmer et al 1986); we wished to investigate whether similar subjects would have evidence of endothelial dysfunction in other large systemic arteries.

4.1.1 SPECIFIC METHODS

Twenty subjects were recruited from the cardiology outpatient clinics at St Bartholomew's Hospital, London. There were 17 men and 3 women, aged 54-74 (63 ± 5) years. All had had either coronary artery bypass surgery or angioplasty. The brachial artery was studied by the standard protocol in 10 cases; all of these were suitable for analysis. The superficial femoral artery was studied in 10 subjects; only 7 of these were later thought to be of a sufficient quality for analysis. Results were compared to those from the control group of adults described in section 3.1, by the unpaired student's t-test. Subjects and controls were matched for sex and vessel type, but not for age, risk factor profile or vessel size (Tables 4.1.1, 4.1.2).

4.1.2 RESULTS

Plaques were seen in the superficial femoral arteries of 4 subjects, causing 20-50% stenosis; no plaques were seen in the brachial arteries. The coronary disease subjects were significantly older, had higher cholesterol levels and had larger vessels than the controls (Tables 4.1.1, 4.1.2). Flow-mediated dilatation was reduced or absent in all cases, and GTN-induced dilatation was also significantly less than in the control subjects, in both the superficial femoral and the brachial artery studies (Figures 4.1.1, 4.1.2).

<u>Table 4.1.1</u>. Superficial femoral artery studies in adults with atherosclerosis.

	Controls (n=14)	Known CAD (n=7)	p-value
Age (yrs)	27±2 (20-50)	61±2 (54-67)	<0.001
Male (%)	10 (72%)	6 (86%)	0.48
Cholesterol (mmol/1)	4.5±0.1 (3.7-5.5)	6.1±0.4 (4.8-8.1)	0.01
Vessel size (mm)	6.8±0.2 (5.2-7.9)	7.7±0.4 (5.9-8.9)	0.06
FMD (%)	4±1 (0-9)	1±0.3 (-1-+2)	<0.001
GTN (%)	10±1 (4-17)	7±1 (3-10)	0.07
Resting flow (ml/min)	303±10	267±26	0.14
Hyperaemia (%↑flow)	495±16	451±29	0.23

Results are expressed as a mean±SE (range). CAD - coronary artery disease, FMD - flow mediated dilatation, GTN - nitroglycerine induced dilatation.

<u>Table 4.1.2</u>. Brachial artery studies in adults with established atherosclerosis.

	Controls (n=19)	Known CAD (n=10)	p-value
Age (yrs)	38±2 (21-57)	60±2 (53-67)	<0.001
Male (%)	15 (79%)	9 (90%)	0.58
Cholesterol (mmol/1)	4.6±0.1 (3.0-5.5)	5.5±0.2 (4.2-6.2)	0.002
Vessel size (mm)	3.7±0.1 (2.5-4.5)	4.4±0.3 (2.7-6.1)	0.04
FMD (%)	11±1 (7-18)	0±1 (-6-+7)	<0.001
GTN (%)	19±1 (15-31)	11±1 (3-14)	<0.001
Resting flow (ml/min)	91±10	95±9	0.80
Hyperaemia (% ↑ flow)	628±44	418±54	0.01

Results are expressed as mean \pm SE (range). CAD - coronary artery disease, FMD - flow mediated dilatation, GTN - nitroglycerine induced dilatation.

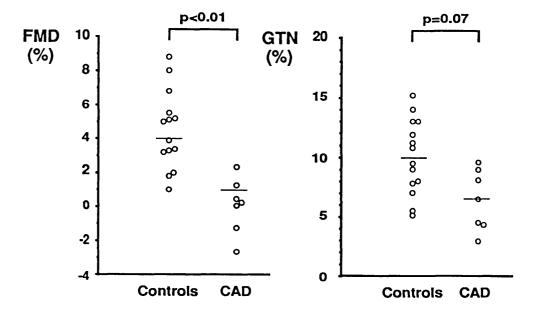


Figure 4.1.1 Flow-mediated dilatation (FMD) in the superficial femoral arteries of control and atherosclerotic (CAD) subjects.

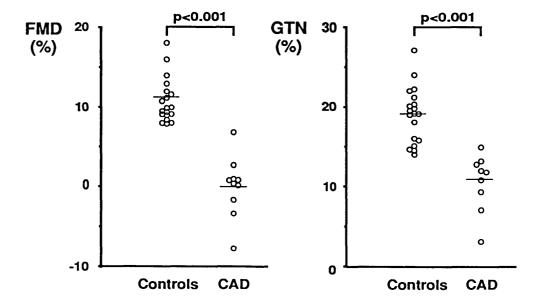


Figure 4.1.2 FMD in the brachial arteries of control and atherosclerotic (CAD) subjects.

4.1.3 DISCUSSION

This study demonstrates that arterial physiology is abnormal in both the brachial and superficial femoral arteries of subjects with established coronary atherosclerosis. Vascular disease is a diffuse process, and often follows a parallel course in the coronary, femoral and carotid arteries. Involvement of the brachial artery is less often clinically manifest, however endothelial dysfunction has been demonstrated in the brachial circulation of asymptomatic subjects with hypercholesterolaemia (Creager et al 1990) and hypertension (Panza et al 1990). Furthermore Vogel et al (1993) have recently shown concordance between the presence of coronary atheroma on angiography and the presence of impaired flow-mediated dilatation of the brachial artery, as measured by a technique similar to that first described by our group (Celermajer et al 1992).

This study was undertaken before the interactions between age (section 3.4), vessel size (section 3.1) and arterial physiology were appreciated. It is possible that the impaired responses we observed were due to atherosclerosis, older age, larger vessel size or a combination of these factors. The impaired response to GTN suggests a vasculopathy that effects endothelial, smooth muscle and/or adventitial physiology. The results are consistent with (but do not prove) endothelial dysfunction being present in the systemic arteries of subjects with clinically symptomatic coronary atherosclerosis. The remainder of this chapter addresses whether arterial physiology is similarly perturbed in younger asymptomatic subjects, prone to atheroma because of the presence of risk factors in early life.

4.2 ENDOTHELIAL DYSFUNCTION IS AN EARLY EVENT IN CHILDREN WITH FAMILIAL HYPERCHOLESTEROLAEMIA, AND IS RELATAED TO THE LP(a) LEVEL

INTRODUCTION

Familial hypercholesterolaemia (FH) is associated with premature atherosclerosis, and is characterised by hypercholesterolaemia, xanthomata, normal triglyceride levels and a similarly affected first degree relative. In FH there is a defect in the low-density lipoprotein (LDL) receptor, leading to high LDL and total cholesterol levels.

Hypercholesterolaemia is an important risk factor for the early structural changes of atherosclerosis (PDAY 1990), and for cardiovascular morbidity and mortality in later life (Kannel 1978). In autopsy studies of young adults, a significant positive correlation between LDL cholesterol and the extent of atherosclerosis in the aorta and coronary circulation has been documented (PDAY 1990). Furthermore, a recent long-term prospective study has shown a strong association between the cholesterol level in young adult life and the later risk of cardiovascular disease (Klag et al 1993). In particular, if levels of LDL cholesterol and Lp(a) are elevated, the risk of coronary disease is markedly increased (Armtrong et al 1986). Lp(a) is a lipoprotein similar in structure to LDL, linked by a disulphide bridge to apoprotein a.

As above, endothelial dysfunction is an important early event in atherogenesis (Ross 1986, Henderson 1991). Endothelial injury has been induced experimentally by hypercholesterolaemia, particularly the LDL component (Galle et al 1990). In clinical studies using invasive techniques, hypercholesterolaemic adults have been shown to have impaired endothelium-dependent responses to a variety of pharmacological stimuli, including infusions of acetylcholine (Drexler et al 1991b).

We have therefore tested whether endothelial dysfunction can be demonstrated at a much earlier stage of the disease process, in the systemic arteries of young children with FH. In addition, we have examined the relationship between disturbed arterial physiology and the major lipid subfractions.

4.2.1 SPECIFIC METHODS

<u>Subjects.</u> Thirty children (17 girls, 13 boys) aged 7-17 years (median 11) with FH were studied. All were asymptomatic, normotensive, non-diabetic and non-smokers. FH was defined as total plasma cholesterol ≥6.8 mmol/l or LDL cholesterol ≥4.5 mmol/l with tendon xanthomas in the patient or in a relative (15). Twenty eight of these children were heterozygotes and 2 were homozygotes. All 30 were prescribed lipid lowering diet. Drug therapy was prescribed at the discretion of each patient's physician, and 12 were receiving lipid lowering drugs (7 cholestyramine, 5 HMG CoA reductase inhibitors) for 1 to 7 years. Thirty healthy children (17 girls, 13 boys, aged 5-15, median 11 years) served as controls. All were normotensive, non-smokers and had no family history of premature vascular disease. Each subject and/or their parents gave informed consent to the study.

<u>Lipid analysis</u>. Total plasma cholesterol was measured in all 60 subjects (FH group and controls) using the cholesterol C-system high performance CHOD-PAP method (Boehringer-Mannheim GmbH, Diagnostica). Measurement of plasma lipids and lipoproteins in the 28 children with heterozygous FH was performed on EDTA plasma after a 14 hour overnight fast, using the methods described in section 3.3.

Study design. Arterial endothelial and smooth muscle function was studied non-invasively, as described in section 2.1. The superficial femoral artery (SFA) was scanned in longitudinal section, and the centre of the vessel was identified when the clearest images of the anterior and posterior walls of the artery were obtained.

<u>Data analysis.</u> Scans were recorded on super-VHS tapes and arterial diameters were measured directly from the tape by two independent observers "blinded" to the scan sequence and the identity of the subject, as described in section 2.1. Vessel diameters were measured from the anterior to the posterior interface between media and adventitia (the "m-line") at a fixed distance from an anatomical marker, usually the flow divider between the superficial and profunda branches of the common femoral artery.

Statistics. Descriptive data are expressed as mean ± standard error. The FH and control groups were compared using two sample t-tests. For the whole group, the relationships between the dependent variables flow-mediated dilatation (FMD) and GTN induced dilatation and sex, vessel size and total cholesterol level were explored by both univariate and multivariate regression analysis. For the 28 children with hetrozygous FH, the relationship between FMD and GTN induced dilatation and age, sex, vessel size, cholesterol, triglycerides, HDL, LDL, apolipoproteins A and B and Lp(a) levels was explored by univariate analysis and multiple regression models. The distribution of Lp(a) levels was normal in this group except for 3 subjects who had very high levels (> 50 mg/dl); therefore the analyses were repeated excluding these 3 children. Statistical significance was inferred at a p-value of <0.05.

RESULTS

Lipid data. Total plasma cholesterol in the control subjects was 4.1 ± 0.3 mmol/l (range 2.8-5.2). Total cholesterol was significantly increased in the FH group (8.0 \pm 0.4 mmol/l, p<0.0001). In the FH children, HDL level was 1.2 ± 0.1 mmol/l (range 0.8-1.6), and LDL level was 5.6 ± 0.3 mmol/l (range 3.8-9.2). Triglyceride levels were normal (1.0 \pm 0.1 mmol/l). Apolipoprotein A was 130 \pm 4 mg/dl (range 96-195). Apolipoprotein B was 132 \pm 6 mg/dl (range 70-215). Lp(a) was 24.8 \pm 3.2 mg/dl (range 7.2-70).

Vascular studies. Vessel size was similar in both groups (4.83 mm in control vs 4.79 mm

in FH subjects, p=0.807). The mean resting blood flow in the SFA was 263 ± 24 ml/min in controls and 232 ± 17 ml/min in the FH group (p=0.876). The mean flow increase observed immediately after cuff deflation was $414 \pm 19\%$ (range 268-662%) (Table 4.2.1).

<u>Table 4.2.1</u>. Vascular study results in children with familial hypercholesterolaemia and controls.

		FH	con	trols	p-value
Age (years)	10.9	(0.46)	10.9	(0.45)	0.92
Vessel size*	4.79	(0.10)	4.83	(0.10)	0.807
Baseline flow (ml/min)*	232	(17)	269	(24)	0.138
Hyperaemia (%)	419	(20)	414	(19)	0.876
FMD (%)	1.2	(0.4)	7.4	(0.6)	<0.0001
GTN (%)	10.0	(0.6)	12.4	(0.8)	0.023
FMD/GTN ratio	0.14	(0.05)	0.55	(0.07)	<0.0001

Results are expressed as mean (SE)

* as measured during the first (resting) scan
M-male, F-female, FMD-flow mediated dilatation, GTN-glyceryl
trinitrate induced dilatation.

In the arteries of the control children, FMD was 7.5 \pm 0.7% and GTN induced dilatation was 12.4 \pm 0.8% (Figures 4.2.1, 4.2.2). FMD was absent (<2%) in 3 of these 30 children. In the FH subjects, FMD was significantly impaired (1.2 \pm 0.4%, range -2.01 to 5.8%, p<0.0001, Table 4.2.1, Figure 4.2.1). In 22 subjects (73%) FMD was absent. GTN induced dilatation was mildly attenuated in FH subjects compared to controls (10.0 \pm 0.6%, range 4.0 - 16.5%, p=0.023) (Figure 4.2.2). The FMD/GTN ratio was also significantly reduced in the FH group (0.14 \pm 0.05 vs 0.62 \pm 0.05, p<0.0001).

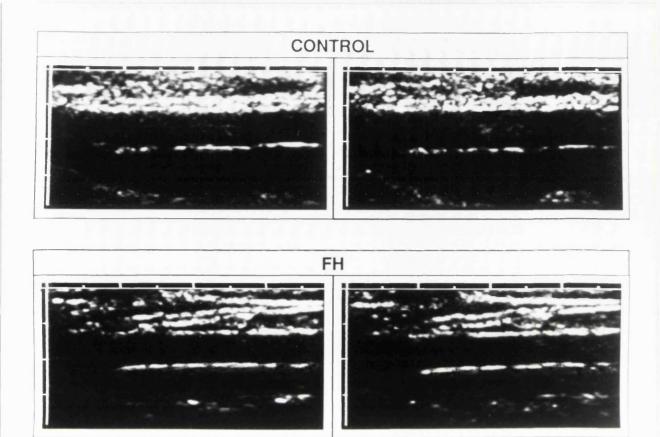


Figure 4.2.1

Scans from the superficial femoral artery in a control child (top panel) showing flow-mediated dilatation, and in a child with FH (lower panel), showing absence of flow-mediated dilatation. Each artery is shown at rest (left) and after reactive hyperaemia (right).

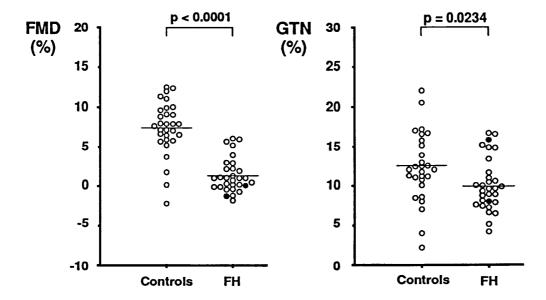


Figure 4.2.2
Flow-mediated (FMD) and GTN-induced dilatation in the controls and children with familial hypercholesterolaemia.
Horizontal lines = group means; • = subjects with homozygous FH.

Twenty-nine of the 30 children with FH had smooth carotid and femoral arteries on ultrasound examination; one of the 2 homozygotes had small bilateral plaques in the internal carotid artery, not causing flow disturbance.

For the whole group (n=60), total cholesterol level was inversely correlated with FMD (r=-0.58, p< 0.0001) (Figure 4.2.3), but not with GTN-induced dilatation. A similar result was obtained on multivariate analysis (total cholesterol related inversely to FMD, r=-0.61, p<0.0001). For the 28 heterozygous children with FH, the only significant correlate of FMD on univariate analysis was Lp(a) (r=-0.51, p<0.01) (Figure 4.2.4). This inverse correlation between FMD and Lp(a) was still observed on multivariate analysis entering age, sex, vessel size, HDL, LDL, triglycerides and Lp(a) (r=-0.61, p=0.027) (Table 4.2.2). Similar results were obtained if total cholesterol was entered into this model instead of HDL and LDL. Furthermore, when these multivariate analyses were repeated excluding the 3 children with very high Lp(a) levels, almost identical results were obtained (Lp(a) inversely related to FMD, r=-0.55, p=0.029).

Table 4.2.2. Multivariate regression analysis for determinants of flow-mediated dilatation in 28 children with heterozygous familial hypercholesterolaemia.

<u>Variable</u>	Partial regression coefficient	p-value
Age	+0.41	0.19
Sex	-0.25	0.34
Vessel size	-0.37	0.18
LDL	-0.11	0.60
HDL	-0.08	0.68
Triglycerides	-0.08	0.74
Lp(a)	-0.61	0.027

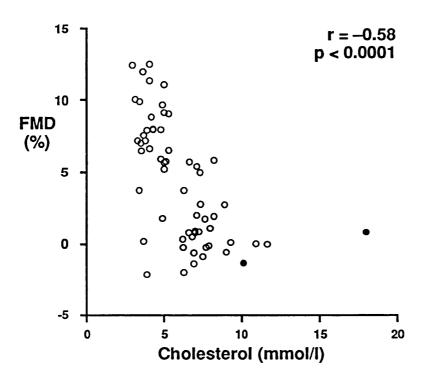


Figure 4.2.3 Relation between total cholesterol level and flow-mediated dilatation (FMD) in the superficial femoral arteries of controls and children with FH (n = 60).

= subjects with homozygous FH.

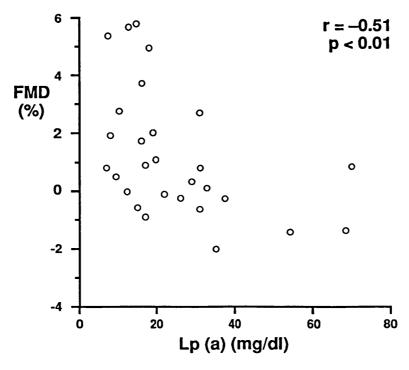


Figure 4.2.4 Relation between Lp(a) level and flow-mediated dilatation (FMD) in the superficial femoral arteries of the children with heterozygous FH (n = 28).

DISCUSSION

Familial hypercholesterolaemia is an autosomal dominant lipoprotein disorder with a prevalence of approximately 1:500 in its heterozygous form (Kwiterovitch 1990). Heterozygotes have a 100-fold increased risk of premature death from coronary heart disease (Simon Broome 1991); in homozygous FH, clinical coronary disease can occur in childhood. The metabolic consequences of the LDL-receptor deficiency are present from birth (Kwiterovitch et al 1973), and our results show that abnormalities of arterial function are already present in children as young as 7 years of age.

In children with FH, FMD, which is an endothelium-dependent phenomenon (Rubanyi et al 1986), was reduced by almost 90% compared with healthy controls. The GTN response, which tests smooth muscle relaxation directly, was only slightly attenuated in the FH group. The ratio of FMD to GTN induced dilatation was significantly reduced in the FH group, consistent with endothelial dysfunction.

A major functional consequence of endothelial dysfunction is the inability to produce and release endothelium-derived relaxing factor (EDRF), a local vasodilator, which also inhibits platelet adherence and aggregation, smooth muscle proliferation and endothelial cell-leukocyte interactions, all of which are important early events in atherogenesis. Recent evidence suggests that administration of L-arginine, the substrate for EDRF production, is not only associated with preserved endothelium-dependent responses, but is also anti-atherogenic (Cooke et al 1992). This implies that endothelial dysfunction is not merely a marker of early atherosclerosis, but may be intimately involved in its pathogenesis.

Hypercholesterolaemia, especially LDL-cholesterol, is known to cause endothelial injury in vitro, with attenuation of the normal endothelium-dependent relaxation occurring long before the formation of atherosclerotic lesions (Cohen et al 1988, Komori et al 1989). Invitro, native LDL-cholesterol appears to impair reversibly endothelium-dependent

dilatation minutes after exposure (Kugiyama et al 1990, Tomita et al 1990), and oxidized LDL-cholesterol causes slowly developing and apparently irreversible inhibition of endothelium-dependent relaxation (Jacobs et al 1990, Simon et al 1990). In experimental animals with diet induced hypercholesterolaemia, arteries show impaired vasodilatation to acetylcholine in the absence of atheromatous lesions (Yamamoto et al 1987). In clinical studies, selective loss of endothelium-dependent vasodilatation to acetylcholine has been documented in hypercholesterolemic adults with angiographically normal coronary arteries (Zeiher et al 1991), and found to be associated with the degree of elevation of serum cholesterol (Vita et al 1990). Our findings show that the damaging interaction between hypercholesterolaemia and the endothelium may begin even earlier, in the first decade of life.

We have investigated which lipid subfractions might be related to early vascular injury in these children. In a relatively small group, HDL and LDL levels were not correlated with impaired endothelial function. However, a significant relation between Lp(a) and FMD was observed, independent of the total cholesterol level. Lp(a) has recently been documented as a risk factor for premature atherosclerosis (Genest et al 1992, Scanu et al 1992). Patients with FH suffering from coronary heart disease have higher Lp(a) levels than FH patients free of coronary artery disease (Seed et al 1990, Wiklund et al 1991). The mechanism by which high levels of Lp(a) are related to atherosclerosis is unclear, but hypotheses include inhibition of plasminogen activity (Eaton et al 1987) and alteration of LDL mediated delivery of cholesterol to the vessel wall (Armstrong et al 1986). Our data represent the first evidence that elevated Lp(a) levels may be related to endothelial injury in-vivo.

Impaired flow mediated dilatation was found in 3 children in the control group. As the process of atherosclerosis begins in childhood and adolescence (Enos et al 1953, Stary 1989), even in normocholesterolaemic subjects, these children may indeed already have abnormal arteries. Serial evaluation during long term follow-up will be required to investigate this possibility.

In contrast, GTN produced vasodilatation in all subjects, by acting directly on smooth muscle. GTN was an effective vasodilator in both controls and FH children, although there was a modest impairment of GTN response in the latter group. A similar trend has been observed previously in small groups of hypercholesterolaemic adults with either angiographically normal coronary arteries (Zeiher et al 1991) or minimal coronary irregularities (Drexler & Zeiher 1991), and also in the superficial femoral artery of patients with mild atherosclerosis (Liao et al 1991). The vasodilator response observed after GTN administration is a function of both direct smooth muscle relaxation, and the response to the hyperaemia caused by GTN-induced dilatation of the resistance vessels (Schnaar & Sparks 1972). Loss of this flow component to the GTN response may explain the mildly impaired response seen in the FH group. However a smooth muscle abnormality in the conduit arteries of these children cannot be excluded. It should be noted, however, that the dilatation to GTN in the FH group was sufficiently great (10±0.6%) so as not to preclude endothelium-dependent dilatation.

In this study we investigated vascular physiology in the superficial femoral artery of children. In section 3.1 we defined the relationship between vessel size and FMD in systemic arteries in man, and found that the diameter of the SFA in children is within the optimal range for resolution and response (17). We have not studied the coronary arteries, as these are not accessible to external ultrasound. Atherosclerosis, however, is a diffuse process, and clinical and autopsy data suggest that the disease often follows a parallel course in the coronary and proximal femoral arteries (Solberg & Strong 1983, Olsson 1991). Other investigators have studied proximal segments of the femoral arteries to monitor the effects of lipid lowering therapy on the regression of atherosclerotic lesions (Blankenhorn 1991). It is likely that impaired endothelium-dependent dilatation in the SFA of our children with FH reflects abnormalities of other sites in the arterial tree, such as the carotid and coronary arteries.

In established disease, cholesterol lowering therapy can attenuate progression of human

coronary and femoral atherosclerosis, and lead to regression of existing plaques (Duffield et al 1986, Lichtlen et al 1992). In early disease, cholesterol lowering therapy might be beneficial if it was associated with an improvement in endothelial function. In a primate model of early atherosclerosis, cholesterol induced endothelial dysfunction has been reversed by reversion to a low-fat diet (Harrison et al 1987), but as yet no reversibility studies in early disease have been undertaken in humans. The reduction in LDL cholesterol produced by a low-fat, low-cholesterol diet is small (Hunninghake et al 1993) and despite the addition of lipid lowering drugs in some of our patients, none had cholesterol or LDL levels as low as those shown to be beneficial in atherosclerosis regression studies in adults. There is legitimate concern about the administration of lifelong therapy with cholesterol lowering agents (Newman et al 1992) and many pediatricians therefore avoid their use (Kimm et al 1992). Our findings suggest that treatment may be especially important in children with FH who also have an elevated Lp(a) level, as this group may have a particularly high risk of atherosclerosis. Whether the early abnormalities in arterial physiology can be reversed with any treatment will require prospective investigation, which is facilitated by the availability of a non-invasive test for the study of endothelial and smooth muscle function.

4.3 CIGARETTE SMOKING IS ASSOCIATED WITH DOSE-RELATED IMPAIRMENT OF ENDOTHELIUM-DEPENDENT DILATATION IN YOUNG ADULTS

INTRODUCTION

Cigarette smoking is a major risk factor for atherosclerosis, and is strongly associated with coronary, cerebral and peripheral vascular disease (US Surgeon General 1982, 1983, Holbrook et al 1984). The public health impact of smoking is enormous. As documented in the 1989 Surgeon General's Report, an estimated 390,000 people in the USA die each year from diseases caused by smoking. This toll includes 115,000 deaths from heart disease; 106,000 from lung cancer; 31,600 from other cancers; 57,000 from chronic obstructive pulmonary disease; 27,500 from stroke; and 52,900 from other conditions related to smoking. More than one of every six deaths in the United States are caused by smoking, mainly related to atherosclerosis. For more than a decade the Public Health Service has identified cigarette smoking as the most important preventable cause of death in our society.

The exact components of cigarette smoke responsible for the association with occlusive vascular disease are not known, although nicotine and carbon monoxide have been implicated, nor is the mechanism of smoking related arterial damage. Experimental evidence has suggested that smoking-induced endothelial damage may mediate increased cardiovascular morbidity (Davis et al 1985), although Vita et al (1990) failed to find an association between smoking and coronary endothelial dysfunction in a study of 12 adult smokers undergoing angiography. Because endothelial dysfunction is an early event in atherogenesis (Henderson 1991), we hypothesised that endothelial dysfunction might be present in the systemic arteries of young adult smokers, and that this might be a dose-dependent phenomenon.

4.3.1 SPECIFIC METHODS

Subjects. One hundred and sixty subjects aged 15-57 years were studied; all were normotensive, non-diabetic, had no family history of premature vascular disease and had plasma total cholesterol ≤6.0mmol/l (range 3.7-6.0 mg/dl). All were clinically well and on no regular cardiovascular medications. Subjects were recruited from amongst hospital staff, families, friends and other volunteers over a 1 year period. There were 80 life-long non-smokers aged 16-56 years (mean 35 years) (controls) and 80 current smokers aged 15-55 years (mean 33 years), with 42 males and 38 females in each of these groups. Current smokers were defined as any who had smoked at least 20 cigarettes per day for 1 year (1 pack year) or the equivalent. Smokers were classified into 4 groups; very light (16 subjects, 0-4 pack years), light (17 subjects, 5-9 pack years), moderate (22 subjects, 10-19 pack years) or heavy (25 subjects, ≥20 pack years).

<u>Salivary cotinine estimations</u>. Salivary cotinine levels were measured by a rapid gasliquid chromatographic method, using a Hewlett Packard model HP5890A gas chromatograph equipped with a nitrogen detector, as described elsewhere (Feyerabend & Russell 1990).

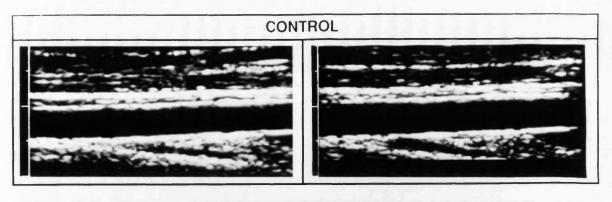
Statistics. Descriptive statistics are expressed as mean \pm standard deviation. The control and smoking groups were compared using two sample t-tests. In the group of current smokers, the relationship between pack years smoked and FMD was assessed by one way of analysis of variance for the subgroups of very light, light, moderate and heavy smokers. The relationship between flow-mediated or GTN induced dilatation and other variables was assessed by univariate and multivariate linear regression analysis, with pack years smoked treated as a continuous variable. Statistical significance was inferred at a p-value ≤ 0.05 .

4.3.2 RESULTS

Control Subjects. In 80 control subjects, baseline flow was 114 ± 50 mls/min (range 32-248), and reactive hyperaemia was $484\pm172\%$ (range 260-900%). The arteries dilated $10\pm3.3\%$ (range 4-22%) in response to this increase in flow. On multivariate analysis, flow-mediated dilatation (FMD) was correlated inversely with resting vessel diameter (p=0.015), but not with subject age or cholesterol level. Four control subjects declined sublingual GTN; in the remaining 76, vessel dilatation to GTN was $20\pm5.2\%$ (range 11-34%). GTN-induced dilatation was also inversely correlated with vessel diameter (p=0.02), with similar findings to those for FMD on multivariate analysis. The ratio of FMD to GTN induced dilatation was 0.52 ± 0.20 .

Smokers. Age, resting vessel size, cholesterol level, baseline flow and degree of reactive hyperaemia were similar in the smokers and the control subjects (Table 4.3.1). FMD was reduced or absent in most of the smokers $(4.0\pm3.9\%, \text{ range } 0 \text{ to } 17\%, \text{p}<0.0001 \text{ compared to controls})$ (Figures 4.3.1, 4.3.2). FMD was $3.0\pm2.8\%$ in the male and $5.3\pm5.9\%$ in the female smokers. Dilatation to GTN was also lower in the smoking group $(17.0\pm5.8\%, \text{ range } 7\text{-}34\%, \text{p}=0.002 \text{ compared to controls})$. The ratio of FMD to GTN induced dilatation was 0.24 ± 0.19 (p<0.0001 compared to controls). No subject had ultrasound evidence of narrowing or plaque in the vessel studied.

Subjects with a wide range of cigarette consumption were studied, with a (self-reported) pack year history of 1-75 pack years (mean 16 ± 1.8 , median 11, interquartile range 6-22; mean 19 ± 12.0 for males and 13 ± 8.8 for females, p=0.10). Salivary cotinine levels measured on the day of study were 5-700 ng/ml (mean 275, median 257, interquartile range 124-387). FMD was $6.6\pm4.0\%$ (range 1-17%) in very light smokers, $4.8\pm3.1\%$ (range 0-16%) in light smokers, $3.2\pm3.2\%$ (range 0 to 16%) in moderate and $2.6\pm1.2\%$ in heavy smokers (range 0 to 8%) (p<0.01 compared to controls for all groups) (Figure 4.3.3). One way analysis of variance showed that FMD was significantly lower in heavier smokers (p=0.006). Similar results were obtained when the male and female smokers were analysed separately. On multivariate analysis of age,



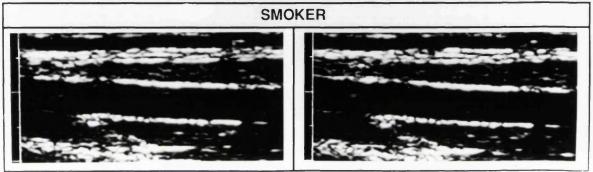


Figure 4.3.1

Scans from the brachial artery of a control adult male (top panels) and from an adult male smoker (lower panels), showing the artery at rest (left) and after reactive hyperaemia (right). Flow mediated dilatation is present in the control subject (artery dilates from 4.1 to 4.6mm) but absent in the smoker (artery measures 4.6mm in both scans). The scale markers to the left of each frame are 5mm apart.

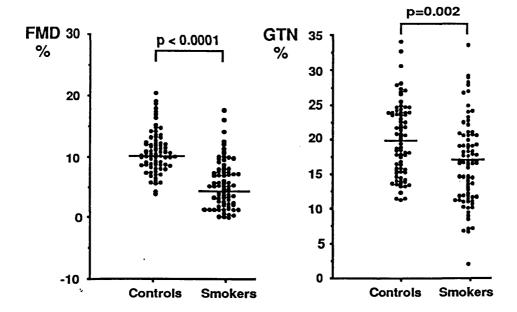


Figure 4.3.2
(a) Flow-mediated dilatation (FMD) and (b) Glyceryl trinitrate (GTN) induced dilatation in smokers and control subjects. FMD, GTN induced dilatation and the ratio of flow mediated to GTN induced dilatation are all significantly lower in the smokers.

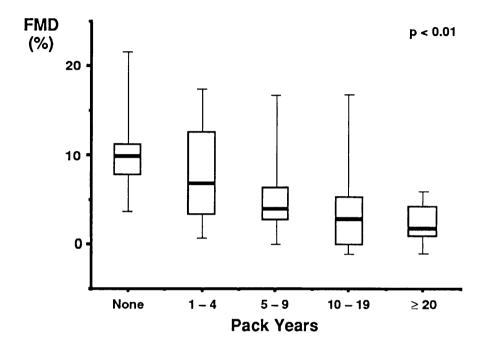


Figure 4.3.3
Relation between amount smoked (in pack years) and flow mediated dilatation (FMD), in control subjects and current smokers. For each value of pack years, the box represents the interquartile range with the mean bar horizontally within each box. The bars outside each box show the entire range of the values.

sex, vessel size, cholesterol, salivary cotinine and number of pack years smoked, only vessel size (p=0.01) and total dose smoked (pack years) (p=0.05) were related to impairment of FMD (Table 4.3.2).

GTN induced dilatation was $18.4\pm6.4\%$ (range 7-28%) in very light smokers, $17\pm5.2\%$ (range 9-29%) in light smokers, $17.8\pm5.7\%$ (range 7-34%) in moderate and $15.5\pm5.6\%$ (range 7-29%) in heavy smokers; analysis of variance showed no significant association between GTN induced dilatation and total smoking dose as measured in pack years. On multivariate analysis of age, sex, vessel size, cholesterol, cotinine level and total smoking dose, there was no significant association between GTN induced dilatation and either smoking dose or cotinine level.

<u>Table 4.3.1</u>. Vascular study results in adult smokers and controls.

	<u>Smokers</u>	<u>Controls</u>	<u>p value</u>
	(n=80)	(n=80)	
Age (years)	33.6±9.0	35.4±9.0	0.23
M:F	42:38	42:38	-
Cholesterol (mmol/l)	4.6±0.7	4.5±0.7	0.32
Vessel size (mm)*	3.70±0.7	3.55±0.6	0.12
FMD (%)	4.0±3.9	10.0±3.3	<0.0001
GTN (%)	17.0±5.8	20.1±5.2	0.002
FMD/GTN ratio	0.24±0.19	0.52±0.20	<0.0001
Baseline Flow (ml/min)*	131±74	114±50	0.10
Hyperaemia (%)	471±181	484±172	0.65

Results are expressed as mean ± standard deviation.

F - female, FMD - flow mediated dilatation, GTN - glyceryl trinitrate induced dilatation, M - male.

^{*}As measured during the first (resting) scan.

Table 4.3.2. Multivariate regression analysis for determinants of flow-mediated dilatation in 80 current smokers.

<u>Variable</u>	<u>Partial</u> <u>regression</u> <u>coefficient</u>	<u>p-value</u>
Age	0.11	0.46
Sex	0.05	0.74
Vessel size	-0.36	0.01
Cholesterol	-0.04	0.75
Cotinine	-0.02	0.94
Pack years	-0.33	0.05

4.3.3 DISCUSSION

Endothelium dependent vasodilatation is impaired in apparently healthy young adult smokers. Our data suggest that endothelial dysfunction may occur in the systemic arteries of even very light smokers, from adolescence onwards, although the likelihood of vascular physiologic abnormalities increases with increasing total amount smoked.

In this study, endothelium dependent (flow-mediated) dilatation was significantly impaired in the group of smokers as a whole, compared to controls, as was the ratio of flow-mediated to GTN induced dilatation. These data are consistent with an association between smoking and endothelial dysfunction. Other studies have demonstrated a direct toxic effect of tobacco smoke on human endothelium. Smoking two tobacco cigarettes approximately doubles the number of nuclear-damaged endothelial cells in circulating blood (Davis et al 1985, Davis et al 1986), whereas non-tobacco cigarettes have little effect. In addition pronounced degenerative changes of umbilical artery endothelium have been found at delivery in smoking but not non-smoking mothers (Asmussen &

Kjeldsen 1975). The mechanism of smoking-associated endothelial damage is not established, but may be related to the effect of smoking on increasing platelet/vessel wall interactions (Pittilo et al 1984, Rival et al 1987) and/or the inverse relation between cigarette consumption and plasma levels of high-density lipoprotein cholesterol (Gordon & Doyle 1986, Freedman et al 1987).

Endothelium independent vasodilatation to GTN was also mildly impaired in smoking adults, suggesting that either less GTN is being delivered to the smooth muscle or that GTN is causing a decreased vasorelaxant effect. There may therefore be a functional or structural abnormality in the arterial smooth muscle and/or the adventitia in these subjects. This is supported by previous studies showing that GTN induced dilatation is less in atherosclerotic compared to control arteries (Ludmer et al 1986, Nabel et al 1990), and is even impaired in hypercholesterolemic patients with angiographically smooth arteries compared to controls (Zeiher et al 1991). GTN induced dilatation is inversely related to vessel size (Feldman et al 1981, Celermajer et al 1992), and so the impaired response to GTN in the smokers in our study may be partially due to the slightly larger arterial diameter observed in the group. It should be noted, however, that smooth muscle relaxation to GTN in all groups was sufficiently great to allow endothelium-dependent dilatation to take place in response to increased flow.

The dose-dependence of smoking related endothelial dysfunction supports a causative role for smoking in atherogenesis. We found a stronger association between impaired FMD and pack years smoked (reflecting chronic exposure), than an association with cotinine levels (reflecting acute exposure); therefore it is unlikely that the effect of smoking on endothelial function is an acute phenomenon. We have observed a threshold for smoking dose and endothelial dysfunction, in that all subjects smoking ≥20 pack years had reduced or absent FMD. In a pathological study of 390 young males (15 to 34 years of age), the prevalence of aortic atherosclerosis was strongly related to serum thiocyanate concentration, a marker of recent smoking (PDAY 1990). Besides endothelial damage, other mechanisms have been suggested as explanations for the

association of smoking and vascular disease, including increased platelet adherence (Pittilo et al 1984) and aggregation (Davis et al 1985), increased fibrinogen (Meade et al 1986b) and decreased plasminogen levels (Wilhelmsen et al 1984), arterial spasm (Sugiishi & Takatsu 1993) and reduced capacity of the blood to deliver oxygen (Galea & Davidson 1985). These studies and our own observations confirm that smoking is an important causative agent for vascular disease, and that the earliest structural and functional changes of atherosclerosis may be demonstrated in adolescent and young adult smokers. Although the overall number of smokers in society is decreasing, the high prevalence of regular smoking in young adults shows no sign of significant decline in the last decade (Royal College of Physicians 1992). Our findings suggest that even light smoking at an early age may damage vascular endothelium.

Follow up for many years would be required to show that young adult smokers with impairment of endothelium-dependent dilatation will indeed go on to develop atherosclerosis. However endothelial dysfunction is an early event leading to atherosclerosis, preceding occlusive vascular disease in both the experimental primate model (Harrison et al 1987) and in human heart transplant recipients (Fish et al 1988). Impaired endothelial function in early life results in abnormal reactions between the vessel wall and platelets and leucocytes, thereby promoting the atherogenic process (Ross et al 1986).

The availability of a non-invasive method to perform serial studies of endothelial and smooth muscle function in asymptomatic young adults will allow prospective investigation of the reversibility of abnormal vascular physiology with smoking cessation, and such studies are being undertaken (see Chapter 5).

4.4 ENDOTHELIAL DYSFUNCTION OCCURS IN THE SYSTEMIC ARTERIES OF CHILDREN WITH HOMOZYGOUS HOMOCYSTINURIA BUT NOT THEIR HETEROZYGOUS PARENTS

INTRODUCTION

Classical homocystinuria is an autosomal recessive disorder caused by a deficiency of cystathionine beta-synthase. Homozygotes commonly have ocular, skeletal and neurobehavioural complications of the disease, and are at high risk of premature atherosclerosis and thrombosis (Figure 4.4.1) (Mudd et al 1985, Mudd et al 1989). Homocyst(e)ine is a highly reactive amino acid and is generally thought to initiate premature atherosclerosis by damaging endothelial cells (Harker et al 1976, Wall et al 1980); endothelial dysfunction may then result in abnormal reactions between the vessel wall and platelets, neutrophils and macrophages, and thereby contribute to the atherogenic and thrombogenic processes (Ross 1986). The age at which endothelial dysfunction is demonstrable in the systemic arteries of children homozygous for homocystinuria is not known.

Recent data have suggested that milder hyperhomocystinaemia, usually associated with the heterozygous state, may be an independent risk factor for atherosclerosis (Boers et al 1985, Kang et al 1986, Clarke et al 1991). Although up to 30 percent of patients with premature vascular disease may have hyperhomocystinaemia (Brattstrom et al 1984, Malinow et al 1989), the incidence of myocardial infarction and stroke in a population of subjects with heterozygous homocystinuria has been shown to be similar to that in controls (Mudd et al 1981). The question of whether heterozygotes for homocystinuria are at increased risk of atherosclerosis is important because the prevalence of heterozygosity is approximately 1-2 per cent of the population (Clarke et al 1991). The risk for such patients is not yet known, and furthermore the mechanism whereby vascular damage may occur is not established.

The non-invasive method to study endothelial function in human systemic arteries may be applied to document endothelial dysfunction in children and adults at increased risk of atherosclerosis. We aimed to investigate at what age abnormalities in vascular physiology might be detectable in homozygous children, and whether endothelial dysfunction was demonstrable in their heterozygous parents.

4.4.1 SPECIFIC METHODS

<u>Subjects</u>. Nine children with homozygous homocystinuria due to cystathionine beta-synthase deficiency were studied, including 2 sets of siblings. The 14 parents of these children (obligate heterozygotes) were also investigated. Each subject was matched with 2 controls of similar age, sex, smoking history, cholesterol level, vessel size and vessel studied; controls were chosen from the database of over 300 subjects investigated using this technique.

<u>Blood chemistries</u>. Plasma and urine amino acids (including free homocystine) were measured by standard liquid column chromatography. Total plasma homocystine was measured by high performance liquid chromatography, by a modification of the method of Ueland et al (1984).

<u>Statistics</u>. Descriptive statistics are expressed as mean \pm standard error. The control groups of children and adults were compared to the children with homozygous homocystinuria and their parents respectively, using two sample t-tests. The relationship between variables was assessed by linear regression analysis. Statistical significance was inferred at a p-value < 0.05.

4.4.2 RESULTS

Homocystinuria children. Age at diagnosis was 4 months - 7.5 years (mean 4 years), and age at time of study was 4-17 years (mean 11 years) (Table 1). Of the 9 children, 7 (78%) had lens dislocation, and 4 (44%) were at a special school because of mental retardation. Eight were taking a low protein diet, 6 were taking pyridoxine, 7 were prescribed betaine and all were on folic acid supplements. At the time of study, total plasma homocystine was raised in all patients (38-104 μ mol/1, mean 63; normal range 7-19), and urinary homocystine was 2-62 μ mol/mmol creatinine (mean 11, normally undetectable). Total plasma cholesterol was 4.1±0.4 mmol/1 (range 2.7-6.3).

No children had symptoms or signs of vascular disease, nor any ultrasound evidence of arterial narrowing or plaque formation in the vessel studied. Flow-mediated dilatation was $2.8\pm0.7\%$ (range 1-7%); degree of flow mediated dilatation was not related to total plasma homocystine (r=0.04), cholesterol levels (r=0.08) or age at diagnosis (r=-0.11). Dilatation after nitroglycerine was $13.1\pm1.2\%$ (range 6-16%). The youngest patient, who was diagnosed at 4 months of age because of an older sibling with the disease, and who had had good metabolic control with no ocular or neurobehavioural complications, had flow mediated dilatation of 5% in the femoral artery. This was the best response for a femoral vessel in the homocystinuria group, but still below the range of values found in the control children (see below).

Control children. In the 18 control children, flow-mediated dilatation was $9\pm0.6\%$ (range 6-12%, p<0.0001 compared to homocystinuria children, see Table 4.4.1, Figure 4.4.2). Dilatation after nitroglycerine was $15.7\pm1.6\%$ (range 10-24%, p=0.27). Baseline flow and degree of reactive hyperaemia were similar between the homocystinuric and control children. Therefore flow-mediated dilatation was impaired in the homocystinuria children compared to controls, whereas nitroglycerine response was similar; this is consistent with endothelial dysfunction in the children with homocystinuria.



Figure 4.4.1

The inferior thyroid artery of a 7 year old child with homozygous homocystinuria, showing abnormal intimal thickening and a plaque.

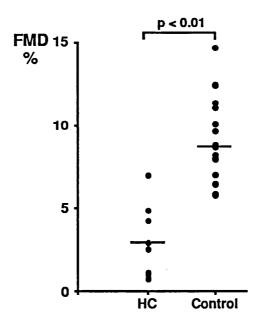


Figure 4.4.2 Flow mediated dilatation (FMD) in children with homocystinuria (HC) and in controls. Horizontal lines indicate group means.

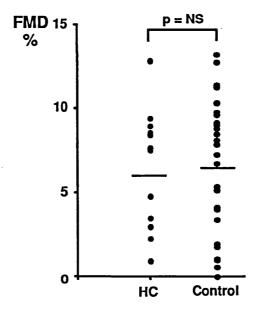


Figure 4.4.3 Flow mediated dilatation (FMD) in adults heterozygous for homocystinuria (HC) and controls. Horizontal lines indicate group means.

Obligate heterozygotes, homocystinuria. Fourteen parents from 7 families investigated had vascular studies performed, all of whom were clinically well. Their age ranged from 34-49 years (mean 41 years). Free plasma homocystine was undetectable and plasma methionine levels were normal in all subjects, all of whom were taking a normal diet. No subject had hypertension or a family history of premature vascular disease, and cholesterol levels were 4.4-7.2 mmol/l (mean 5.6). Four out of 14 (29%) were current smokers and 2 were reformed smokers. Flow mediated dilatation was $6.3\pm0.9\%$ (range 1-13%) and dilatation following nitroglycerine was $17\pm1.4\%$ (range 9-27%).

Control adults. Each obligate heterozygote was matched with 2 control adults of similar age, sex, cholesterol level, smoking history and vessel size (Table 4.4.2). In the controls, flow mediated dilatation was $6.8\pm0.7\%$ (range 0-13%, p=0.68) and nitroglycerine induced dilatation was $20.7\pm1.7\%$ (range 7-34%, p=0.17) (Figure 4.4.3). Resting flow values and degree of reactive hyperaemia were similar in the 2 adult groups.

<u>Table 4.4.1</u>. Arterial studies in homocystinuric and control children.

-	HC children (n=9)	Controls (n=18)	p-value
Age (years)	10.9±1.3	12.1±0.7	0.36
Males (%)	3 (33)	6(33)	1.0
Cholesterol (mmol/l)	4.1±0.4	4.2±0.2	0.74
Vessel size (mm)	4.0±0.3	4.2±0.2	0.45
FMD (%)	2.8±0.7	9.0±0.6	<0.0001
GTN (%)	13.1±1.2	15.7±1.6	0.27
<pre>Flow at rest (ml/min)</pre>	219±42	204±34	0.78
% Hyperaemia	349±25	391±49	0.55

FMD - flow mediated dilatation

GTN - nitroglycerine induced dilatation

HC - homozygous homocystinuria

<u>Table 4.4.2</u>. Arterial studies in obligate heterozygotes for homocystinuria and their adult controls.

]	HC parents (n=14)	Controls (n=28)	<u>p-value</u>
Age (years)	41.2±1.3	40.4±1.0	0.66
Males (%)	7 (50%)	14 (50%)	1.0
Cholesterol (mmol/1)	5.6±0.3	4.9±0.2	0.03
Vessel size (mm)	3.9±0.2	3.7±0.1	0.32
FMD (%)	6.3±0.9	6.8±0.7	0.68
GTN (%)	17±1.4	20.7±1.7	0.17
<pre>Flow at rest (ml/min)</pre>	165±28	123±11	0.10
% Hyperaemia	425±56	459±36	0.60

FMD - flow mediated dilatation

GTN - nitroglycerine induced dilatation

HC - heterozygous homocystinuria

4.4.3 DISCUSSION

Endothelial function is impaired in children with homocystinuria as young as 4 years of age, before clinical or anatomical evidence of atherosclerosis. In contrast, the endothelium- dependent responses of an adult group of obligate heterozygotes is similar to control subjects, suggesting that mild impairments of homocystine metabolism may not cause important endothelial damage.

Children with homozygous homocystinuria are at risk of severe premature vascular disease (Mudd et al 1989). Marked hyperhomocystinaemia is found in homozygotes, and is directly toxic to endothelium (Wall et al 1980), induces smooth muscle proliferation (Harker et al 1976), potentiates the auto-oxidation of low density lipoprotein cholesterol

(Parthasarathy 1987, Heinecke et al 1987) and promotes thrombosis (Graeber et al 1982, McCully et al 1987). The mechanism of homocyst(e)ine-induced injury is not established, but may be due to abnormal generation of H_2O_2 by way of a sulphydryl group, or due to direct toxicity of an oxidised product of homocyst(e)ine, homocyst(e)ine thiolactone (Stamler at al 1993). This study is the first to examine vascular physiology in homocystinuria, and demonstrates that children homozygous for this disease also have endothelial damage in early life, despite appropriate medical management from an early age.

When populations with premature peripheral, cerebral and coronary artery disease have been studied, about one-third have been shown to have mild hyperhomocystinaemia after a methionine load, and most of these subjects were heterozygous for cystathionine synthase deficiency (Boers et al 1985, Clarke et al 1991). This has led some authors to propose that heterozygotes are at increased risk for premature atherosclerosis, and cite hyperhomocystinaemia as an independent risk factor (Sardharwalla et al 1974, Wilcken et al 1983). Few studies, however, have examined a population of heterozygotes to assess the frequency of premature vascular disease. A survey of over 1000 parents and grandparents of children with severe cystathionine synthase deficiency failed to demonstrate a significant increase in the incidence of premature vascular disease (Mudd et al 1981). No more than 1 of 20 such heterozygotes was likely to have an adverse vascular event by the age of 50. These results are consistent with our study of obligate heterozygotes with low plasma homocystine levels (without methionine loading), in whom systemic arterial endothelial and smooth muscle physiology were similar to control subjects, despite slightly higher cholesterol levels in the heterozygote group. Therefore any increased atherosclerosis risk in heterozygotes may not be mediated through abnormal homocystinaemia, and may be due to a mechanism other than endothelial damage, such as a prothrombotic effect, or an increased susceptibility to other atherogenic factors.

Children with homozygous homocystinuria have impaired endothelial function in their systemic arteries, despite dietary and pharmacologic therapy. More aggressive homocystine lowering regimes or newer agents may be required to retard the progression to atherosclerosis. In contrast, endothelial function was preserved in the adults we studied with heterozygous homocystinuria. Neither attempts to screen the general adult population for mild hyperhomocystinaemia nor the treatment of such heterozygotes with pyridoxine, folic acid, betaine or low methionine diets seem justified on the basis of these data.

4.5 ARTERIAL REACTIVITY IS MARKEDLY IMPAIRED IN NORMOTENSIVE YOUNG ADULTS FOLLOWING SUCCESSFUL REPAIR OF AORTIC COARCTATION IN CHILDHOOD

INTRODUCTION

Despite successful repair of coarctation of the aorta (CoA), life expectancy is not normal, with the mean age of death being reported as 38 years in 2 long term follow-up studies (Presbitero et al 1987, Cohen et al 1989). The increased mortality is due to cardiovascular complications related to hypertension, the risk increasing with later age at repair (Bergdahl et al 1983, Clarkson et al 1983). Even in normotensive subjects without residual coarctation, 20% have a hypertensive response to exercise (Simsolo et al 1988). The reasons for these late vascular complications are unclear; upper limb hypertension that exists before CoA repair may cause long-term baroreceptor dysfunction, or structural and functional abnormalities in the vessel wall of large and small arteries. This may be similar to the changes associated with essential hypertension, such as intimal and medial hypertrophy of large and small arteries (Folkow 1987) and/or impaired endothelial function in forearm resistance vessels (Panza et al 1990).

We have therefore used the non-invasive method to examine vascular physiology in normotensive young adults following satisfactory end to end repair of CoA, to determine whether abnormal endothelial or smooth muscle function is present in the right brachial circulation (pre-coarctation site); whether normal lower limb reactivity is preserved; whether age at repair influences arterial physiology, and whether blood pressure response to exercise correlates with age at repair or with abnormal arterial function.

4.5.1 SPECIFIC METHODS

<u>Subjects</u>. Between 1971 and 1985, 114 subjects had isolated coarctation of the aorta repaired at The Hospital for Sick Children, London. We identified 77 who fulfilled the

following criteria for inclusion; age 14 - 30 years; isolated coarctation of the aorta (except for associated bicuspid aortic valve with no stenosis); repair by resection with end to end anastomosis at least 7 years previously; no history of recoarctation, and patients who were normotensive and on no medication at their last visit. Those with Turner's syndrome were excluded. Seventy-seven patients fulfilled the inclusion criteria; 13 < 1 yr; 42 between the ages of 1 and 8, and 22 older than 8 years. Of the 13 repaired during infancy, 8 who could be traced all agreed to participate. Nine of those repaired after 8 years of age responded and 8 were studied; one was working abroad. Half of the 42 suitable subjects in the middle group were approached. They were selected because of geographical proximity to London. Ten were untraceable, 2 wished to postpone the study for several months and therefore 9 were studied. In total, 50% (28/56) of suitable patients responded. Each CoA subject was matched for age, sex and smoking history with 2 healthy normotensive subjects for the brachial studies and 1 matched control for the femoral studies. Each subject gave informed consent.

<u>Protocol</u>. Resting blood pressure was measured in the right arm by a mercury sphygmomanometer after the subject had been lying supine for ≥ 15 minutes. A mean of 3 readings was taken. A blood sample was taken for total plasma cholesterol.

The adequacy of arch repair was assessed by cross sectional echocardiography with colour flow Doppler. Doppler estimation of maximum flow velocity in the descending aorta and the presence or absence of a "diastolic tail" were noted (Carvalho et al 1990). Left ventricular wall thickness and systolic function were studied using m-mode. All subjects then underwent an exercise test using a modified Bruce protocol (2 minute stages) until stage 5, or until 90% of predicted heart rate was achieved. Right arm blood pressure was measured at rest, at maximal exertion, and again after 8 minutes' recovery.

After at least 30 minutes rest, arterial endothelial and smooth muscle function was studied using high resolution vascular ultrasound. The right brachial artery was studied in all 25 subjects. Changes in arterial diameter in response to reactive hyperaemia (increased flow producing endothelium - dependent vasodilatation), and to nitroglycerine (GTN, an endothelium - independent vasodilator) were measured, as described in section 2.1. Twelve subjects subsequently returned for femoral artery study; in these cases, scans were recorded at rest and after GTN only (we have previously shown that flow-mediated dilatation is small even in normal adult femoral arteries, because of their larger resting diameter) (section 3.1).

Statistical Analysis. Descriptive data are expressed as mean \pm standard deviation. Subjects and controls were compared using two-sample t-tests. Univariate and multivariate linear regression analyses were performed on the 25 post coarctation subjects, with flow mediated dilatation, GTN - induced response, and reactive hyperaemia as the dependent variables. Significance was taken at the p<0.05 level.

4.5.2 RESULTS

Twenty five patients (16 males and 9 females) aged 19.6 (14 - 27) years were studied. The median age at repair was 52 months (0 - 167), including 8 subjects who had undergone repair in the first year of life, 7 in the neonatal period. Nine subjects were repaired between 1 and 8 years, and 8 at age greater than 8 years. The median time after surgery was 15 years (7 - 21). Four of the subjects smoked cigarettes. All subjects were clinically well, had no signs of recoarctation, and were taking no medications. There was no significant difference in cholesterol level, vessel size or resting blood pressure between cases and controls (Table 4.5.1).

<u>Table 4.5.1</u>. Baseline characteristics of the coarctation subjects and controls.

	CoA Subjects	<u>Controls</u>	p-value
Age (years)	19.6±2.2	19.6±4.1	0.81
Sex	16:9	32:18	0.1
Cholesterol (mg/dl)	170±32	163±26	0.82
BA size (mm)	3.35±0.5	3.32±0.5	0.81
Resting BP (mmHg)	119±17	114±14	0.15

BA - brachical artery BP - blood pressure CoA - coarctation

On echocardiography, no subject had flow velocity $> 3 \text{ms}^{-1} \ (2.2 \text{ ms}^{-1} \pm 0.1)$ in the descending aorta, nor diastolic flow > 200 ms. All subjects had normal left ventricular systolic function (fractional shortening > 28%) and wall thickness (posterior wall $\le 10 \text{mm}$). All study subjects exercised until stage 5 and/or their heart rate was > 90% predicted. No study was limited by symptoms and no arrhythmia was noted. Maximum blood pressure on exercise was $180 \pm 30 \text{ mmHg} \ (120 - 230)$, and on recovery was $130 \pm 20 \text{ mmHg} \ (95 - 160)$. Maximal systolic BP on exercise was not correlated with age at repair (p = 0.27).

Hyperaemic response in the right brachial artery of CoA subjects was significantly reduced compared to controls (mean $343 \pm 130 \text{ v }471 \pm 162\%$; p < 0.001) (Figure 4.5.1). Both flow-mediated and GTN induced dilatation of the right brachial artery were impaired in the CoA subjects compared with the controls (FMD $3.8 \pm 3.3 \text{ vs } 8.8 \pm 3.6\%$ p < 0.001; GTN $13.3 \pm 6.0 \text{ vs } 20.5 \pm 6.1\%$ p < 0.001) (Figure 4.5.2). In

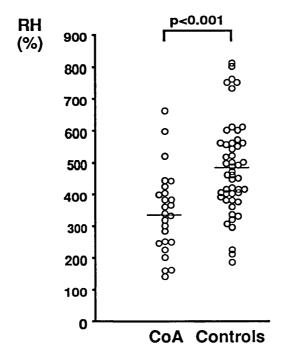


Figure 4.5.1
Reactive hyperaemia (RH) in the 25 subjetcs studied late after coarctation (CoA) repair and in 50 age and sex-matched controls.

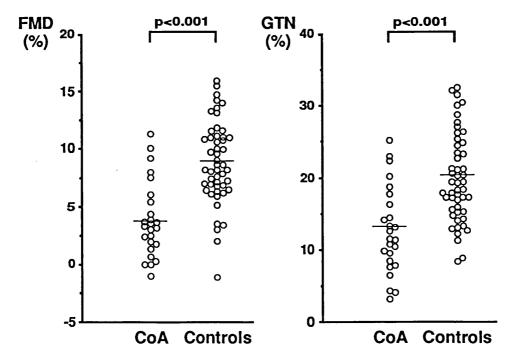


Figure 4.5.2 Flow-mediated dilatation (FMD) and GTN-induced responses of the right brachial artery in the 25 subjects studied late after coarctation (CoA) repair and in 50 age and sex-matched controls.

contrast femoral artery response to GTN was normal in the coarctation patients (9.5 \pm 2.6 v 10.1 \pm 4.1, p = 0.7) (Figure 4.5.3). In the 12 subjects who had both brachial and femoral studies, GTN response was impaired in the arm (14.1 \pm 5.8%).

On univariate analysis of the 25 coarctation subjects, GTN response in the brachial artery was inversely correlated with maximum systolic blood pressure on exercise (r = -0.61; p = 0.01, Figure 4.5.4), and with vessel size (r = -0.46; p = 0.05) but not with patient age, sex, age at repair, length of follow-up or smoking status. On multivariate analysis, GTN response was inversely correlated with exercise blood pressure only (r = -0.52, p = 0.04, Table 4.5.2). There was no correlation between FMD or RH and any of the independent variables on univariate or multivariate analysis.

Table 4.5.2. Multivariate analysis of determinants of GTN-induced response in the brachial artery of 25 subjects following successful repair of coarctation of the aorta.

<u>Variable</u>	Partial regression coefficient	p-value
Age	0.15	0.13
BP max*	-0.51	0.04
Repair age	-0.39	0.27
Follow-up	-0.24	0.42
Smoking	-0.41	0.11
Vessel size	-0.33	0.12
Sex	0.02	0.94

^{*}Blood pressure at maximal exercise

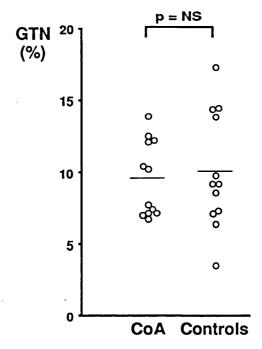


Figure 4.5.3
GTN response of the superficial femoral artery in 12 of the 25 subjects studied late after coarctation (CoA) repair, and in 12 age and sex-matched controls.

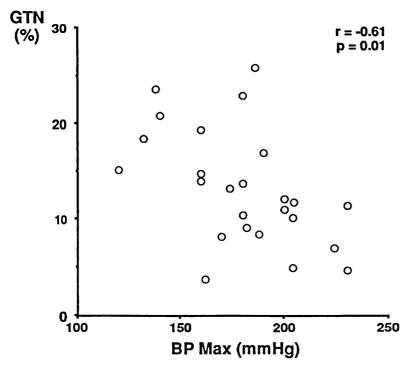


Figure 4.5.4
The association between GTN response in the right brachial artery in the 25 subjects studied late after coarctation (CoA) repair and the systolic blood pressure at peak exercise.

4.5.3 DISCUSSION

This study has shown marked abnormalities in arterial physiology in young adult patients up to 20 years after apparently successful paediatric repair of isolated coarctation of the aorta, including those who were operated on in infancy. The reduced hyperaemic response suggests abnormalities in the resistance vessels, and the impaired response to GTN is consistent with large vessel changes. Those subjects with impaired GTN response had a hypertensive blood pressure response to exercise. These abnormalities were present in the precoarctation but not in the femoral arteries.

Reactive hyperaemia occurs as a result of metabolic and neural responses to ischaemia and reperfusion. The reduced degree of hyperaemia in the coarctation subjects suggests abnormal vasodilatory capacity of the forearm resistance vessels, however the pathophysiologic basis for this impaired small vessel response is unclear. Gidding and colleagues have previously reported augmented vasoconstrictor response to norepinephrine in the arms of hypertensive patients with mild residual CoA (1985). Our findings show an impaired vasodilator response, even in normotensive subjects.

The impaired vasodilatation of the brachial arteries in response to GTN, but not of the femorals, suggests that there is also abnormal vasodilator capacity of the conduit vessels in the pre-coarctation bed. In-vitro studies of segments of aorta have shown that the pre-coarctation aorta is significantly less contractile to potassium, norepinephrine and prostaglandin F2a than either sections below the coarctation, or proximal and distal aortic ring preparations from controls (Sehested et al 1982). This is likely to be due to the altered composition of the arterial wall proximal to the coarctation, with increased scleroprotein content (collagen and elastin), and less smooth muscle compared to distal segments (Berry et al 1976). These changes may contribute to exercise-induced hypertension. Our finding of abnormal arterial reactivity even in young adults who were repaired in the neonatal period suggests that some structural arterial changes may persist after operative correction.

Impaired FMD was also seen in the brachial artery of the CoA patients. This has been demonstrated in other groups of young subjects with risk factors for atherosclerosis, such as cigarette smoking and hypercholesterolaemia (see sections 4.2, 4.3). In the presence of a normal degree of reactive hyperaemia and preserved responses to GTN, an impaired response to increased flow indicates endothelial dysfunction (Celermajer et al 1992). In this study, however, similar inferences cannot be made because of the coexistent small and large vessel abnormalities.

The patients in this study represent the best end of the spectrum of coarctation repair, having normal resting blood pressures and no clinical or echocardiographic evidence of residual arch obstruction. One might expect that vascular function might be even more abnormal in those followed up after less successful surgery.

These abnormal vascular responses were not related to age at operation, and were even found in subjects repaired in the neonatal period. Samanek and colleagues have previously demonstrated abnormal forearm vascular resistance both at rest and on exercise in 58 young adults after successful repair of coarctation from age 5 years; the degree of abnormality was unrelated to age at surgery or duration of follow-up (Samanek et al 1976). No studies have examined vascular function in patients who had earlier repair. Our findings also show no correlation between age at surgery and abnormal arterial responses at late follow-up. This is consistent with other evidence of persistent cardiovascular abnormalities long after successful coarctation repair in early life; left ventricular mass and diastolic function may also be abnormal late after successful childhood surgery (Moskowitz et al 1990).

We used a non-invasive method to measure vascular responses by detecting small changes in arterial diameter and by calculating Doppler - derived flow. The ability of this technique to measure small changes in arterial diameter accurately and reproducibly has been established previously in phantom and clinical experiments (sections 2.2, 2.3).

Although measurement of absolute flow by Doppler techniques may not be precise, relative changes, such as the degree of flow increase following cuff inflation and release, have been validated (Levenson et al 1981, Chauveau et al 1985). The same method for measuring reactive hyperaemia was used for patients and controls.

In this study the abnormal GTN response was independently related to exercise - induced hypertension. Although a hypertensive response to exercise is commoner in patients repaired late (Maron et al 1973, Bergdahl et al 1983), an exaggerated response to exercise also occurs in 20% of patients repaired early (Simsolo et al 1988). The fact that marked physiologic abnormalities of the large and small arteries of the precoarctation bed may still be found many years after successful repair of coarctation in childhood suggests that structural and/or functional arterial abnormalities are not completely reversible after operation. In such cases, early repair may not necessarily prevent late hypertension or other cardiovascular complications.

4.6 ENDOTHELIUM-DEPENDENT DILATATION IN THE SYSTEMIC ARTERIES OF ASYMPTOMATIC SUBJECTS RELATES TO CORONARY RISK FACTORS AND THEIR INTERACTION

INTRODUCTION

Large prospective epidemiologic studies have established that several major factors contribute to an individual's risk of coronary heart disease and its complications (Castelli 1984, Lipid Research Clinics 1984). Some of these are modifiable, including hypercholesterolaemia, cigarette smoking and hypertension, whereas others, such as age, gender and family history are not. Many of these risk factors operate from early life (childhood and adolescence), and may cause vascular damage long before clinically evident disease (see section 1.1). If the effect of these factors on the arterial wall could be studied in this long preclinical phase of atherogenesis, this might present opportunities to intervene in high risk individuals with the aim of altering the natural history.

Using the non-invasive method, we have shown that endothelium-dependent dilatation is abnormal in young, asymptomatic subjects, such as children with familial hypercholesterolaemia (section 4.2) or young adults who smoke heavily (Section 4.3), before clinical evidence of disease. However the relative importance of these risk factors in the general population of apparently healthy subjects and the way in which they might interact to damage the arterial wall have not been investigated. We have therefore studied endothelial and smooth muscle physiology in 500 healthy men and women aged 5-73 years with a wide range of cholesterol levels and tobacco consumption. This information might refine the ability to identify groups at particularly low and high risk, and therefore permit more appropriate targeting of anti-atherogenic interventions.

4.6.1 SPECIFIC METHODS

Subjects. Between January 1991 and June 1993, 712 people aged 4-78 years were studied by external ultrasound imaging of the brachial or femoral artery. We excluded patients who had been recruited because of known disease, such as coronary atherosclerosis, diabetes, hypertension, those with repaired aortic coarctation and those with inborn errors of metabolism, such as familial hypercholesterolaemia and homocystinuria. The remaining 500 subjects were clinically well and formed the study group. They were recruited from hospital staff, friends and families, and volunteers from the community, and none were taking any regular cardioactive medications (including aspirin).

Study design. Each subject was asked details of their smoking history and family history of premature vascular disease. Cigarette smokers were defined as subjects who had smoked ≥ 1 cigarette daily for ≥ 1 year. Former smokers were defined as those who had not smoked for ≥ 3 months. Smokers were grouped into 4 categories on the basis of self-reported life-time total of pack years (PY) smoked; very light (1-4 PY), light (5-9 PY), moderate (10-19 PY), and heavy (≥ 20 PY). Family history was considered positive if a first degree relative had had clinical evidence of coronary artery disease, such as angina, myocardial infarction or bypass surgery, at age ≤ 55 years. Resting supine blood pressure using a standard sphygomanometer and non-fasting total serum cholesterol were then measured in each subject.

Risk factor score. A composite risk factor score was calculated for each subject, with 1 point for each of the following: cholesterol \geq 240 mg/dl; current or former smoker; current smoker with a consumption \geq 10 PY; family history; male gender; age \geq 50 years.

Arterial physiologic testing. Each subject then underwent non-invasive ultrasound study of a systemic artery, to assess endothelial and smooth muscle responses, as previously described (section 2.1). As optimal vessel size for these studies has been established as ≤6.0 mm (section 3.1), the superficial femoral artery was scanned in the 46 pre-pubertal subjects and the brachial artery in the 454 adolescents and adults. Arterial diameter was measured at rest, during reactive hyperaemia (induced by inflation and then deflation of a pneumatic tourniquet placed on the limb, distal to the target artery, to 300 mmHg), again at rest and after sublingual nitroglycerin (GTN).

<u>Statistics</u>. Flow mediated dilatation was calculated for each subject as the percentage increase in arterial diameter during the condition of increased flow compared to the average of the two control scans, and GTN induced dilatation was calculated similarly for the post-GTN scan diameter increase. The measurements of the two observers were averaged for each result for each subject. Descriptive data are expressed as mean \pm standard deviation.

Univariate analysis of the effects of each potential risk factor on FMD and GTN response was performed with linear regression for continuous variables (cholesterol level, mean blood pressure, age, vessel size, blood flow and hyperaemia) and with one-way analysis of variance for categorical variables (smoking category, family history, gender and vessel type). The interaction between risk factors and FMD or GTN was then examined using multiple stepwise regression analysis. These analyses were then repeated considering males and females separately and then for the subjects aged <50 years. Statistical significance was inferred at a p-value <0.05.

4.6.2 RESULTS

Subjects. There were 252 men and 248 women, whose mean age was 36±15 years (median 33, interquartile range 25-45, range 5-73 years). The mean cholesterol level was 191±40 mg/dl (median 188, interquartile range 163-214, range 94-330 mg/dl). There were 321 subjects (64%) who had never smoked regularly, 72 former smokers and 107 current smokers. Of the 179 smokers, 35 had a history of very light, 49 light, 42 moderate and 53 heavy cigarette consumption. The average systolic blood pressure was 121±10 mmHg and diastolic was 77±7 mmHg; in no case was blood pressure over 150 mmHg systolic or 90 mmHg diastolic. Fifty-two subjects (10%) had a family history of premature vascular disease. The risk factor score was 1.4±1.1: 107 subjects had a score of 0, 181 a score of 1, 124 a score of 2, 66 a score of 3, 17 a score of 4, and 5 a score of 5 or 6.

Risk factor correlations. There were several significant correlations between the risk factors themselves. A higher total cholesterol level was significantly correlated with cigarette smoking (r=0.14, p=0.002), higher blood pressure (r=0.31, p<0.0001), positive family history (r=0.11, p=0.02) and older age (r=0.48, p<0.0001), but not with gender, vessel size or vessel type. More males had a higher blood pressure (p<0.0001), positive family history (14% vs 7%, p=0.004) and more males smoked cigarettes (41% vs 30%, p<0.001). Older age was associated with heavier smoking (r=0.15, p=0.001), higher mean blood pressure (r=0.50, p<0.001) and total risk factor score (r=0.44, p<0.0001), but not with either family history or gender.

<u>Vascular study results</u>. Artery size was 3.75 ± 0.9 mm (range 2.0-7.3 mm). Resting blood flow was estimated as 122 ± 87 ml/min and reactive hyperaemia after cuff deflation was $485\pm191\%$ (range 180-1460%). Flow mediated dilatation was $6.3\pm4.2\%$ (-1 to 17%) and GTN-induced dilatation was $17.2\pm6.4\%$ (1 to 38%).

Risk factors and vessel responses. Univariate analysis revealed a significant correlation between reduced FMD and high cholesterol level (r=0.21, p<0.001), smoking (r=0.38, p<0.001), high blood pressure (r=0.29, p<0.001), positive family history (p=0.01), older age (r=0.11, p<0.01), male gender (p<0.001), larger vessel size (r=0.39, p<0.001) and total number of risk factors (r=0.48, p<0.001). smokers, FMD was inversely related to the "amount" of cigarettes smoked, in pack years (p<0.001). FMD was not correlated with resting blood flow nor degree of hyperaemia. On stepwise multiple regression analysis, impaired FMD was significantly and independently correlated with cigarette smoking (p=0.0004), older age (p=0.0002), male gender (p=0.004) and larger vessel size (p<0.0001), but not with cholesterol level, blood pressure, family history or vessel type. Risk factor score was strongly and independently related to FMD (p < 0.0001) (Table 4.6.1, Figure 4.6.1). The addition of this score to the multivariate model did not change the levels of significance of the other risk factors examined, suggesting risk factor interaction. The r²-value for the model of FMD using all the independent variables was 0.40 (implying that only 40% of the variability of FMD in this study could be explained by the risk factors measured).

Univariate analysis of risk factors and GTN response showed a significant correlation between GTN response and smoking (p=0.01), female gender (p<0.001), smaller vessel size (r=0.53, p<0.001), and total number of risk factors (r=-0.21, p<0.01). On stepwise multiple regression analysis, however, the only variable significantly correlated with GTN response was vessel size (p<0.0001).

Multivariate analysis for males and females separately produced similar results. In both cases, impaired FMD was independently correlated with smoking, older age, larger vessel size and higher total risk factor score (p < 0.01 for each). When analysis was confined to subjects < 50 years old, impaired FMD was still independently correlated with smoking, male gender, larger vessel size, and higher risk factor score (p < 0.01 for each). For each of these subgroup analyses, when GTN was the dependent variable, only vessel size was independently correlated.

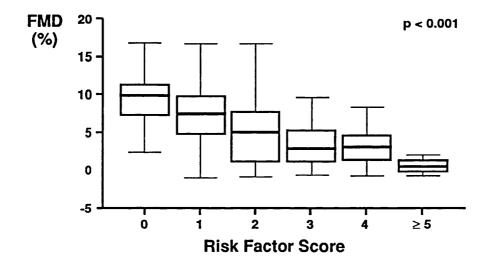


Figure 4.6.1
Risk factor score is strongly and independently related to flow-mediated dilatation (FMD). Each box represents the interquartile range, with the horizontal bar indicating the mean value. The bars encompass the entire range of values for each risk factor score.

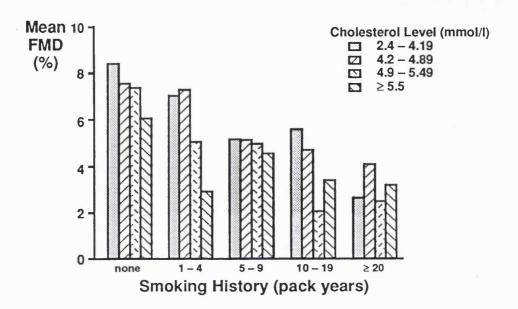
<u>Table 4.6.1</u>. Multiple regression analysis* for determinants of flow-mediated dilatation in 500 clinically well subjects.

<u>Variable</u>	Partial	regression	coefficient	p-value
Cholesterol		0.01		0.75
Smoking**		-0.20		0.0004
Mean blood pres	ssure+	-0.04		0.41
Family History		-0.001		0.98
Age		-0.20		0.0002
Sex		-0.16		0.004
Vessel size		-0.38		<0.0001
Vessel type		0.03		0.57
Risk Score		-0.30		<0.0001

^{*}Multiple-r value = 0.63, $r^2 = 0.40$

+Similar results were obtained if systolic or diastolic were entered instead of mean blood pressure.

^{**}Smoking was entered as a categorical variable (non-smokers, very light, light, moderate or heavy) based on the history of pack years smoked (see Methods).



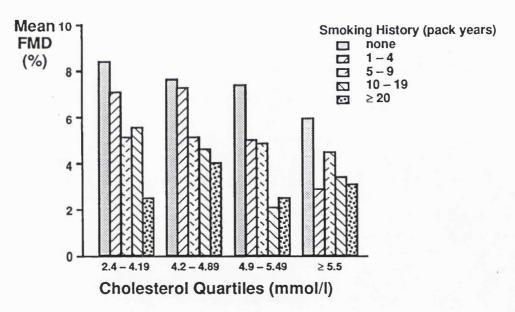


Figure 4.6.2
The interaction between smoking and cholesterol. In non- and light smokers, higher cholesterol is associated with lower flow-mediated dilatation (FMD). In heavy smokers, FMD is low, whatever the cholesterol level (top panel). In the lowest cholesterol quartile, there is a significant association between smoking and reduced FMD, however in the highest quartile, even very light smokers have impaired endothelial function (bottom panel).

Figure 4.6.2 demonstrates an interaction between cholesterol levels and life-time amount smoked. In those who have never smoked, cholesterol levels exert an important influence on FMD (lower cholesterol levels associated with better endothelium-dependent responses, r=-0.27, p<0.001). In contrast, heavy smokers have impaired FMD, whatever the cholesterol level (p=0.38). In the lowest cholesterol quartile (≤ 160 mg/dl), even moderate smokers could have a normal FMD, whereas in the highest quartile (≥ 215 mg/dl), FMD was impaired, even in very light smokers.

4.6.3 DISCUSSION

In this study, we have shown that the same factors which increase the risk of clinical vascular disease are associated with endothelial dysfunction in asymptomatic subjects, often decades earlier. In this group of 500 clinically well men and women, all vessels dilated to GTN, a direct smooth muscle dilator, and the risk factors that were correlated with FMD were not related to GTN response. This suggests that impaired FMD was due to impaired endothelial function. Furthermore these risk factors interacted to increase the likelihood of early vascular damage, even in young subjects, in the same way as they are known to act synergistically to increase the risk of cardiovascular morbidity and mortality, usually in middle-age or later. We have demonstrated the possibility of making objective measurements of the effects of these damaging interactions at an early stage of the atherogenic process. This may enable the selection of individuals most likely to benefit from active intervention, and the assessment of response, at a time when the disease process is most likely to be reversible (Kwiterovitch 1990).

Two previous studies have investigated the relationship between risk factors and arterial wall damage. In a multicentre autopsy study of 390 young adults (aged 15-34 years) who died of violent causes, the amount of aortic and coronary atherosclerosis was correlated with serum levels of low density lipoprotein and thiocyanate (a marker for smoking), although the interaction between these variables was not examined. Our results confirm these observations, which showed that hyperlipidaemia and smoking damage arteries from

an early age. Vita et al have examined the association between risk factors and vascular physiology, rather than pathology, in 34 adults with chest pain syndromes and angiographically smooth coronary arteries. In this relatively small study of patients, all of whom had clinical suspicion of coronary disease, risk factors such as high cholesterol and older age (but not cigarette smoking) were independently associated with impaired coronary vasomotor responses. We have now extended these observations to examine the interaction of the same risk factors in apparently well subjects.

The method we have used to study endothelium-dependent dilatation is non-invasive and well tolerated, allowing investigation of a large number of such subjects, with and without risk factors. We have studied the influence of and the interaction between potentially modifiable risk factors such as smoking and cholesterol. We excluded patients with hypertension, as most are on medications that might alter their endothelium and/or smooth muscle dependent responses. This is probably why blood pressure level was not independently related to endothelium-dependent dilatation in this study group. Subjects with identified illnesses known to predispose to vascular disease, such as diabetes mellitus and familial hypercholesterolaemia, were also excluded. Although we have studied the femoral and brachial arteries, atherosclerosis is a diffuse process, and Vogel et al have recently reported good correlation between abnormal brachial vasomotor responses and coronary atherosclerosis (1993).

In this study, total cholesterol level was not independently associated with impaired endothelium-dependent dilatation. This may be due to the relatively low cholesterol levels of the population we studied; only 25% of the subjects had cholesterol \geq 215 mg/dl; in contrast, in a previous study of of hypercholesterolemic children, many of whom had a total cholesterol level \geq 300 mg/dl, we found an inverse relation between the cholesterol level and endothelium-dependent dilatation. In addition, our physiologic study is considerably smaller than the epidemiologic studies which have shown that total cholesterol level is related to cardiovascular mortality. Measurement of cholesterol subfractions, such as high- and low-density lipoprotein levels, might have identified

groups at a higher risk of impaired endothelial function. Total cholesterol level, however, was relevant when interactions with other risk factors, such as smoking, were examined. Total cholesterol was inversely related to FMD in life-long non-smokers, but this relationship was not present in heavy smokers.

In contrast, smoking was very powerfully associated with endothelial damage in a dose-related manner. In the light smokers, however, there was wide variability of FMD, which was influenced by the cholesterol level; those with a low cholesterol were less likely to have impaired endothelium-dependent dilatation. In the heavy smokers, FMD was impaired, regardless of the cholesterol level. The impact of cholesterol may therefore be less relevant in the context of moderate or heavy smoking.

The multivariate model used to predict endothelium-dependent dilatation entered cholesterol level, smoking, blood pressure, family history, age and gender as independent variables. The fact that risk factor score was strongly and independently related to FMD, even when its constituent variables are represented in the model, suggests that there is an interaction between some or all of the variables making up the score. The mechanism of impaired endothelium-dependent dilatation subjects with risk factors is uncertain, but may include reduced release and/or synthesis of endothelium derived relaxing factor (EDRF) impaired membrane receptor sensitivity to shear stress and/or increased scavenging of EDRF by free radicals (Verbeuren et al 1986, Bossaller et al 1987, Egashira et al 1993). The relative importance of these effects may differ between risk factors.

Interestingly, this model only accounted for 40% of the observed variability between subjects, despite the fact that most of the risk factors were strongly associated with impaired arterial physiology. This suggests that other unidentified factors may play an important role in atherogenesis; candidates include certain genes, such as the angiotensin converting enzyme genotype (Cambien et al 1992), and certain lipid subfractions, such as lipoprotein(a) levels (Genest et al); neither of these were measured in this study.

Important risk factors for atherosclerosis may still be unknown, some of which may be modifiable.

Atherosclerosis is a life-long disease process that begins in childhood but does not usually cause clinical complications until middle age or later. During this long preclinical phase, the same risk factors that have been identified in large epidemiologic studies are associated with loss of endothelium-dependent vasodilatation. As these harmful interactions may even occur in young asymptomatic subjects, this emphasizes the importance of risk factor control in the long-range prevention of adult atherosclerotic disease. Objective measurement of early vascular damage enables the identification of high risk subgroups most likely to benefit from intervention, and allows serial assessment of any treatment benefits.

CHAPTER 5 - STUDIES OF REVERSIBILITY

5.1 BACKGROUND

The previous 3 chapters have described the development of a non-invasive method for the study of endothelial and smooth muscle physiology, its application to the investigation of "normal" subjects, and the demonstration of abnormal vascular reactivity in children and young adults at high risk of atherosclerosis. The availability of this test provides a unique opportunity to evaluate whether appropriate risk factor modification can reverse endothelial dysfunction at an early stage of the atherosclerotic process, when maximum benefit might be expected (Kwiterovitch 1990). Given that endothelial dysfunction predisposes to local vasoconstriction, thrombosis and abnormal intercellular interactions with platelets and leucocytes (Ross 1986), reversal of endothelial dysfunction might restrict atherogenesis and reduce the incidence of important clinical outcomes.

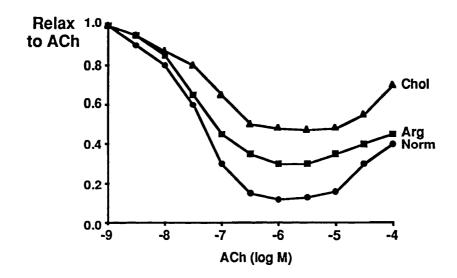
Several investigators have studied the potential reversibility of endothelial dysfunction, in experimental animals. Four interventions have been shown to be effective in improving endothelium-dependent responses; cholesterol lowering in hyperlipidaemic monkeys (Harrison et al 1987), administration of L-arginine to hypercholesterolaemic rabbits (Cooke et al 1992), antihypertensive treatment of rats with salt-induced hypertension (Luscher et al 1987) and administration of oestrogen to post-menopausal monkeys (Williams et al 1990). Recently three groups have studied similar interventions using invasive techniques on small numbers of humans prone to atherosclerosis; the effect of intravenous oestrogen on endothelium-dependent coronary responses in post-menopausal women (Williams et al 1992), the effect of intravenous L-arginine on the coronary microcirculation (Drexler et al 1991b) and the effect of antihypertensive treatment on vascular responses in forearm resistance vessels (Panza et al 1993). A non-invasive approach is required, however, to allow serial study of these potentially anti-atherogenic

strategies in large numbers of men and women. Some details of the strategies of intervention assessed to date are presented below.

- (a) Cholesterol lowering therapy. Hypercholesterolaemia is a common, important and modifiable risk factor, associated with endothelial dysfunction, as outlined in section 4.2. In monkeys with diet-induced hypercholesterolaemia, reversion to a normal diet results in recovery of endothelium-dependent vasodilatation after 18 months (Harrison et al 1987). Trials of cholesterol lowering therapy in subjects with established atherosclerosis have shown some evidence that plaque progression may be halted in treated patients, and that cardiovascular mortality may be lower, but there has been little impact on total mortality (Arntzenius et al 1985). Similar interventions at an earlier stage in the natural history might be expected to have a greater impact.
- (b) <u>Smoking cessation</u>. In section 4.3, we demonstrated that smoking causes dose-dependent endothelial damage, even in otherwise healthy young adults. Cardiovascular risk is lower in smokers who give up after a first myocardial infarction compared to those who continue smoking (Rosenberg et al 1985), possibly via favourable effects on thrombogenesis. There is no conclusive evidence that smoking cessation influences the size or number of existing atherosclerotic plaques.
- (c) Oestrogen replacement therapy. Pre-menopausal women are at lower risk of coronary heart disease than men of similar age, although after menopause the incidence of vascular disease rises steadily (British Heart Foundation 1992). Oestrogen replacement reduces the risk of first cardiovascular events by about 50% in this group (Knopp 1988). Oestrogens increase high density and decrease low density lipoprotein cholesterol levels, however beneficial effects on endothelial function may also be important in modulating cardiovascular risk. Long-term unopposed oral oestrogen replacement prevents endothelial dysfunction in surgically menopausal monkeys (Williams et al 1990), and recently the same investigators have shown that short-term

intravenous administration of oestrogen to post-menopausal women with atherosclerosis acutely improves endothelium-dependent dilatation in the coronary arteries (Williams et al 1992).

- (d) L-arginine therapy. L-arginine is the precursor of nitric oxide, and recent data has suggested that the reduced synthesis of EDRF in hypercholesterolaemic animals may be substrate limited. Intravenous administration of L-arginine to hypercholesterolaemic rabbits has been shown to improve endothelium dependent vasodilatation of aortic rings (Cooke et al 1991). In man, intra-arterial L-arginine has been shown to improve endothelium-dependent dilatation in both the coronary and brachial microcirculations of a small number of hypercholesterolaemic patients, but no influence on large artery physiology was found (Drexler et al 1991b, Creager et al 1992). In rabbits fed a high-cholesterol diet, oral supplementation with dietary L-arginine for 10 weeks has been shown to improve endothelium-dependent vasodilatation, and also to reduce the formation of atherosclerotic plaques in the aorta by about 80% (Cooke et al 1992) (Figure 5.1.1). The effect of oral L-arginine on arterial physiology in man has not yet been studied.
- (e) <u>Blood pressure lowering therapy</u>. Hypertension is associated with impaired endothelium-dependent small vessel relaxation (Panza et al 1990). In experimental animals, this endothelial injury is reversible (Luscher et al 1987, Sugimoto et al 1988). In section 4.5, we showed that successful repair of coarctation, a condition which produces upper limb hypertension, is not necessarily associated with normal endothelial function, even in normotensive subjects years after surgery. Panza et al (1993) have recently studied 15 hypertensive adults, and found that clinically effective antihypertensive therapy did not restore normal endothelial function. Therefore the identification of which hypertensive subjects might have reversible vascular damage, and over what time course, is not yet clear.



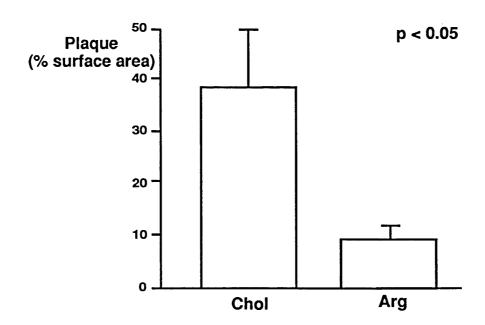


Figure 5.1.1
In rabbits with diet-induced hypercholesterolaemia (Chol), supplementation with oral L-arginine (Arg) preserves endothelium-dependent relaxation to acetylcholine (ACh), (upper panel), and also decreases atheroma formation (lower panel).

Prospective crossover or parallel group trials would be ideally suited to the investigation of reversibility of endothelial dysfunction in man. In section 2.3, we presented data on the reproducibility of arterial responses in the same subject studied on different occasions. The within-patient and between-patient variability could be quantified. These data can be used to allow the rational design of studies to investigate whether potentially anti-atherogenic interventions, such as risk factor modification, are associated with significant improvements in endothelium-dependent arterial responses. These results are presented in Section 5.2.

5.2 POWER FUNCTION ANALYSES

INTRODUCTION

The non-invasive method for measuring arterial physiology in-vivo is ideally suited for prospective studies of disease progression or reversibility, including assessing the effects of potentially anti-atherogenic strategies, in asymptomatic subjects at high risk of atherosclerosis. In Section 2.3, we presented data on endothelium-dependent arterial responses acquired from 40 adults studied on 4 occasions each. This quantitative information about the natural variability of vascular physiology provides the basis for rational design of clinical trials, to assess reversibility of abnormal endothelial pathophysiology.

5.2.1 SPECIFIC METHODS

The within-patient variability obtained in Section 2.3 allows an estimate of the percentage improvement in FMD that would have to be observed in an individual patient in order to be confident that the change was not simply due to spontaneous variation. It was then assumed that investigators would wish to design studies with 80% statistical power and a confidence level where p=0.05 (two-sided test). The number of individuals required to detect a range of hypothesised benefits could then be calculated for various study designs using the results from the analysis-of-variance (technical details are provided below; this analysis was performed by Dr D Spiegelhalter of the MRC Biostatistics Unit, Cambridge). Results were calculated for crossover and parallel-group trial designs, for different numbers of studies performed in each subject before and after intervention.

Since interventional studies primarily would target individuals with low or absent FMD, this estimate was made for the whole group and for the subset of "non-responders" (17 subjects in whom the mean FMD was less than 5%).

Detailed methods for generating power function curves. Let T be the statistic that will be used to estimate the mean difference in FMD between control and treatment periods, and V_T/I be the variance of T, where I is the total number of patients in the study. Let V_p and V_w denote the between and within-patient variance respectively.

When a single patient is measured on K days, their mean response will have variance $W=V_w/K$. In a crossover trial, assuming no carry-over and no treatment-period interaction, $V_T/I=2W/I$. In a parallel study with I/2 patients per group measured on K days, $V_T/I=4(W+V_p)/I$. Note the parallel study both introduces between-patient variability and halves the number of patients per treatment.

Suppose we hypothesise a reduction due to treatment of d%. Under this alternative hypothesis T has a distribution with mean d and variance V_T/I , and the number of patients in the study to have 80% power to correctly reject the hypothesis of no effect with a 2-sided test at the 5% level is given by

$$I = (0.842 + 1.96)^2 V_{\tau}/d^2$$

Plotting I against d provides the Figures 5.2.1 and 5.2.2.

For a single patient the mean response has a standard deviation of $\sqrt{2W}$, and hence from random variation alone there is a 5% chance the response will exhibit a change of at least $\pm d = 1.96 \sqrt{2W}$. This provides the entries in Table 3 for the reduction that might be expected by chance.

5.2.2 RESULTS

<u>Individual variation</u>. Table 5.2.1 shows the minimum percent increase in FMD required to demonstrate a significant improvement in endothelium-dependent arterial physiology at the 95% confidence level. For example, a subject who is studied once before and once after an intervention would need to show a 5.2% improvement in FMD to be confident

that the change is due to a true beneficial effect rather than to natural variability. By increasing the number of studies before and after intervention, the amount of improvement required decreases (if a subject is studied 4 times before and after intervention, an improvement of only 2.6% would be required). In subjects who are "non-responders" (FMD <5% over 4 studies), less improvement is needed to detect a significant change.

Table 5.2.1. Percentage improvement in flow-mediated dilatation (FMD) required for statistical confidence (p<0.05, 2-sided) that the change in FMD for an individual studied before and after an intervention is real rather than due to spontaneous variation.

Number of scans*	1	2	4	
For any subject**	5.2	3.6	2.6	
For a non-responder***	4.5	3.2	2.3	

^{*} number of studies before and after the intervention

**calculated using data from all subjects (n=40)

***a "non-responder" was any subject in whom the mean FMD

over 4 studies was < 5% (n=17)</pre>

Clinical trial design

(i) Cross-over protocol. Figure 5.2.1 shows the relationship between hypothesised percentage improvement in FMD and the number of subjects required for a trial with 80% power at the 5% level. Curves are drawn to represent this relationship for 1, 2 and 4 studies being performed in each subject before and after intervention. For example, if a new cholesterol-lowering therapy improved FMD by 2.5% in subjects with

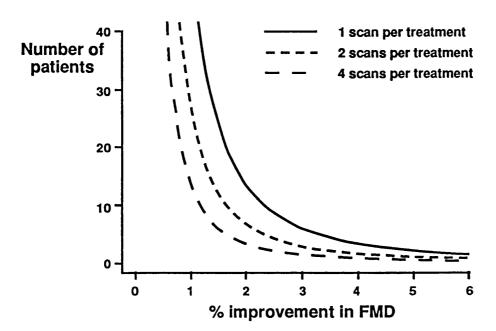


Figure 5.2.1
Relationship between hypothesised improvement in flow-mediated dilatation (FMD) and number of subjects required in a cross-over trial design (80% power, 95% confidence level). The 3 curves represent 3 different monitoring strategies, as shown.

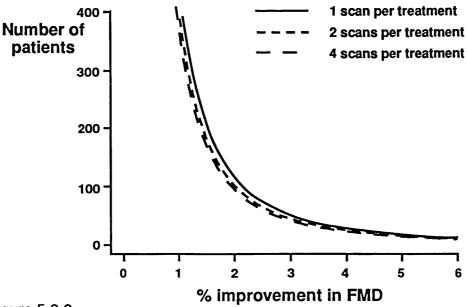


Figure 5.2.2
Relationship between hypothesised improvement in flow-mediated dilatation (FMD) and number of subjects required in a "parallel-group" trial design (80% power, 95% confidence level). The 3 curves represent 3 different monitoring strategies, as shown.

hypercholesterolaemia, and these subjects were studied once before and after each treatment period, a sample size of 10 subjects would have an 80 % power of demonstrating a significant benefit.

(ii) Parallel trial. Figure 5 shows the same relationship for a parallel-group trial design. Much larger numbers of subjects are required; in the example above, a sample size of approximately 90 subjects (45 in each group) would be needed to have the same statistical power.

5.2.3 DISCUSSION

Endothelial dysfunction, which can now be measured non-invasively in-vivo, is thought to represent an early stage in the atherogenic process. Risk factors that have a proven relationship with late atherosclerosis and its complications have now been shown to be associated with endothelial dysfunction in asymptomatic individuals who smoke (Section 4.3), have hypercholesterolaemia (Section 4.2), or who are hypertensive (Panza et al 1993). The recent demonstration that L-arginine, the substrate for the production of endothelium-derived relaxing factor, not only protects endothelium-dependent relaxation but also inhibits atheroma formation (Cooke et al 1992), is provocative. It suggests that loss of normal endothelial function is not simply a marker of early disease, but may be intimately involved in the pathogenesis of atherosclerosis. It is therefore timely and appropriate to test whether the endothelial dysfunction that we and others have shown in clinical studies can be modified or reversed in high risk asymptomatic subjects by appropriate interventions, such as risk factor modification or L-arginine administration, in prospective clinical studies.

Our non-invasive method is ideally suited to perform prospective studies of this kind. The effect of interventions can be assessed in individual patients, and performing several studies before and after treatment will maximise the chances of demonstrating a real benefit. The power function analyses presented allow rational design of trials, as the

number of patients required is dependent on the hypothesised improvement in endothelial function and the type of study (cross-over or parallel). Improvements of at least 1-2 % in FMD would be required in any trial for a significant benefit to be found, no matter how many patients were enrolled. The more studies performed pre-and post-intervention on each patient, the fewer patients are required, particularly in a cross-over study design. In parallel trials, the numbers required are much greater, because of between patient variation.

5.3 FORMER SMOKERS; CROSS-SECTIONAL AND PROSPECTIVE STUDIES

INTRODUCTION

A large amount of evidence indicates that smoking cessation yields substantial benefits for both the individual smoker and for public health. Former smokers live longer than those who continue to smoke, and smoking cessation clearly decreases the risk of lung and other cancers, chronic obstructive lung disease and atherosclerotic complications (US Surgeon General 1990). Among persons with diagnosed coronary disease, smoking cessation markedly reduces the risk of recurrent infarction and cardiovascular death (Aberg et al 1983, Rosenberg et al 1985). In asymptomatic subjects, smoking quitters have substantially lower risk of peripheral, cerebral and coronary disease than those who continue to smoke (Doll & Peto 1976, Rogot & Murray 1980). These excess risks in smokers are reduced by about half after 1 year of smoking abstinence, and then decline gradually; after 15 years of abstinence, the risk of vascular disease is similar to that of the general population.

Despite these overwhelming indications for smoking cessation, many are unable or unwilling to quit. Because of the strength of nicotine addiction, smoking cessation programs have relapse rates of over 75% at 12 months (Thompson 1978, Chapman 1985).

The reasons why smoking cessation lowers the cardiovascular event rate are unclear. There are favourable effects of smoking cessation on the coagulation cascade, including return of fibrinogen levels towards normal (Wilhelmsen et al 1984). Whether smoking cessation also has an anti-atherogenic effect is unknown.

In Section 4.3, we found that smoking is associated with a dose-dependent alteration in arterial physiology in otherwise healthy young adults. We have since then conducted 2

small studies to assess whether smoking-related impairment of endothelium-dependent dilatation is potentially reversible; the first on 40 former smokers, to compare their endothelial function with that of current smokers, the second on 10 current smokers, followed prospectively as they quit.

5.3.1 STUDY OF 40 FORMER SMOKERS

5.3.1.1 SPECIFIC METHODS

Data from the 80 current smokers studied in section 4.3 was compared to that from 40 former smokers. All subjects were clinically well, taking no cardiovascular medications, were normotensive, had plasma total cholesterol ≤ 6.2 mmol/l and had no known vascular risk factors, other than smoking. The former smokers were recruited from amongst hospital staff, families, friends and other volunteers. A former smoker was defined as any reformed regular smoker, who had not smoked cigarettes for ≥ 3 months. All had a standard non-invasive study of the right brachial artery.

The current and former smokers were compared using two-sample t-tests. Descriptive statistics are expressed as mean \pm standard deviation. In addition multivariate analysis of predictors of FMD was performed, and included age, sex, cholesterol, vessel size as well as group (current or former smokers). Statistical significance was inferred at a p-value ≤ 0.05 .

5.3.1.2 RESULTS

The former smokers were aged 25-57 (38±8) years, and there were 23 men and 17 women. Average time since cessation of smoking was 6 ± 3.8 years (range 3 months to 14 years), and was similar for men and women. Cigarette consumption had ranged from 1-75 pack years (mean 15 ± 14.1 for male and 9 ± 7.6 for females, p=0.15). Compared to the group of current smokers, the former smokers had similar age, sex distribution,

cholesterol levels, number of pack years smoked, vessel sizes, baseline flows and hyperaemia values (Table 5.3.1). FMD for the overall group of former smokers was $5.1\pm4.0\%$ ($5.2\pm2.8\%$ for men and $5.1\pm5.2\%$ for women), and was not correlated with time since smoking cessation. GTN induced dilatation was $17.4\pm5.5\%$.

In a multivariate analysis comparing the 40 former and the 80 current smokers after adjustment for other variables, former smokers had higher FMD values (p=0.07). When this analysis was performed for male subjects only, FMD was significantly higher in the former smokers (p=0.001) (Figure 5.3.1); for female subjects, FMD was similar in both groups (p=0.24). Similar analysis for GTN-induced dilatation showed no significant difference by group (p=0.73).

Table 5.3.1. Vascular study results in 80 current and 40 former smokers

	Current Smokers	Former Smokers	<u>p-value</u>
	(n=80)	(n=40)	
Age (years)	33.6±9.0	38.1±8.4	0.11
M:F	42:38	23:17	0.77
Cholesterol (mmol/1)	4.6±0.7	4.9±0.5	0.28
Pack years	16±15	13±13	0.10
Vessel size	3.70±0.7	3.80±0.6	0.51
FMD (%)	4.0±3.9	5.1±3.8	0.07
GTN (%)	17.0±5.8	17.4±5.4	0.88
Baseline Flow (ml/mir	n) 131±74	123±88	0.73
Hyperaemia (%)	471±181	457±195	0.61

F - female, FMD - flow mediated dilatation, GTN - glyceryl trinitrate induced dilatation, M - male.

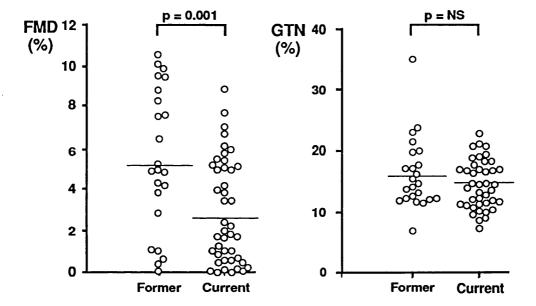


Figure 5.3.1 Flow-mediated (FMD) and GTN-induced dilatation in male former compared to current smokers. FMD is better in the former smokers, but not normal.

5.3.1.3 DISCUSSION

We have found that endothelium-dependent dilatation tended to be better in former than in current smokers, although flow-mediated dilatation was not normal, compared to lifelong non-smokers (section 4.3). A study of coronary arterial pathology has demonstrated similar findings; more severe atherosclerosis in current than non-smokers, with former smokers having intermediate levels (Auerbach et al 1976). These data are consistent with other studies suggesting that the effects of cigarette smoking on vascular physiology may be reversible (Aberg et al 1983). Epidemiological studies investigating patients with established atherosclerosis have indicated that the risk of subsequent cardiovascular events is lower in smokers who give up cigarettes (Rosenberg et al 1985); this may be mediated by a reversible effect of smoking on thrombogenesis and/or atherogenesis. The maximal potential for reversibility would be expected, however, with risk factor modification at a much earlier stage of the disease process, before plaques are established (Kwiterovich 1990).

FMD was significantly higher in male former compared to current smokers, but not females. Given that the male current smokers had lower FMD than females (accounted for on multivariate analysis by higher pack year consumption and larger vessel size rather than by a sex difference), one may need a larger sample size of female former smokers to demonstrate a significant improvement towards normal. Nevertheless the data on this sample of former smokers suggests, but does not prove, that endothelial function may improve with smoking cessation. Prospective serial studies of endothelial and smooth muscle function in asymptomatic young adults will allow investigation of the reversibility of abnormal vascular physiology with smoking cessation.

5.3.2 PROSPECTIVE STUDY OF SMOKING CESSATION

We studied 10 young adults (5 male, 5 female, aged 27-51 years) who had enrolled in a quit-smoking program, and investigated their arterial responses once before and at 2-

monthly intervals after smoking cessation, until relapse or 6 months of abstinence. No subjects used nicotine patches or gum.

At enrolment, they had smoked 3-45 (22 \pm 10) pack years, and had salivary cotinine levels of 118-436 (292 \pm 89) ng/ml. Vessel size was 2.6-4.5 (3.7 \pm 0.4) mm. FMD was 0-7 (3.4 \pm 1.6)%. All were normotensive, non-diabetic, had no family history of premature vascular disease, and had total plasma cholesterol \leq 6.2 mmol/l.

Eight of the smokers relapsed early (1-8 weeks), before the first post-cessation scan was performed, despite professional support from a quit-smoking organisation. The two other subjects, both men, have remained abstinent for 6 months. This was confirmed by serial measurement of salivary cotinine levels.

One man, a 43 year old with a 30 pack year history, has had no change in his arterial responses over the 6-month follow-up period. FMD was 0-3% on each of the 4 occasions he was studied.

The other successful quitter, a 42 year old man with a 20 pack year history, showed unequivocal improvement in endothelium-dependent dilatation (Figure 5.3.2). Over the series of studies his FMD improved from 2-3% to 9-10%, whereas his GTN-induced dilatation stayed the same (18-20%). FMD in 10 age-matched non-smoking controls was $8.2\pm2.0\%$. This improvement in FMD is large enough to be confident that the change was not due to spontaneous within-patient variability (see Table 5.2.1).

This single case of improved arterial physiology after smoking cessation suggests that in addition to the documented beneficial effects of smoking cessation on decreasing thrombogenesis (Kannel et al 1987), some benefit may also be mediated by improved endothelial function.

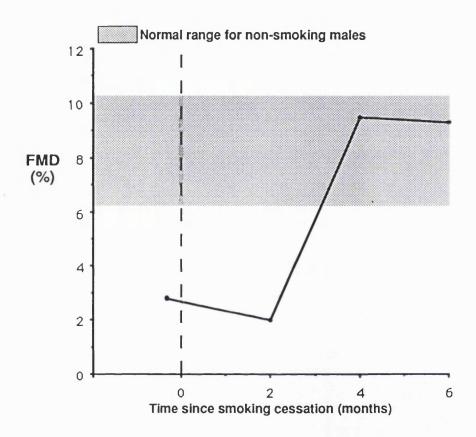


Figure 5.3.2 Flow-mediated dilatation (FMD) in a 42 year old heavy smoker who was studied before and after quitting. Four and six months after smoking cessation, his FMD had improved and was in the normal range for his age, sex and vessel size.

CHAPTER 6 - SUMMARY AND GENERAL DISCUSSION

6.1 SUMMARY

The major original findings from this work are summarised below.

- 1. A novel method for non-invasive assessment of arterial physiology in children and adults has been developed, based on high resolution ultrasound. The diameter of an artery is measured at rest, during a condition of increased flow, and after sublingual nitroglycerine. This technique allows investigation of both endothelium-dependent and independent arterial responses. After clinical application of the method in over 600 subjects studied on 1-8 occasions each, the method appears to be widely applicable and well tolerated.
- 2. The precision of B-mode ultrasound for measuring small differences in vessel diameter has been defined. Using an ultrasound phantom, diameter changes of as little as 0.1mm can be detected using commercially available high-resolution vascular equipment. Differences between results obtained by experienced operators and observers are negligible.
- 3. Endothelium-dependent responses are reproducible when measured in the same patient studied on different occasions, over a period of up to 4 months. Using 40 healthy young adults studied on 4 separate occasions each, we found a between-scan coefficient of variation for measurement of flow-mediated dilatation of approximately 2%.
- 4. In normal subjects (defined as those with no known vascular risk factors), the degree of flow-mediated and nitroglycerine-induced dilatation is inversely proportional to resting vessel diameter, over the range 2.0-8.0mm. Normal ranges for brachial and femoral artery responses to increased flow and nitroglycerine were defined, in children

and adults.

- 5. Ageing is associated with a progressive loss of normal endothelium-dependent dilatation in healthy adults. The age at which endothelial function begins to decline is different in men and women; endothelial dysfunction is present in men from age 40-45 years, and in women from age 50-55 years. This parallels the known sex difference in susceptibility to cardiovascular morbidity and mortality in our society.
- 6. Arterial physiology is markedly impaired in adults with established coronary atherosclerosis.
- 7. Endothelium-dependent arterial responses are impaired in children with familial hypercholesterolaemia as young as 7 years of age. The degree of endothelial dysfunction in these children appears to be inversely correlated with the Lp(a) level.
- 8. Cigarette smoking causes dose-related impairment of endothelium-dependent dilatation in healthy young adults.
- 9. Endothelial dysfunction is present in young children with homozygous homocystinuria, but not in their heterozygous parents.
- 10. Arterial physiology is markedly abnormal in normotensive young adults long after successful repair of aortic coarctation in childhood. Both small and large vessels in the pre-coarctation bed have abnormal vascular reactivity, suggesting that structural and/or functional arterial damage may persist long after repair. The impaired vasodilator capacity in these subjects may be related to an abnormal hypertensive response to exercise.
- 11. Risk factors, such as smoking and hypercholesterolaemia, may interact to produce endothelial dysfunction in asymptomatic children and adults.

12. Preliminary evidence suggests that endothelial dysfunction may be reversible in cigarette smokers who quit. The availability of the non-invasive method and data on the natural variability of endothelium-dependent arterial responses will allow rational design and performance of clinical trials to investigate the potential reversibility of early signs of vascular injury, in children and young adults prone to atherosclerosis.

LIMITATIONS OF THE STUDY

The major limitation of this work is the assumption that subjects with impaired endothelium-dependent dilatation in the brachial artery and/or femoral arteries have a higher risk of later developing clinically important atherosclerosis. This assumption is based on experimental evidence that endothelial dysfunction is a key early event in atherogenesis; certainly as a marker of early disease and perhaps also as an important pathogenetic mechanism. Furthermore we presume that atherosclerosis is a diffuse disease of systemic arteries, at least in its early stages, a view supported by laboratory and clinical evidence, and recently Vogel et al have reported that brachial artery vasomotor responses are well correlated with coronary atherosclerosis (1993). thesis also provides support for the importance of endothelial dysfunction in early atherosclerosis, as the same risk factors that are known to predispose to late occlusive vascular disease, such as ageing, hypercholesterolaemia and smoking, have been shown to be associated with impaired endothelial function in the arteries we studied. Nevertheless, long term prospective follow-up would be required to confirm that asymptomatic subjects with endothelial dysfunction are at increased risk of symptomatic vascular disease.

The second important limitation is that the use of high-resolution vascular ultrasound is confined to the study of relatively superficial arteries. Coronary vessels cannot be imaged using this technique. Furthermore the extracranial carotid arteries would be difficult to assess using our method, despite their superficial course, because hyperaemia

cannot be produced easily in these vessels. Hyperventilation followed by CO₂ rebreathing may increase carotid flow, but not to the same degree as the hyperaemia induced in limb vessels by distal cuff occlusion and release. Therefore the 2 arterial territories most often involved in clinical disease, the coronary and carotid, are currently not easy to investigate using our method, and "surrogate" arteries are usually studied.

The final important limitation relates to equipment, staff and data analysis. The ultrasound machine and transducer described are expensive, although widely available. A highly trained vascular technician is needed to obtain good quality images. Experienced ultrasound observers are required for scan measurement. These conditions are potentially reproducible in many hospitals or research laboratories, although each new department may have to conduct accuracy and reproducibility studies when commencing their own programs. At least 3 groups in the United States have started using this technique recently (in Boston, Chicago and Baltimore), and their preliminary results from control and atherosclerotic subjects appear very similar to our own data. The technique, however, will only be widely utilised when an automatic edge-detection system for diameter analysis becomes available. The technology and algorithms for this exist, and have been applied successfully to the much more difficult problem of endocardial edge detection for left ventriculography by ultrasound. Such "acoustic" edge detection should be relatively straightforward for a smooth vessel which runs perpendicular to the ultrasound beam, and may soon be available.

6.3 THE FUTURE

An accurate and reproducible non-invasive test of early vascular injury has enormous potential for both clinical use and for research. Along with carotid intima-media thickness and arterial compliance measurements, our test of endothelial and smooth muscle pathophysiology has excited much interest amongst physicians and scientists concerned with early detection of atherosclerosis. All 3 techniques require development of computerised analysis systems to facilitate their widespread use. As carotid intima-

media thickness measurement has a higher inter-observer error and is only abnormal relatively late in the natural history of atherosclerosis, I believe it is the least likely to be important for detection of early disease, although it is the easiest to perform, and may be an important marker of established disease in older adult subjects. Compliance measurements are complex and await rigorous validation studies. Our method may therefore prove to be an attractive technique for monitoring early vascular disease in individuals and/or populations.

The next stage of this work is to study potential reversibility of endothelial dysfunction in children and young adults at risk of atherosclerosis. The most exciting areas for prospective study include the effects of cholesterol-lowering therapy or dietary L-arginine in hypercholesterolaemia, and the effects of hormone replacement therapy in post-menopausal women. Such investigations are currently being undertaken in our department. Results from these and similar early-intervention trials will represent an important advance in the effort to prevent or retard the development of atherosclerosis.

REFERENCES

- Aberg A, Bergstrand R, Johansson S et al. Cessation of smoking after myocardial infarction. Br Heart J 1983;49:416-22
- Allen DR, Browse NL, Rutt DL. Effects of cigarette smoke, carbon monoxide and nicotine on the uptake of fibrinogen by the canine arterial wall. Atherosclerosis 1989;77:83-8
- American Heart Association. Heart and Stroke facts. American Heart Association, Dallas, 1991
- Amezcua JL, Palmer RMJ, de Souza BM, Moncada S. Nitric oxide synthesised from L-arginine regulates vascular tone in the coronary circulation of the rabbit. Br J Pharm 1989;97:1119-24
- Anderson EA, Mark AL. Flow-mediated and reflex changes in large peripheral artery tone in humans. Circulation 1989;79:93-100
- Anderson KM, Castelli WP, Levy DL. Cholesterol and mortality: 30 years of follow-up from the Framingham study. JAMA 1987;257:2176-80
- Anggard E, Galton S, Rae G et al. The fate of radio-iodinated endothelin-1 and endothelin-3 in the rat. J Cardiovasc Pharmacol 1989;13(suppl 5):s46-9
- ARIC investigators. The Atherosclerosis Risk In Communities (ARIC) study; design and objectives. Am J Epidemiol 1989;129:687-99

- Armstrong VM, Cremer P, Eberle P et al. The association between serum Lp(a) concentrations and angiography assessed coronary atherosclerosis: dependence on serum LDL levels. Atherosclerosis 1986;62:249-57
- Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3:5'-cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci 1977;74:3203-7
- Arntzenius AC, Kromhout D, Barth JD et al. Diet, lipoproteins, and the progression of coronary atherosclerosis: The Leiden Intervention Trial. N Engl J Med 1985; 312:805-11
- Asmussen G, Kjeldsen K. Intimal ultrastructure of human umbilical arteries.

 Observations on arteries from newborn children of smoking and nonsmoking mothers. Circ Res 1975;36:579-89
- Auerbach O, Carter HW, Garfinkel L, Hammond EC. Cigarette smoking and coronary artery disease. A macroscopic and microscopic study. Chest 1976;70:697-705
- Belcaro G, Barsotti A, Nicolaides AN. Ultrasonic biopsy a non-invasive screening technique. Vasa 1991;20:40-50
- Bergdahl L, Bjork VO, Jonasson R. Surgical correction of coarctation of the aorta.

 Influence of age on late results. J Thorac Cardiovasc Surg 1983;85:532-6
- Bernstein EF, Dilley RB, Goldberger LE, Gosink BB, Leopold GR. Growth rates of small aortic aneurysms. Surgery 1976;8:765-70
- Berry CL, Greenwald SE. Effects of hypertension on the static mechanical properties and chemical composition of the rat aorta. Cardiovasc Res 1976;10:437

- Best LC, Martin TJ, Russell RGG, Preston FE. Prostacyclin increases cyclic AMP levels and adenylate cyclase activity in platelets. Nature 1977;267:850-2
- Blankenhorn DH, Chinn HP, Strinwerda S et al. Work in progress: Common carotid artery contours reconstructed in three dimensions from parallel ultrasonic images.

 Radiology 1983;148:533-45
- Blankenhorn DH, Nessim SA, Johnson RL, Sanmarco ME, Azen SP, Cshin-Hemphill L.

 Beneficial effects of combined colestipol-niacin therapy on coronary atherosclerosis and coronary venous bypass grafts. JAMA 1987;257:3233-40
- Blankenhorn DH, Azen SP, Crawford DW et al. Effects of colestipol-niacin therapy on human femoral atherosclerosis. Circulation 1991;83:438-47
- Boers GH, Smals AGH, Trijbels FJM et al. Heterozygosity for homocystinuria in premature peripheral and cerebral occlusive arterial disease. N Engl J Med 1985; 313:709-15
- Boggousslavsky J, Despland P-A, Regli F. Asymptomatic tight stenosis of the internal carotid artery: Long term prognosis. Neurology 1986;36:861-3
- Bossaller C, Habib GB, Yamamoto H, Williams C, Wells S, Henry PD. Impaired muscarinic endothelium-dependent relaxation and cyclic guanosine 5'-monophosphate formation in atherosclerotic human coronary artery and rabbit aorta. J Clin Invest 1987;79:170-4
- Boulanger C, Luscher TF. Release of endothelin from the porcine aorta: inhibition by endothelium-derived nitric oxide. J Clin Invest 1990;85:587-90

- Brand FN, McGee DL, Kannel WB et al. Hyperuricemia as a risk factor of coronary heart disease: The Framingham study. Am J Epidemiol 1985;121:11-8
- Brattstrom LE, Hardebo JE, Hultberg BL. Moderate hyperhomocysteinemia a possible risk factor for arteriosclerotic cerebrovascular disease. Stroke 1984;15:1012-6
- Bredt DS, Synder SH. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme.

 Proc Natl Acad Sci 1990;87:682-5
- Brensike JF, Levy RI, Kelsy SF et al. Effects of therapy with cholestyramine on progression of coronary atherosclerosis: Results of the NHLBI Type II Coronary Intervention Study. Circulation 1984;69:313-24
- Breslau PJ, Fell G, Phillips DJ, Thiele BL, Strandness DE Jr. Evaluation of carotid bifurcation disease: The role of common carotid artery velocity patterns. Arch Surg 1982;117:58-60
- British Heart Foundation Statistics Database. Coronary Heart Disease Statistics, 1992.

 The Coronary Prevention Group, London, 1992
- Brown BG, Bolson EL, Dodge HT. Arteriographic assessment of coronary athersclerosis. Review of current methods, their limitations and clinical applications. Arteriosclerosis 1982;2:1-15
- Brown BG, Bolson EL, Dodge HT. Quantitative computor techniques for analyzing coronary arteriograms. Prog Cardiovas Dis 1986;28:403-18

- Brown G, Albers JJ, Fisher LD et al. Regression of coronary artery disease as a result of intensive lipid lowering therapy in men with high levels of apolipoprotein B. N Engl J Med 1990;323:1289-98
- Brown MS, Kovanen PT, Goldstein JL. Regulation of plasma cholesterol by lipoprotein receptors. Science 1981;212:628-35
- Buchwald H, Varco RL, Matts JP et al. Effect of partial ileal bypass surgery on mortality and morbidity from coronary heart disease in patients with hypercholesterolemia. N Engl J Med 1990;323:946-55
- Bush TL, Barrett-Connor E, Cowan LD et al. Cardiovascular mortality and noncontraceptive use of estrogen in women: results from the lipid research clinics program follow-up study. Circulation 1987;75:1102-9
- Cambien F, Poirier O, Lecerf L et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction.

 Nature 1992;359:641-4
- Caracci BF, Zuckowski AJ, Hurley JJ et al. Asymptomatic severe carotid stenosis. J Vasc Surg 1989;9:361-6
- Castelli WP. Epidemiology of coronary heart disease. The Framingham Study. Am J Med 1984;76:4-12
- Castellot J, Addonizo ML, Rosenberg R et al. Cultured endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. J Cell Biol 1981;90:372

- Celermajer DS, Sorensen KE, Gooch VM et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet 1992;340: 1111-5
- Celermajer DS, Sorensen KE, Ryalls M et al. Endothelial dysfunction occurs in the systemic arteries of children with homozygous homocystinuria but not in their heterozygous parents. J Am Coll Cardiol 1993a; in press
- Celermajer DS, Cullen S, Deanfield JE. Impairment of endothelium dependent pulmonary artery relaxation in children with congenital heart disease and abnormal pulmonary hemodynamics. Circulation 1993b;87:440-6
- Celermajer DS, Sorensen KE, Georgakopoulos D et al. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. Circulation 1993c; in press
- Chand N, Altura BM. Acetylcholine and bradykinin relax intrapulmonary arteries by acting on endothelial cells: Role in lung vascular disease. Science 1981;213:1376-9
- Chapman JM, Goerke LS, Dixon W et al. Measuring the risk of coronary heart disease in adult population groups: IV. The clinical status of a population group in Los Angeles under observation for two of three years. Am J Public Health 1957;47: 33-42
- Chapman S. Stop-smoking clinics: a case for their abandonment. Lancet 1985;1:918-20

- Chauveau M, Levy B, Dessanges JF, Savin E, Bailliart O, Martineaud JP. Quantitive Doppler blood flow measurement method and in vivo calibration. Cardiovasc Res 1985;19:700-6
- Clarke R, Daly L, Robinson K et al. Hyperhomocysteinemia: an independent risk factor for vascular disease. N Engl J Med 1991;324:1149-55
- Clarke WR, Schortt HG, Leaverton PE, Connor WE, Lauer RM. Tracking of blood lipids and blood pressure in school age children: The Muscatine Study. Circulation 1978;56:626-34
- Clarkson PM, Nicholson MR, Barratt-Boyes BG, Neutze JM, Whitlock RM. Results after repair of coarctation of the aorta beyond infancy: A 10 to 28 year follow-up with particular reference to late systemic hypertension. Am J Cardiol 1983;51:1481-6
- Clarkson TB, Weingard KW, Kaplan JR, Adams MR. Mechanisms of atherogenesis.

 Circulation 1987;76(suppl I):I-20-8
- Cobbe S, Shepherd J. Management guidelines for hyperlipidaemia. Br Heart J 1993; 69(Suppl):S69
- Cody RJ. The potential role of endothelin as a vasoconstrictor substrate in congestive heart failure. Eur Heart J 1992;13:1573-8
- Cohen M, Fuster V, Steele PM, Driscoll D, McGoon DC. Coarctation of the aorta:

 Long-term follow-up and prediction of outcome after surgical correction.

 Circulation 1989;80:840-5

- Cohen RA, Zitnay KM, Haudenschild CC, Cunningham LD. Loss of selective endothelial vasoactive functions caused by hypercholesterolemia in pig coronary arteries. Circ Res 1988;63:903-10
- Colditz GA, Willett WC, Stampfer MJ, Rosner B, Speizer FE, Hennekens CH.

 Menopause and the risk of coronary heart disease in women. N Engl J Med
 1987;316:1105-10
- Cole JS, Harley CJ. The pulsed Doppler coronary artery catheter. Circulation 1977;56:18-25
- Collins P, Griffith TM, Henderson AH, Lewis MJ. Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: a cyclic guanosine monophosphate-mediated effect. J Physiol Lond 1986;381:427-37
- Comerota AJ, Cranley JJ, Cook SE. Real time B-mode carotid imaging in diagnosis of cerebrovascular disease. Surgery 1981;89:719-25
- Cooke JP, Andon NA, Girerd XJ, Jirsch AT, Creager MA. Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta. Circulation 1991;83:1057-62
- Cooke JP, Singer AH, Zera P, Rowan RA, Billingham ME. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest 1992;90:1168-72
- Cox DA, Vita JA, Treasure CB et al. Atherosclerosis impairs flow-mediated dilation of coronary arteries in humans. Circulation 1989;80:458-65
- Coy KM, Maurer G, Siegel RJ. Intravascular ultrasound imaging: a current perspective. J Am Coll Cardiol 1991;18:1811-23

- Crawford T, Levene C. Medial thinning in atheroma. J Pathol Bacteriol 1953;66:19-23
- Creager MA, Cooke JP, Mendelsohn ME et al. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. J Clin Invest 1990;86:228-34
- Creager MA, Gallagher SH, Girerd XJ, Coleman S, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. J Clin Invest 1992;90:1248-53
- Crouse JR, Thompson CJ. An evaluation of methods for imaging and quantifying coronary and carotid lumen stenosis and athersclerosis. Circulation 1993; 87(suppl II):II17-33
- Dart AM, Lacombe F, Yeoh JK et al. Aortic distensibility in patients with isolated hypercholesterolaemia, coronary artery disease, or cardiac transplant. Lancet 1991;338:270-88
- Davenport AP, Ashley MJ, Easton P et al. A sensitive radioimmunoassay measuring endothelin-like immunoreactivity in human plasma: comparison of levels in patients with essential hypertension and normotensive control subjects. Clin Sci 1990;78:261-4
- Davies ML, Woolf N. Atherosclerosis: What is it and why does it occur? Br Heart J 1993;69(suppl):s3-11
- Davis JW, Shelton L, Eigenberg DA, Hignite CE, Watanabe IS. Effects of tobacco and non-tobacco cigarette smoking on endothelium and platelets. Clin Pharmacol Ther 1985;37:529-33

- Davis JW, Shelton L, Hartman CR, Eisenberg DA, Ruttinger HA. Smoking-induced changes in endothelium and platelets are not affected by hydroxyethylrutosides.

 Br J Exp Pathol 1986;67:765-71
- Dawber TR, Moore FE, Mann GV. Measuring the risk of coronary heart disease in adult population groups: II. Coronary heart disease in the Framingham study.

 Am J Public Health 1957;47:4-24
- Deanfield JE, Shea MJ, Wilson RA et al. Direct effects of smoking in the heart: Silent ischemic disturbances of coronary flow. Am J Cardiol 1986;57:1005-9
- DeGroot PG, Willems C, Boers GHJ et al. Endothelial cell dysfunction in homocystinuria. Eur J Clin Invest 1983;13:405-10
- DeRouen TA, Murray JA, Owen W. Variability in the analysis of coronary arteriograms. Circulation 1977;55:324-8
- Detre KM, Kelsey SF, Passamani ER et al. Reliability of assessing change with sequential coronary arteriography. Am Heart J 1982;104:816-23
- DiCorleto PE, Bowen-Pope DF. Cultured endothelial cells produce a platelet-derived growth factor-like protein. Proc Nat Acad Sci 1983;80:1919-23
- Dinh-Xuan AT, Higenbottam TW, Clelland CA et al. Impairment of endothelium-dependent pulmonary artery relaxation in chronic obstructive lung disease. N Engl J Med 1991;324:1539-47
- Doll R, Peto R. Mortality in relation to smoking: 20 years' observations on male British doctors. Br Med J 1976;2:1525-36

- Doyle JT, Heslin AS, Hilboe HE et al. Measuring the risk of coronary heart disease in adult population groups: III. A prospective study of degenerative cardiovascular disease in Albany: Report of three years' experience. Am J Public Health 1957;47:25-32
- Drake RM, Beuchley RW, Breslow L. Measuring the risk of coronary heart disease in adult population groups: V. An epidemiological investigation of coronary heart disease in the California health survey population. Am J Public Health 1957;47:43-57
- Drexler H, Zeiher AM. Endothelial function in human coronary arteries in vivo. Hypertension 1991a;18:II-90-9
- Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine.

 Lancet 1991b;338:1546-50
- Duffield RGT, Lewis B, Miller NE, Jamieson CW, Brunt JN, Colchester AC.

 Treatment of hyperlipidaemia retards progression of symptomatic femoral atherosclerosis. Lancet 1986;2:639-42
- Duguid JB. Thrombosis as a factor in the pathogenesis of coronary atherosclerosis. J Pathol Bacteriol 1946;58:207-12
- Duling BR, Berne RM. Propagated vasodilation in the micro-circulationion of the hamster cheek pouch. Circ Res 1970;26:163-70
- Eaton DL, Fless GM, Kohn WJ. Partial amino-acid sequence of apolipoprotein (a) shows that it is homologous to plasminogen. Proc Natl Acad Sci 1987;84:3224-8

- Edelman RR, Warach S. Magnetic resonance imaging (second of two parts). N Engl J Med 1993;328:785-90
- Edwards DH, Griffiths TM, Ryley HC, Henderson AH. Haptoglobin-haemoglobin complex in human plasma inhibits endothelium-dependent relaxation: evidence that endothelium-derived relaxing factor acts as a local autacoid. Cardiovasc Res 1986;20:549-56
- Ellis S, Sanders W, Goulet C et al. Optimal detection of the progression of coronary artery disease: comparision of methods suitable for risk factor intervention trials. Circulation 1986;74:1235-42
- Enos WF, Holmes RH, Beyer J. Coronary disease among United States soldiers killed in action in Korea: preliminary report. JAMA 1953;152:1090-3
- Esmon NL. Thrombomodulin. Seminars Haemostasis Thrombosis 1987;13:454-63
- Evans HG, Ryley H, Hallot I, Lewis MJ. Human red blood cells inhibit endotheliumderived factor activity. Eur J Pharmacol 1989;163:361-4
- Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate: I. Changes that lead to fatty streak formation. Arteriosclerosis 1984;4:323-40
- Farmer JA, Gotto AM. Risk factors for coronary artery disease. In: Heart Disease (4th edn). Braunwald E (ed). WB Saunders Company, Philadelphia, 1992;1125-60

- Feinleib M, Kaplin NM, Stamler J(eds). Prevention of coronary heart disease: Practical management of the risk factors. WB Saunders Company Philadelphia, 1983;120-9
- Feldman RL, Pepine CJ, Conti CR. Magnitude of dilatation of large and small coronary arteries by nitroglycerin. Circulation 1981;64:324-33
- Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine smooth muscle. Br J Pharmacol 1988;93:515-24
- Feyerabend C, Russell MAH. A rapid gas-liquid chromatographic method for the determination of cotinine and nicotine in biological fluids. J Pharm Pharmacol 1990;42:450-2
- Fields S, Dunn F. Correlation of echographic visualizability of tissue with biological composition and physiological state. J Acoust Soc Am 1973;54:809-12
- Fish RD, Nabel EG, Selwyn AP et al. Responses of coronary arteries of cardiac transplant patients to acetylcholine. J Clin Invest 1988;81:21-31
- FitzGerald GA, Brash AR, Falardeau P, Oates JA. Estimated rate of prostacyclin secretion into the circulation of normal man. J Clin Invest 1981;68:1272-5
- Fleisch A. Les reflexes nutritifs ascendants producteurs de dilatation arterielle. Arch Int Physiol 1935;41:141-67
- Folkow B. Structure and function of the arteries in hypertension. Am Heart J 1987;114:938-48

- Franke RP, Grafe M, Schnittler H, Seiffge D, Mittermayer C. Induction of human vascular endothelial stress fibres by fluid shear stress. Nature 1984;307:648-9
- Franklin FA, Brown RF, Franklin CC. Screening, diagnosis, and management of dyslipoproteinemia in children: lipid disorders. Endocrin Metab Clin North Am 1990;19:399-449
- Freedman DS, Shear CL, Srinivasan SR, Webber LS, Berenson GS. Tracking of serum lipids and lipoproteins in children over an eight-year period. Bogalusa Heart Study. Prev Med 1985;14:203-16
- Freedman DS, Srinivasan SR, Shear CL, Webber LS, Chiang YK, Berenson GS.

 Correlates of high density lipoprotein cholesterol and apolipoprotein A-1 levels in children. The Bogalusa Heart Study. Arteriosclerosis 1987;7:354-60
- Frick MH, Elo O, Haapa K et al. Helsinki Heart Study: Primary prevention trial with Gemfibrozil in middle aged men with dyslipidemia. N Engl J Med 1987;317: 1237-45
- Friedwald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma without the use of the preparative ultracentrifuge. Clin Chem 1972;18:499-502
- Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetycholine. Nature 1980;288:373-6

- Furchgott RF. Studies on relaxation of rabbit aorta by soduim nitrate: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrate and the endothelium-derived relaxing factor is nitric oxide. In Vasodilation. Vanhoutte PM, Leusen I(eds). Raven Press, New York, 1988;401-14
- Gajdusek CM, Schwartz SM. Ability of endothelial cells to condition culture medium. J Cell Physiol 1982;110:35-42
- Galea G, Davidson RJL. Haematological and haemorheological changes associated with cigarette smoking. J Clin Pathol 1985;38:978-84
- Galle J, Bassenge E, Busse R. Oxidized low density lipoproteins potentiate vasoconstrictions to various agonists by direct interaction with vascular smooth muscle. Circ Res 1990;66:1287-93
- Gambrell RD Jr, Teran AZ. Changes in lipids and lipoproteins with long-term estrogen deficiency and hormone replacement therapy. Am J Obstet Gynecol 1991;165:307-15
- Ganger KF, Vyas S, Whitehead M, Crook D, Heire H, Campbell S. Pulsatility index in internal carotid artery in relation to transdermal oestradiol and time since menopause. Lancet 1991;338:839-42
- Garcia MJ, McNamara PM, Gorden T, Kannel WB. Sixteen year follow-up study.

 Morbidity and mortality in diabetics in the Framingham population. Diabetes 1976;23:105-12
- Geer JC. Fine structure of human aortic intimal thickening and fatty streaks. Lab Invest 1965;14:1764-83

- Genest Jr J, McNamara JR, Ordovas JM et al. Lipoprotein cholesterol, apolipoprotein A-1 and B and lipoprotein(a) abnormalities in men with premature coronary artery disease. J Am Coll Cardiol 1992;19:792-802
- Gerova M, Smiesko V, Gero J, Bartha E. Dilatation of conduit coronary artery induced by high blood flow. Physiol Bohemoslov 1984;32:55-63
- Gerrity RG, Naito HK, Richardson M, Schwartz CL. Dietary induced atherogenesis in swine: Morphology of the intima in prelesion stages. Am J Pathol 1979;95:775-80
- Gidding SS, Rocchini AP, Moorehead C, Schork MA, Rosenthal A. Increased forearm vascular reactivity in patients with hypertension after repair of coarctation. Circulation 1985;71:495-9
- Gimbrone MA. Culture of vascular endothelium. Prog Haem Thomb 1976;3:1-28
- Glagov S, Weisenberd E, Zarins C, Stankunavicius R, Kolettis G. Compensatory enlargement of human atherosclerotic coronary arteries. N Engl J Med 1987; 316:1371-5
- Godfrey RC, Stenhouse NS, Cullen KJ, Blackman V. Studies of the cholesterol levels of Busselton school children and their parents. Aust Paediatr 1972;8:72-8
- Godsland IF, Wynn V, Crook D, Miller NE. Sex, plasma lipoproteins, and atherosclerosis: prevailing assumptions and outstanding questions. Am Heart J 1987;114:1467-503

- Goldman L, Cook EF. The decline in ischemic heart disease mortality rates: an analysis of the comparative effects of medical interventions and changes in lifestyle. Ann Intern Med 1984; 101:825-36
- Gordon DJ, Knoke J, Probstfield JL, Superko R, Tyroler HA. High density lipoprotein cholesterol and coronary heart disease in hypercholesterolemic men: The Lipid Research Clinics Coronary Primary Prevention Trial. Circulation 1986;74: 1217-25
- Gordon JB, Ganz P, Nabel EG et al. Atherosclerosis and endothelial function influence the coronary vasomotor response to exercise. J Clin Invest 1989;83:1946-52
- Gordon T, Doyle JT. Alcohol consumption and its relationship to smoking, weight, blood pressure, and blood lipids. The Albany Study. Arch Int Med 1986;146: 262-5
- Gotto AM, La Rosa JC, Hunninghake D et al. The cholesterol facts. Circulation 1990; 81:1721-31
- Graeber JE, Slott JH, Ulane RE, Schulman JD, Stuart MJ. Effect of homocysteine and homocystine on platelet and vascular arachidonic acid metabolism. Pediatr Res 1982;16:490-3
- Gray-Weale AC, Graham J, Burnett JR, Bryne K, Lusby RJ. Carotid artery atheroma.

 Comparision of preoperative B-mode ultrasound appearance with carotid endarterectomy specimen pathology. J Cardiovasc Surg 1988;29:676-83
- Green MS, Jucha E, Luz Y. Blood pressure in smokers and non-smokers:

 Epidemiologic findings. Am Heart J 1986;111:932-40

- Griffith TM, Edwards DH, Lewis MJ, Newby AC, Henderson AH. The nature of endothelium-derived relaxant factor. Nature 1984;308:645-7
- Griffith TM, Edwards DH, Collins P, Lewis MJ, Henderson AH. Endothelium derived relaxant factor. J Royal Coll Phys 1985;19:74-9
- Gross JL, Moscatelli D, Jaffe EA et al. Plasminogen activator and collagenase production by cultured capillary endothelial cells. J Cell Biol 1982;95:974-81
- Gruetter CA, Gruetter DY, Lyon JE, Kadowitz PJ, Ignarro LJ. Relationship between cyclic guanosine 3':5'-monophosphate formation and relaxation of coronary arterial smooth muscle by glyceryl trinitrate, nitroprusside, nitrite and nitric oxide: Effects of methylene blue and methemoglobin. J Pharmacol Exp Ther 1981;219:181-6
- Haarbo J, Leth-Espensen P, Stender S, Christiansen C. Estrogen monotherapy and combined estrogen/progestogen replacement therapy attenuate aortic accumulation of cholesterol in ovariectomized cholesterol-fed rabbits. J Clin Invest 1991;87: 1274-9
- Harker LA, Ross R, Slichter SJ, Scott CR. Homocystine-induced arteriosclerosis: the role of endothelial cell injury and platelet response in its genesis. J Clin Invest 1976;58:731-41.
- Harrison DG, Armstong ML, Freiman PC, Heistad DD. Restoration of endothelium-dependent relaxation by dietary treatment of atherosclerosis. J Clin Invest 1987; 80:1808-11
- Hartley CJ, Cole JS. An ultrasonic pulsed Doppler system for measuring blood flow in small vessels. J Appl Physiol 1974;34:626-9

- Healy B. Endothelial cell dysfunction: an emerging endocrinopathy linked to coronary disease. J Am Coll Cardiol 1990;16:357-8
- Heinecke JW, Rosen H, Suzuki LA, Chait A. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. J Biol Chem 1987;262:1098-103
- Heistad DD, Abboud FM, Eckstein JW. Vasoconstrictor response to simulated diving in man. J Appl Physiol 1968;25:542-9
- Hekman CM, Loskutoff DJ. Fibrinolytic pathways and the endothelium. Seminars Haemostasis Thrombosis 1987;13:514-27
- Henderson AH. Endothelium in control. Br Heart J 1991;65:116-25
- Hinkley DV. Inference in two-phase regression. J Am Stat Assoc 1971;66:736-43
- Hintze TH, Vatner SF. Reactive dilation of large coronary arteries in conscious dogs. Circ Res 1984;54:50-7
- Hirai T, Sasayama S, Kawasaki T, Yagi S. Stiffness of systemic arteries in patients with myocardial infarction. Circulation 1989;80:78-86
- Hokanson DE, Mozersky DJ, Sumner DS et al. Ultrasonic arteriography: A new approach to arterial visualisation. Biomed Eng 1971;6:420

- Holbrook JS, Grundy SM, Hennekens CH, Kannel WB, Strong JP. Cigarette smoking and cardiovascular diseases: A statement for health professionals by a task force appointed by the steering committee of the American Heart Association. Circulation 1984;70:1114A-7A
- Holman RL, McGill HC Jr, Strong JP, Geer JC. The natural history of atherosclerosis: the early aortic lesions as seen in New Orleans in the middle of the 20th century.

 Am J Pathol 1958;4:209-35
- Holtz J, Forstermann U, Pohl U, Giesler M, Bassenge E. Flow-dependent, endothelium-mediated dilation of epicardial coronary arteries in conscious dogs: effects of cyclooxygenase inhibition. J Cardiovasc Pharmacol 1984;6:1161-9
- Hunninghake DB, Stein EA, Dujovne CA et al. The efficacy of intensive dietary therapy alone or combined with lovastatin in outpatients with hypercholesterolemia. N Engl J Med 1993;328:1213-19.
- Ignarro LJ, Byrns RE, Wood KS. Biochemical and pharmacological properties of endothelium-derived relaxing factor and its similarity to nitric oxide radical. In Vasodilation: Vascular smooth muscle, peptides, autonomic nerves and endothelium. Vanhoutte PM (ed).Raven Press, New York, 1988;427-36
- Ingebrigtsen R, Leraand S. Dilatation of a medium-sized artery immediately after local changes of blood pressure and flow as measured by ultrasonic techniques. Acta Physiol Scand 1970;79: 552-8
- Jacobs HS, Loeffler FE. Postmenopausal hormone replacement therapy. Br Med J 1992;305:1403-8

- Jacobs M, Plane F, Bruckdorfer KR. Native and oxidized low-density lipoproteins have different inhibitory effects on endothelium-derived relaxing factor in the rabbit aorta. Br J Pharmacol 1990;100:21-6
- Jaffe EA (ed). Biology of endothelial cells. Martinus Nijhoff, Boston, 1984
- Jaffe EA. Cell biology of endothelial cells. Human Pathol 1987;18:234-9
- Jager KA, Phillips DJ, Martin RL et al. Noninvasive mapping of lower limb arterial lesions. Ultrasound Med Biol 1985;11:515-21
- Jenner JL, Ordovas JM, Lamon-Fava S et al. The effects of age, sex and menopausal status on plasma Lp(a) levels. Circulation 1993;87:1135-41
- Johnson EL, Yock PG, Hargrave VK et al. Assessment of severity of coronary stenosis using Doppler catheter. Circulation 1989;80:625-35
- Johnson JM, Kenelly MM, Decasare D, Morgan S, Sparrow A. Natural history of asymptomatic carotid plaque. Arch Surg 1985;120:1010-2
- Jorde LB, Williams RR. Relation between family history of coronary disease and coronary risk variables. Am J Cardiol 1988;62:708-13
- Kaiser L, Hull SS, Sparks NV Jr. Methylene blue and ETYA block flow-dependent dilation in canine femoral artery. Am J Physiol 1986;250:348-52
- Kang S-S, Wong PWK, Cook HY, Norusla M, Messer JV. Protein-bound homocyst(e)ine: a possible risk factor for coronary artery disease. J Clin Invest 1986;77:1482-6

- Kannel WB. Hypertension, blood lipids and cigarette smoking as co-risk factors for coronary heart disease. Ann NY Acad Sci 1978;304:128-39
- Kannel WB, D'Agostino RB, Belanger AJ. Fibrinogen, cigarette smoking, and the risk of cardiovascular disease. Am Heart J 1987;13:1006-10
- Kaplan NM. Cardiovascular risk reduction: The role of antihypertensive treatment. Am J Med 1991; 90:19s-20s
- Kaufman DW, Helmrich SP, Rosenberg L et al. Nicotine and carbon monoxide content of cigarette smoke and the risk of myocardial infarction in young men. N Engl J Med 1983;308:409-13
- Keys A. Coronary heart disease in seven countries. Circulation 1970;41(suppl I)
- Kimm SYS, Payne GH, Lakatos E, Webber LS, Greenblatt J. Primary care physicians and children's blood cholesterol. Prev Med 1992;21:191-202
- Klag MJ, Ford DE, Mead LA, He J, Whelton PK, Liang K-Y, Levine DM. Serum cholesterol in young men and subsequent cardiovascular disease. N Engl J Med 1993;328:313-18
- Kleinman KC, Donahue RP, Harris MI et al. Mortality among diabetics in a National Health sample. Am J Epidemiol 1988;128:389-401
- Knopp RH. The effects of postmenopausal estrogen therapy on the incidence of arteriosclerotic vascular disease. Obstet Gynecol 1988;72(suppl):s23-30

- Kohler TR, Nance DR, Cramer MM, Vandenburghe N, Strandness DE. Duplex scanning for diagnosis of aortoiliac and femoropopliteal disease: a prospective study. Circulation 1987;76:1074-80
- Kohn RR. Heart and Cardiovascular system. In: Handbook of the Biology of Ageing. Finch CE, Hayflink L (eds). Van Nostrand Reinhold Company, New York, 1977.
- Komori K, Shimokawa H, Vanhoutte PM. Hypercholesterolemia impairs endothelium-dependent relaxations to aggregating platelets in porcine iliac arteries. J Vasc Surg 1989;10:318-25
- Koren MJ, Devereux RB, Casale PN et al. Relation of left venticular mass and geometry to morbidity in uncomplicated essential hypertension. Ann Intern Med 1991;114: 345-52
- Koseki C, Imai M, Hirata Y, Yanagisawa M, Masaki T. Autoradiographic distribution in rat tissues of binding sites for endothelin: a neuropeptide? Am J Physiol 1989; 256:R858-66
- Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent relaxation by lysolecithin in modified low-density lipoproteins. Nature 1990;344:160-2
- Kuller LH. Epidemiology of cardiovascular disease: Current perspectives. Am J Epidemiol 1976;104:425-96
- Kwiterovich PO, Levy RI, Fredrickson DS. Neonatal diagnosis of familial type II hyperlipoproteinemia. Lancet 1973:1:118-21

- Kwiterovich PO Jr. Diagnosis and management of familial dyslipoproteinemia in children and adolescents: current issues in pediatric and adolescent endocrinology. Pediatric Clin North Am 1990;37:1489-1523
- Lauer RM, Connor WE, Leaverton PE, Reiter M, Clarke WR. Coronary heart disease risk factors in school children The Muscatine Study. J Pediatr 1975;86:697-706
- Laurent S, Lacolley P, Brunel P, Laloux B, Pannier B, Safar M. Flow-dependent vasodilation of brachial artery in essential hypertension. Am J Physiol 1990;258:H1004-11
- Leary T. The genesis of atherosclerosis. Arch Pathol 1941;32:505-18
- Levenson JA, Peronneau PP, Simon A, Safar ME. Pulsed Doppler: determination of diameter, blood flow velocity and volumic flow of brachial artery in man. Cardiovasc Res 1981;15: 164-70
- Lewis BD, James EM, Welsh TJ. Current applications of duplex and color Doppler ultrasound imaging: carotid and peripheral vascular system. Mayo Clin Proc 1989;64:1147-57
- Liao JK, Bettman MA, Sandor T, Tucker JI, Coleman SM, Creager MA. Differential impairment of vasodilator responsiveness of peripheral resistance and conduit vessels in humans with atherosclerosis. Circ Res 1991;68:1027-34
- Lichtlen PR, Nikutta P, Jost S, Deckers J, Wiese B, Rafflenbeul W. Anatomical progression of coronary artery disease in humans as seen by prospective, repeated, quantitated coronary angiography. Circulation 1992;86:828-38

- Lie M, Sejersted OM, Kiil F. Local regulation of vascular cross section during changes in femoral arterial blood flow in dogs. Circ Res 1970;27:727-37
- Lipid Research Clinics Program. The Lipid Research Clinics Primary Prevention Trial results. JAMA 1984;251:351-64
- Ludmer PL, Selwyn AP, Shook TL et al. Paradoxical vasoconstriction induced by acetycholine in atherosclerotic coronary arteries. N Engl J Med 1986;315:1046-51
- Luscher TF, Vanhoutte PM, Raij L. Antihypertensive treatment normalizes decreased endothelium-dependent relaxations in rats with salt-induced hypertension. Hypertension 1987;9(suppl III): III193-7
- Macho P, Hintze TH, Vatner SF. Regulation of large coronary arteries by increases in myocardial metabolic demands in conscious dogs. Circ Res 1981;76:594-9
- Machovich R. Choices among the possible reaction routes catalysed by thrombin. Ann N Y Acad Sci 1986;485:170-83
- Madri JA, Pratt BM, Yannariello-Brown J. Matrix-driven cell size change modulates aortic endothelial cell proliferation and sheet migration. Am J Pathol 1988;132: 18-27
- Magness RR, Rosenfeld CR. Local and systemic estradiol-17 beta: effects on uterine and systemic vasodilation. Am J Physiol 1989;256:E536-42
- Malinow MR, Kang SS, Taylor LM, et al. Prevalence of hyperhomocyst(e)inemia in patients with peripheral arterial occlusive disease. Circulation 1989;79:1180-8

- Manning WJ, Li W, Edelman RR. A preliminary report comparing magnetic resonance coronary angiography with conventional angiography. N Engl J Med 1993;328: 828-32
- Mantovani A, Dejana E. Modulation of endothelial function by interleukin-1. A novel target for pharmacolgical intervention? Biochem Pharmacol 1987;36:301-5
- Maron BJ, Humphries JO, Rowe RD, Mellits ED. Prognosis of surgically corrected coarctation of the aorta: A 20-year postoperative appraisal. Circulation 1973;47: 119-26
- Martin W, Villani GM, Jothianandan D, Furchgott RF. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. J Pharmacol Exp Ther 1985;232:708-16
- Masaki T. The discovery, the present state, and the future prospects of endothelin. J Cardiovasc Pharmacol 1989;13(suppl 5);s1-4
- Masaryk AM, Ross JS, DiCello MC, Modic MT, Parandi L, Masaryk TJ. 3DFT MR angiography of the carotid bifurication: potential and limitations as a screening examination. Radiology 1991;179:797-804
- Matthews KA, Meilahn E, Kuller LH et al. Menopause and risk factors for coronary artery disease. N Engl J Med 1989;321:641-6
- McCully KS, Carvalho AC. Homocysteine thiolactone, N-homocyteine thiolactonyl retinamide, and platelet aggregation. Res Commun Chem Pathol Pharmacol 1987;56:349-60

- McGill HC Jr. The geographic pathology of atherosclerosis. Williams and Wilkins, Baltimore, 1968
- McGill HC Jr. Persistent problems in the pathogenesis of atherosclerosis.

 Arteriosclerosis 1984;4:443-51
- McGill HC Jr. The cardiovascular pathology of smoking. Am Heart J 1988;115:250-7
- McLenachan JM, Vita J, Fish RD et al. Early evidence of endothelial vasodilator dysfunction at coronary branch points. Circulation 1990;82:1169-73
- McLenachan JM, Williams JK, Fish RD, Ganz P, Selwyn AP. Loss of flow-mediated endothelium-dependent dilation occurs early in the development of atherosclerosis. Circulation 1991;84:1273-8
- McPherson DD, Hiratzka LF, Lamberth WC et al. Delineation of the extent of coronary atherosclerosis by high-frequency epicardial echocardiography. N Engl J Med 1987;316:304-9
- Meade TW, Mellows S, Brozovic M et al. Haemostatic function and ischaemic heart diseases. Principle results of the Northwick Park Heart Study. Lancet 1986a;2: 533-7
- Meade TW, Vickers MV, Thompson SG, Stirling Y, Haines AP, Miller GJ.

 Epidemiological characteristics of platelet aggregability. Br Med J 1986b;290:

 428-32
- Meade TW, Imeson J, Stirling Y. Effects of changes in smoking and other characteristics on clotting factors and the risk of ischemic heart disease. Lancet 1987;2:986-90

- Meister JJ, Tardy Y, Stergiopulos N, Hayoz D, Brunner HR Etienne J. Non-invasive method for the assessment of non-linear elastic properties and stress of forearm arteries in vivo. J Hypertens 1992;10(suppl 6):S23-6
- Melkumyants AM, Balashov SA, Veselova ES, Khayutin VM. Continuous control of the lumen of feline conduit arteries by blood flow rate. Cardiovasc Res 1987;21: 863-70
- Mickelson JK, Simpson PJ, Lucchesi BR. Ischemic Heart Disease: pathophysiology and pharmacologic management. In: Cardiovascular Pharmacology (3rd edn). Antonaccio M (ed). Raven Press, New York, 1990;293-340.
- Miller VM, Aarhus LL, Vanhoutte PM. Modulation of endothelium-dependent responses by chronic alterations of blood flow. Am J Physiol 1986;251:H520-7
- Minick CR, Stemmerman MB, Insull W. Effect of regenerated endothelium on lipid accumulation in arterial wall. Proc Nat Acad Sci 1977;74:1724-8
- Mintz GS, Potkin PN, Cooke RH et al. Intravascular ultrasound imaging in a patient with unstable angina. Am Heart J 1992;123:1692-4
- Mjos OD. The lipid effects of smoking. Am Heart J 1988;115:272-5
- Moncada S, Herman AG, Higgs EA, Vane JR. Differential formation of prostacyclin (PGX or PGI2) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium. Thromb Res 1977;11:323-44
- Moncada S. Biological importance of prostacyclin. Br J Pharmacol 1982;76:3-31

- Moncada S, Palmer RMJ, Gryglewki RJ. Mechanism of action of some inhibitors of endothelium-derived relaxing factor. Proc Natl Acad Sci 1986;83:9164-8
- Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Reviews 1991;43:109-42
- Moneta GL, Taylor DC, Nicholls SC et al. Operative versus non-operative management of asymptomatic high-grade internal carotid artery stenosis: Improved results with endarterectomy. Stroke 1987;18:1005-10
- Moneta GL, Taylor DC, Zieler RE, Kazmers A, Beach K, Strandness DE Jr.

 Asymptomatic high-grade intermal carotid stenosis: Is stratification according to risk factors or duplex speectral analysis possible? J Vasc Surg 1989;10:475-83
- Moore S. Thromboatherosclerosis in normolipemic rabbits; a result of continued endothelial damage. Lab Invest 1973;29:478-87
- Mooser V, Etienne JD, Farine PA et al. Non-invasive measurement of internal diameter of peripheral arteries during the cardiac cycle. J Hypertens 1988;6(suppl 4):179-81
- Morrison JA, de Groot I, Edwards BK et al. Plasma cholesterol and triglyceride levels in 6775 school children, ages 6-17. Metabolism 1977;26:1199-1211
- Moskowitz WB, Schieken RM, Mosteller M, Bossano R. Altered systolic and diastolic function in children after "successful" repair of coarctation of the aorta. Am Heart J 1990;120:103-9

- MRFIT Research Group. The Multiple Risk Factor Intervention Trial (MRFIT). A national study of primary prevention of coronary heart disease. JAMA 1976;235: 825-7
- MRFIT Research Group: Multiple risk factor intervention trial: risk factor changes and mortality results. JAMA 1982;248:1465-77
- Mudd SH, Havlik R, Levy HL et al. A study of cardiovascular risk in heterozygotes for homocystinuria. Am J Hum Genet 1981;33:883-93
- Mudd SH, Skovby F, Levy HL et al. The natural history of homocystinuria due to cystathionine β-synthase deficiency. Am J Hum Genet 1985;37:1-31
- Mudd SH, Levy HL, Skovby FD. Disorders of transulfuration. In: The metabolic basis of inherited disease. 6th edn. Vol 1. Scriver C, Beaudet AL, Sly WS, Valle D (eds). McGraw-Hill, New York, 1989;693-734
- Mugge A, Heublin B, Kuhn M et al. Impaired coronary dilator responses to substance P and impaired flow-dependent dilator responses in heart transplant patients with graft vasculopathy. J Am Coll Cardiol 1993;21:163-70
- Murad F, Mittal CK, Arnold WP, Katsuki S, Kimura H. Guanylate cyclase: activation by azide, nitro compounds, nitric oxide and hydroxyl radical and inhibition by hemoglobin and myoglobin. Adv Cyclic Nucleotide Res 1978;9:145-58
- Myers PR, Minor RL, Guerra R Jr, Bates JN, Harrison DG. Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature 1990;345:161-3

- Nabel EG, Ganz P, Gordon JB, Alexander RW, Selwyn AP. Dilation of normal and constriction of atherosclerotic coronary arteries caused by the cold pressor test.

 Circulation 1988;77:43-52
- Nabel EG, Selwyn AP, Ganz P. Large coronary arteries in humans are responsive to changing blood flow: an endothelium-dependent mechanism that fails in patients with atherosclerosis. J Am Coll Cardiol 1990;16:349-56
- Nase-Hueppmeier S, Uebis R, Doerr R, Hanrath P. Intravascular ultrasound to assess aortocoronary venous bypass grafts in vivo. Am J Cardiol 1992;70:455-8
- Nathan CF, Murray HW, Cohn ZA. Current concepts: The macrophage as an effector cell. N Engl J Med 1980;303:622-4
- Newman TB, Browner WS, Hulley WS. Childhood cholesterol screening:
 Contraindicated. JAMA 1992;267:100-1
- Nikkila EA, Viikinkoski P, Valle M, Frick MH. Prevention of progression of coronary atherosclerosis by treatment of hyperlipidaemia: A seven year prospective angiographic study. Br Med J 1984;289:220-3
- Nishimura RA, Reeder GS. Intravascular ultrasound-Research techinique or clinical tool? Circulation 1992;86:322-4
- Nolsoe CP, Engel U, Karstrup S, Torp-Pedersen S, Garre K, Holm HH. The aortic wall: an in vitro study of the double-line pattern in high-resolution US.

 Radiology 1990;175:387-90

- Nowak J, Murray JJ, Oates JA, FitzGerald GA. Biochemical evidence of a chronic abnormality in platelet and vascular function in healthy individuals who smoke cigarettes. Circulation 1987; 76:6-14
- Olesen SP, Clapham DE, Davies PF. Haemodynamic shear stress activates a K+ current in vascular endothelial cells. Nature 1988;331:168-70
- Olsson AG. Regression of femoral atherosclerosis. Circulation 1991;83:698-700
- Ornish D, Brown SE, Scherwitz LW et al. Can lifestyle changes reverse coronary heart disease? The Lifestyle Heart Trial. Lancet 1990;336:129-33
- Osborne JA, Siegman MJ, Sehar AW, Mooers SV, Lefer AM. Lack of endothelium-dependent relaxation in coronary resistance arteries of cholesterol-fed rabbits.

 Am J Physiol 1989;256:C591-7
- Owen RS, Carpenter JP, Baum RA, Perloff LJ, Cope C. Magnetic resonance imaging of angiographically occult runoff vessels in peripheral arterial occlusive disease.

 N Engl J Med 1992;326:1577-81
- Paffenbarger RS Jr, Wing AL, Hyde RT. Physical activity as an index of heart attack risk in college alumni. Am J Epidemiol 1978;108:161-7
- Palade GE. Fine structure of blood capillaries. J Appl Physiol 1953;24:1424-30
- Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cells synthesise nitric oxide from L-arginine. Nature 1988;333:664-6

- Panza JA, Quyyumi AA, Brush JE Jr, Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N Engl J Med 1990; 323:22-7
- Panza JA, Quyyumi AA, Callahan TS, Epstein SE. Effect of antihypertensive treatment on endothelium-dependent vascular relaxation in patients with essential hypertension. J Am Coll Cardiol 1993;21:1145-51
- Parker JO, Vankoughnett KA, Farrell B. Nitroglycerin lingual spray: clinical efficacy and dose-response relation. Am J Cardiol 1986;57:1-5
- Parthasarathy S. Oxidation of low desnity lipoprotein by thiol compounds leads to its recognition by the acetyl LDL receptor. Biochem Biophys Acta 1987;917:337-40
- PDAY investigators. Relationship of atherosclerosis in young men to serum lipoprotein cholesterol concentrations and smoking. A preliminary report from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group. JAMA 1990;264:3018-24
- Pearson AC, Gudipati CV, Labovitz AJ. Effects of aging on left ventricular structure and function. Am Heart J 1991;121:871-5
- Pearson JD, Gordon JL. Nucleotide metabolism by endothelium. Ann Rev Physiol 1985;47:617-27
- Petty RG, Pearson JD. Endothelium-the axis of vascular health and disease. J Royal Coll Phys 1989; 23:92-101

- Pignoli P, Tremoli E, Pili A, Oreste P, Paoletti R. Intimal plus medial thickness of the arterial wall: a direct measurement with ultrasound imaging. Circulation 1986; 74:1399-406
- Pittilo RM, Clarke JM, Harris D, Mackie IJ, Rowles PM, Machin SJ, Woolf N. Cigarette smoking and platelet adhesion. Br J Haematol 1984;58:627-32
- Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow in vivo. Hypertension 1986;8:37-44
- Presbitero P, Demarie D, Villani M et al. Long term results (15-30 years) of surgical repair of aortic coarctation. Br Heart J 1987;57:462-7
- Proudfit WL, Shirley EK, Sones FM. Selective cine coronary arteriography correlation with findings in 1,000 patients. Circulation 1966;33:901-10
- Raby KE, Goldman L, Creager MA et al. Correlation between preoperative ambulatory ischaemia and major cardiac events after peripheral vascular surgery. N Engl J Med 1989;321:1296-300
- Ragland DR, Brand RJ. Type A behavior and mortality from coronary heart disease. N Engl J Med 1988;318:65-9
- Ratliff DA, Gallagher PJ, Hames TK et al. Characterization of carotid artery disease:

 Comparision of duplex scanning with histology. Ultrasound Med Biol 1985;11:
 835-40
- Reidy MA, Schwartz SM. A technique to investigate surface morphology and endothelial cell replication of small arteries; a study in acute angiotensin-induced hypertension. Microvasc Res 1982;24:158-67

- Reilly LM, Lusby RJ, Hughes L, Ferrell LD, Stoney RJ, Ehrenifeld WK. Carotid plaque histology using real time ultrasonography. Clinical and Therapeutic Implications. Am J Surg 1983;146:188-93
- Reneman RS, Van Merode T, Hick P, Muytjens MM, Hoeks APG. Age-related changes in carotid wall properties in man. Ultrasound Med Biol 1986;12:465-71
- Renkin EM. Multiple pathways of capillary permeability. Circ Res 1977;41:735-43
- Richardson P, Davies M, Born G. Influence of plaque configuration and stress distribution on fissuring of coronary atherosclerotic plaques. Lancet 1989;2:941-4
- Ricketts HJ, Abrams HL. Percutaneous selective coronary cine arteriography. JAMA 1962;181:620-4
- Ricou FJ, Murata K, Oh BH, Kambayashi M, Peterson KL. Evaluation of inotropic effect of endothelin-1 in vivo. J Cardiovasc Pharmacol 1992;20:671-7
- Rival J, Riddlddle JM, Stein PD. Effects of chronic smoking on platelet function.

 Thrombosis Res 1987;45:75-85
- Roederer GO, Langois YE, Lusiani L et al. Natural history of carotid artery disease on the side contralateral to endarterectomy. J Vasc Surg 1984a;1:62-72
- Roederer GO, Strandness DE Jr. A simple spectral parameter for the classification of severe carotid disease. Bruit 1984b;8:174-8
- Rogot E, Murray JL. Smoking and causes of death among US veterans: 16 years of observation. Pub Health Rep 1980;95:213-22

- Rosenberg L, Kaufman DW, Helmrich SP, Shapiro S. The risk of myocardial infarction after quitting smoking in men under 55 years of age. N Engl J Med 1985;313: 1511-4
- Rosenthal J. Aging and the cardiovascular system. Gerontology 1987;33(Suppl I):3-8
- Ross R, Glomset JA. Atherosclerosis and the arterial smooth muscle cell. Science 1973;180:1332-39
- Ross R, Glomset JA. The pathogenesis of atherosclerosis. N Engl J Med 1976;295:369-
- Ross R. Atherosclerosis-a problem of the biology of arterial wall cells and their interaction with blood components. Arteriosclerosis 1981;1:293-311
- Ross R, Wright TN, Strandness E, Thiele B. Human atherosclerosis: I. Cell constitution and characterisics of advanced lesions of the superficial femoral artery. Am J Pathol 1984;114: 79-93
- Ross R. The pathogenesis of atherosclerosis an update. N Engl J Med 1986;8:488-500
- Ross R. The pathogenesis of atherosclerosis. In: Heart Disease (4th edn). Braunwald E (ed). WB Saunders Company, Philadelphia, 1992;1106-24
- Royal College of Physicians. Smoking and the Young. A report of a working party of the Royal College of Physicians. London 1992;41-53
- Rubanyi GM, Lorenz RR, Vanhoutte PM. Bioassay of endothelium-derived relaxing factor(s): inactivation by catecholamines. Am J Physiol 1985a;249:H95-101

- Rubanyi GM, Vanhoutte PM. Hypoxia releases a vasoconstrictor substance from the canine vascular endothelium. J Physiol 1985b;364:46-56
- Rubanyi GM, Romero C, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 1986;250:1115-9
- Rubanyi GM, Freay AD, Kauser K, Johns A, Harder DR. Mechanoreception by endothelium: mediators and mechanisms of pressure-and flow-induced vascular responses. Blood Vessels 1990;27:246-57
- Ruffer MA. On arterial lesions found in Egyptian mummies. J Pathol Bacteriol 1911;15:453-62
- Ryan JW, Ryan US. Endothelial surface enzymes and the dynamic processing of plasma substrates. Int Rev Exp Pathol 1984;26:1-43
- Safar ME, Peronneau PA, Levenson JA, Toto-Moukouo JA, Simon AC. Pulsed Doppler: Diameter, blood flow velocity and volumic flow of the brachial artery in sustained essential hypertension. Circulation 1981;63:393-400
- Sage H. Collagen synthesis by endothelial cells in culture. In Jaffe EA (ed). Biology of endothelial cells. Martinus Nijhoff, Boston, 1984:161-77
- St Goar FG, Pinto FJ, Alderman EL et al. Intracoronary ultrasound in cardiac transplant recipients. Circulation 1992;85:979-87
- Salonen JT, Salonen R. Ultrasound B-mode imaging in observational studies of atherosclerotic progression. Circulation 1993;87(suppl II):II56-65

- Salonen R, Salonen JT. Determinants of carotid intima-media thickness: a population based ultrasonography study in Eastern Finnish men. J Int Med 1991;229:225-31
- Samanek M, Goetzova J, Fiserova J, Skovranek J. Differences in muscle blood flow in upper and lower extremities of patients after correction of coarctation of the aorta. Circulation 1976;54:377-81
- Sardharwalla IB, Fowler B, Robins AJ, Komrower GM. Detection of heterozygotes for homocystinuria: study of sulphur-containing amino acids in plasma and urine after L-methionine loading. Arch Dis Child 1974;49:553-9
- Scanu AM. Lipoprotein(a): A genetic risk factor for premature coronary heart diasease.

 JAMA 1992;267:3326-9
- Schnaar RL, Sparks HV. Response of large and small coronary arteries to nitroglycerine, NaNO₂, and adenosine. Am J Physiol 1972;223:223-8
- Schretzenmayr A. Uber kreislaufregulatorische Vorgange an den grossen Arterien bei der Muskelarbeit. Pfugers Arch 1933;232:743-8
- Schultz KD, Schultz K, Schultz G. Sodium nitroprusside and other smooth muscle relaxants increase cyclic GMP levels in rat ductus deferens. Nature 1977;265: 750-1
- Schymer TE, Popma JJ, Kent KM, Leon MB, Eldridge S, Mintz GS. Use of intracoronary ultrasound to identify the "true" coronary lumen in chronic coronary dissection treated with intracoronary stenting. Am J Cardiol 1992;69: 1107-8

- Seed M, Hopplicher F, Reaveley D et al. Relation of serum lipoprotein(a) concentration and apolipoprotein(a) phenotype to coronary heart disease in patients with familial hypercholesterolemia. N Engl J Med 1990;322:1494-9
- Sehested J, Baandrup U, Mikkelsen E. Different reactivity and structure of the prestenotic and poststenotic aorta in human coarctation: Implications for baroreceptor function. Circulation 1982;65:1060-5
- Sellke FW, Armstrong ML, Harrison DG. Endothelium-dependent vascular relaxation is abnormal in the coronary microcirculation of atherosclerotic primates. Circulation 1990;81:1586-93
- Shirasaki Y, Su C, Lee TJ-K, Kolm P, Cline WH, Nickols GA. Endothelial modulation of vascular relaxation to nitrovasodilators in aging and hypertension. J Pharmacol Exp Therap 1986;239:861-6
- Sibley DH, Millar HD, Harley CJ, Whitlow PL. Subselective measurement of coronary blood flow velocity using a steerable Doppler catheter. J Am Coll Cardiol 1986; 8:1332-40
- Simon BC, Cunningham LD, Cohen RA. Oxidized low density lipoprotein causes contraction and inhibits endothelium-dependent relaxation in the pig coronary artery. J Clin Invest 1990;86:75-9
- Simon Broome Register. Scientific Steering Committee on behalf of the Simon Broome Register Group. Risk of fatal coronary heart disease in familial hypercholesterolaemia. Br Med J 1991;303:893-6
- Simons LA. Interrelations of lipids and lipoproteins with coronary artery disease mortality in 19 countries. Am J Cardiol 1986;57:5G-10G

- Simsolo R, Grunfeld B, Gimenez M et al. Long-term systemic hypertension in children after successful repair of coarctation of the aorta. Am Heart J 1988;115:1268-73
- Sinaiko AR, Wells TG. Childhood hypertension. In: Hypertension. Laragh JH, Brenner BM(eds). Raven Press, New York, 1990;1855-68
- Singer HA, Peach MJ. Calcium-and endothelial-mediated vascular smooth muscle relaxation in rabbit aorta. Hypertension 1982;4(suppl II):II19-25
- Sinoway LI, Hendrickson C, Davidson WR, Prophet S, Zelis R. Characteristics of flow-mediated brachial artery vasodilation in human subjects. Circ Res 1989;64:32-42
- Smiesko V, Kozik J, Dolezel S. Role of endothelium in the control of arterial diameter by blood flow. Blood Vessels 1985;22:247-51
- Smulyan H, Csermely TJ, Mookherjee S, Warner RA. Effect of age on arterial distensibility in asymptomatic humans. Arteriosclerosis 1983;2:199-205
- Solberg LA, Strong JP. Risk factors and atherosclerotic lesions. Arteriosclerosis 1983;3:187-98
- Sones FM Jr. Cine-cardio-angiography. Pediatr Clin N America 1958;5:945-79
- Sones FM Jr, Shirey EK Cine coronary arteriography. Mod Concepts Cardiovasc Dis 1s 1962;31: 735-8
- Spears JR, Sandor T, Als AV et al. Computerized image analysis for quantitative measurement of vessel diameter from cineangiograms. Circulation 1983;68:453-61.

- Stamler J, Berkson DM, Lindberg HA. Risk factors: Their role in the etiology and pathogenesis of the atherosclerotic diseases. The Pathogenesis of Atherosclerosis. Williams and Wilkins, Baltimore, 1962;41
- Stamler J, Osborne JA, Jaraki O et al. Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen.

 J Clin Invest 1993;91:308-18
- Stamler J, Wentworth D, Neaton JD et al. Is relationship between serum cholesterol and risk of premature death from coronary heart disease continuous and graded?

 JAMA 1986;256:2823-8
- Stary HC. Evolution of atherosclerosis plaques in the coronary arteries of young adults.

 Arteriosclerosis 1983;3:471a-80a
- Stary HC. Evolution and progression of atherosclerotic lesions in coronary arteries in children and young adults. Arteriosclerosis 1989;99(Suppl I):I-19-32
- Steinberg DS. Lipoproteins and the pathogenesis of atherosclerosis. Circulation 1987;76: 508-14
- Stern D, Brett J, Harris IC, Nawroth PP. Participation of endothelial cells in the protein C-protein S anticoagulant pathway. J Cell Biol 1986;102:1971-8
- Stiel G, Steil L, Schofer J, Konath K, Mathey D. Impact of compensatory enlargement of atherosclerotic coronary arteries on angiographic assessment of coronary artery disease. Circulation 1989;80:1603-9
- Stiles CD. The molecular biology of platelet-derived growth factor. Cell 1983;33:653-5

- Stolar MW. Atherosclerosis in diabetes. The role of hyperinsulinemia. Metabolism 1988;37 (suppl 1):J1-9
- Strong JP, McGill HC Jr. The pediatric aspects of atherosclerosis. J Atheroscler Res 1969;9:251-65
- Strong JP, Richards ML. Cigarette smoking and atherosclerosis in autopsied men. Atherosclerosis 1976;23:451-76
- Sugiishi M, Takatsu F. Cigarette smoking is a major risk factor for coronary spasm.

 Circulation 1993;87:76-9
- Sugimoto T, Tobian L, Ganguli MC. High potassium diets protect against dysfunction of endothelial cells in stroke-prone spontaneously hypertensive rats. Hypertension 1988;11:579-85
- Svendsen KH, Kuller LH, Martin MJ, Ockene JK. Effects of passive smoking in the Multiple Risk Factor Intervention Trial. Am J Epidemiol 1987;126:783-95
- Tamir I, Heiss G, Glueck CJ et al. Lipid and lipoprotein distribution in white children aged 6-19 years. The Lipid Research Clinics program prevalence study. J Chronic Dis 1981;34:27-39
- Tanaglia AN, Buller CE, Kisso KB, Stack RS, Davidson CJ. Mechanisms of balloon angioplasty and directional coronary atherectomy as assessed by intracoronary ultrasound. J Am Coll Cardiol 1992;20:685-91
- Taylor SG, Weston AH. Endothelium-derived hyperpolarizing factor: a new endogenous inhibitor from the vascular endothelium. Trends Pharmacol Sci 1988;9:272-4

- Thompson EL. Smoking education programmes, 1960-76. Am J Public Health 1978; 68:250-7
- Tiwari AK, Gode JD, Dubey GP. Effect of cigarette smoking on serum total cholesterol and HDL in normal subjects and coronary heart disease patients. Indian Heart J 1989;41:92-4
- Tobis JM, Mallery J, Mahon D et al. Intravascular ultrasound imaging of human coronary arteries in vivo. Circulation 1991;83:913-26
- Tomita T, Ezaki M, Miwa M, Nakamura K, Inoue Y. Rapid and reversible inhibition by low density lipoprotein of the endothelium-dependent relaxation to hemostatic substances in porcine coronary arteries. Circ Res 1990;66:18-27
- Trap-Jensen J. Effects of smoking on the heart and peripheral circulation. Am Heart J 1988;115:263-7
- Treasure CB, Vita JA, Cox DA et al. Endothelium-dependent dilation of the coronary microvasculature is impaired in dilated cardiomyopathy. Circulation 1990;81: 772-9
- Ueland PM, Helland S, Broch OJ, Schanche J-S. Homocysteine in tissues of the mouse and rat. J Biol Chem 1984;259:2360-5
- US Surgeon General. The Health Consequences of Smoking: Cancer. A report of the Surgeon General. US Department of Health and Human Services, Public Health Service, Office on Smoking and Health, Washington DC, 1982, DHHS (PHS); 82-50179

- US Surgeon General. The Health Consequences of Smoking: Cardiovascular Disease.

 A report of the Surgeon General. US Department of Health and Human Services,

 Office on Smoking and Health, Washington DC, 1983, DHHS (PHS);84-50204
- US Surgeon General. The Health Benefits of Smoking Cessation. A report of the Surgeon General. US Department of Health and Human Services, Office on Smoking and Health, Washington DC, 1990. DHHS (PHS);191-7
- Vallance P, Collier J, Moncada S. Effects of endothelium-derived nitric oxide on peripheral arteriolar tone in man. Lancet 1989;2:103-10
- Van Grondelle A, Worthen GS, Ellis D et al. Altering hydrodynamic variables influences PGI₂ production by isolated lungs and endothelial cells. J Appl Physiol 1984;57:388-95
- Van Merode T, Hick PJJ, Hoeks APG, Reneman RS. Noninvasive assessment of artery wall properties in children aged 4-19 years. Pediatr Res 1989;25:94-6
- Verbeuren TJ, Jordaens FH, Zonnekeyn LL, Van Hove CE, Herman AG. Effect of hypercholesterolemia on vascular reactivity in the rabbit. I. Endothelium-dependent and endothelium-independent contractions and relaxations in isolated arteries of control and hypercholesterolemic rabbits. Circ Res 1986;58:552-64
- Virchow R. Phlogose und thrombose in gefassustem, gesammelte abhandlungen zur wissenschaftlichen medicin. Meidinger Sohn and Co, Frankfurt-am-Main, 1856; 458
- Vita JA, Treasure CB, Nabel EG et al. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. Circulation 1990;81:491-7

- Vogel RA, Vaitkevicius PV, Plotnick GD. Ultrasound assessment of brachial artery endothelial-dependent vasoactivity as a means for diagnosing coronary artery disease (abstr). J A Coll Cardiol 1993;21:345A
- Wagner JD, Clarkson TB, St Clair RW, Schwenke DC, Shively CA, Adams MR. Estrogen and progesterone therapy reduces low-density lipoprotein accumulation in the coronary arteries of surgically postmenopausal cynomolgus monkeys. J Clin Invest 1991;88:1995-2002
- Wall RT, Harlan JM, Harker LA, Striker GE. Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. Thromb Res 1980;18: 113-21
- Waller BF, Pinkerton CA, Slack JD. Intravascular ultrasound: a histological study of vessels during life. Circulation 1992;85:2305-10
- Warrens AN, Cassidy MJD, Takahashi K, Ghatei MA, Bloom SR. Endothelin in renal failure. Nephrol Dial Transplant 1990;5:418-22
- Weinstein DB, Neiden JG. Antiatherogenic properties of calcium antagonists. State of the art. Am J Med 1989;17:27-32
- Wendelhag I, Gustavsson T, Suurkula M, Berglund G, Wikstrand U. Ultrasound measurement of wall thickness in the carotid artery: fundamental principles and description of computerised analysing system. Clin Physiol 1991;11:565-77
- Wendelhag I, Wiklund O, Wikstrand J. Arterial wall thickness in familial hypercholesterolemia. Ultrasound measurement of intima-media thickness in the common carotid artery. Arterioscler Thromb 1992;12:70-7

- White CW, Wright CB, Doty DB et al. Does visual interpretation of the coronary arteriogram predict the physiologic importance of a coronary stenosis? N Engl J Med 1984;310:819-24
- Wiklund O, Angelin B, Olufson S et al. Apolipoprotein(a) and ischaemic heart disease in familial hypercholesterolemia. Lancet 1991;2:1360-3
- Wikstrand J, Warnold I, Olsson G et al. Primary prevention with metoprolol in patients with hypertension. Mortality results from the MAPHY study. JAMA 1988;259: 1976-82
- Wilcken DEL, Reddy SG, Gupta VJ. Homocysteinemia, ischemic heart disease, and the carrier state for homocystinuria. Metabolism 1983;32:363-70
- Wilhelmsen L, Svardsudd K, Korsan-Bengtsen K, Larsson B, Welin L, Tibblin G. Fibrinogen as a risk factor for stroke and myocardial infarction. N Engl J Med 1984;311:501-5
- Williams JK, Adams MR, Klopfenstein HS. Estrogen modulates responses of atherosclerotic coronary arteries. Circulation 1990;81:1680-7
- Williams JK, Adams MR, Herrington DM, Clarkson TB. Short-term administration of oestrogen and vascular responses of atherosclerotic coronary arteries. J Am Coll Cardiol 1992;29:452-7
- Williams JK. Oestrogen therapy for myocardial ischaemia in women. Lancet 1993;342:128

- Williams RR, Hunt SC, Hopkins PH. Familial dyslipidemic hypertension. Evidence of 58 Utah families for a syndrome present in approximatly 12% of patients with essential hypertension. JAMA 1988;259:3579-86
- Wilson RF, Laughlin DE, Ackell PH et al. Transluminal, subselective measurement of the coronary artery blood flow velocity and vasodilator reserve in man. Circulation 1985;72:82-92
- Wines PA, Schmitz JM, Pfister SI et al. Augmented vasoconstrictor responses to serotonin precede development of atherosclerosis in aorta of WHHL rabbit.

 Arteriosclerosis 1989;9:195-202
- Winniford MD, Wheelan KR, Kremers MS et al. Smoking-induced coronary vasoconstriction in patients with atherosclerotic coronary artery disease: Evidence for adrenergically mediated alterations in coronary artery tone. Circulation 1986; 73:662-7
- Witteman JCM, Grobbee DE, Kok FJ, Hofman A, Valkenburg HA. Increased risk of atherosclerosis in women after menopause. Br Med J 1989;298:642-4
- Yamamoto H, Bossaler C, Cartwright J Jr, Henry PD. Videomicroscopic demonstration of defective cholinergic arteriolar vasodilation in atherosclerotic rabbits. J Clin Invest 1987;81:1752-8
- Yanagisawa M, Kurihara H, Kimura S et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988;332:411-15
- Yasue H, Matsuyama K, Matsuyama K et al. Responses of angiographically normal human coronary arteries to intracoronary injection of acetylcholine by age and segment. Circulation 1990;81:482-90

- Yeung AC, Vekshtein VI, Krantz DS et al. The effects of atherosclerosis on the vasomotor response of coronary arteries to mental stress. N Engl J Med 1991;325:1551-6
- Young MA, Vatner SF. Blood flow and endothelium-mediated vasomotion of iliac arteries in conscious dogs. Circ Res 1987;61(Suppl II):II-88-93
- Zeiher AM, Drexler H, Wollschlager H, Just H. Modulation of coronary vasomotor tone in humans. Progressive endothelial dysfunction with different early stages of coronary atherosclerosis. Circulation 1991;83:391-401