MD thesis

Coronary artery calcification in male and female subjects with and without type 1 diabetes mellitus: a pharmacological dissection of the role of the L-arginine: nitric oxide: cGMP pathway

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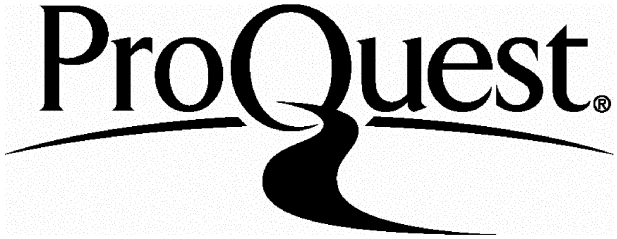

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Statement of Authorship

I confirm that I have carried out the research project described in this thesis. Following previous research work by Dr. Helen Colhoun (my supervisor), Professor Patrick Vallance (my co-supervisor), Dr. Colhoun and myself conceived and designed this study. With the help of Dr. Colhoun and Professor Vallance, I obtained a Junior British Heart Foundation Research Fellowship to fund this study. I wrote the study protocol and carried out this research study. Data cleaning and statistical analysis with the help of Dr Colhoun and our statistician D. Lambrou. Dr Michael Rubens read all the EBCT scans at the Royal Brompton Hospital.

Following completion of this research project, several papers have already been published and the data have been presented in various national and international meetings, including the American Heart Association Annual Conference, the British Diabetic association and British Endocrine Society Meetings.

Coronary artery calcification in male and female subjects with and without type 1 diabetes mellitus: a pharmacological dissection of the role of the L-arginine: nitric oxide: cGMP pathway

Abstract

Background: Defective nitric oxide (NO) release/response may contribute to increased risk in coronary heart disease (CHD) and the loss of sex difference in CHD in diabetes. Evidence for defective NO release/response in type 1 diabetes has been conflicting possibly due to the small sample sizes in previous studies. We conducted a large study to determine whether (1) NO release/response is impaired in type 1 diabetes, (2) whether there is a sex difference in NO release/response in the general population that is altered in diabetes and (3) whether defective NO release/response is associated with coronary artery calcification (CAC).

Methods and Results: Forearm blood flow response was assessed by plethysmography in 88 diabetic and 69 non-diabetic subjects aged 30-53 years without clinical CHD. All participants had CAC measured by electron beam computed tomography. Acetylcholine (ACh) and bradykinin (BK) were used to assess agonist-stimulated NO release. Glyceryl trinitrate (GTN) was used to assess vascular smooth muscle cell (VSMC) response. N^G-monomethyl-L-arginine (L-NMMA) was used to assess basal NO release and noradrenaline as vasoconstrictor control. In diabetic patients, vascular response was reduced by 18% for ACh ($p=0.0008$); 7% for BK ($p=0.042$) and 17% for GTN

(p=0.0001). No difference in response to L-NMMA (p=0.49) or noradrenaline (p=0.55) was observed. Women did not have a greater response than men to all drugs after adjusting for sex differences in basal flow and forearm volume. The effect of diabetes on vasodilator response was the same in men and women. There was no association between CAC and drug responses.

Conclusions: The primary defect in type 1 diabetes is impaired response to exogenous NO donor. The effect of diabetes on vascular function is the same in men and women and does not underlie the loss of the sex difference in CAC in diabetic patients. Vascular dysfunction is not associated with coronary atheroma.

List of abbreviations

ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
AGE	Advanced glycation endproduct
ANOVA	Analysis of variance for repeated measures
AUC	Area under the curve
BH_4	Tetrahydrobiopterin
BK	Bradykinin
BM	Basement membrane
BMI	Body mass index
CAC	Coronary artery calcification
CHD	Coronary heart disease
cGMP	cyclic guanosine monophosphate
CV	Coefficient of variance
DAG	Diacylglycerol
DDAH	Dimethylarginine dimethylaminohydrolase
DCCT	Diabetes control and complication trial
EBCT	Electron beam computed tomography
EDC	Epidemiology of Diabetes Complications
EDHF	Endothelium-derived hyperpolarising factor
EDRF	Endothelium-derived relaxing factor
FBF	Forearm blood flow

FMD	Flow-mediated dilatation
GC	Guanylyl cyclase
GCa	Active guanylyl cyclase
GCi	Inactive guanylyl cyclase
GTN	Glyceryl trinitrate
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
L-NAME	nitro-L-arginine methylester
L-NMMA	N^G monomethyl-L-arginine
NO	Nitric oxide
NOS	Nitric oxide synthease
NADPH	Nicotinamide-adenine dinucleotide phosphate
PKC	Protein kinase C
SNP	Sodium nitroprusside
SPECT	Single photon emission computed tomography
STZ	Streptozotocin
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell

Chapter 1. Introduction

1.1 Background to the loss of sex difference in CHD risk in diabetes

1.1.1 Excess risk of cardiovascular disease in diabetes mellitus

Diabetic microvascular and macrovascular complications are the principal cause of morbidity and mortality in patients with diabetes mellitus (Head & Fuller, 1990; Fuller et al, 1983). Patients with type 1 diabetes have a three to six-fold increased risk of cardiovascular death before the age of 60 compared with non-diabetic subjects (Krolewski et al, 1987). Furthermore, coronary atherosclerosis occurs earlier in individuals with diabetes and its severity is often greater and distribution more diffuse compared to non-diabetic individuals, which may lead to incomplete revascularisation or increase the risk of surgical or percutaneous intervention in diabetic patients (The BARI investigators, 1996; O'Neill, 1998).

1.1.2 The sex difference in coronary heart disease is lost in type 1 diabetes

Type 1 diabetes increases the risk of both coronary heart disease (CHD) events and CHD mortality much more in women than men thereby abolishing the sex difference in CHD among diabetic patients. In the London cohort of the WHO Multinational Study of Vascular Disease in Diabetes, type 1 diabetic women had a higher CHD incidence and mortality rate than men at 8-year follow up (Morrish et al, 1991). In the EURODIAB Prospective Complications Study, the incidence of CHD was the same in men and women at 8-year follow up (Koivisto et al. 1996). Furthermore, in the Pittsburgh Insulin Dependent Diabetes Mellitus Morbidity and Mortality Study, there was again no

difference in the prevalence of cardiovascular complications between men and women (Orchard et al. 1990) and mortality rates were also similar in men and women (Dorman et al, 1984). In the U.K., the relative mortality from CHD below aged 40 years was 26 in diabetic women and 10 in diabetic men compared to the general population (Swerdlow & Jones 1996).

1.1.3 Conventional risk factors and the loss of the sex difference in CHD in type 1 diabetes?

1.1.3.1 Risk factors for CHD in type 1 DM

The principal risk factors for CHD in the non-diabetic population i.e. total cholesterol, HDL-cholesterol, LDL-C, and hypertension also confer an increased risk of CHD in type 1 diabetic patients (Morrish et al, 1991; Koivisto et al, 1996; Lloyd et al, 1996). Cross-sectional and prospective studies have found that raised fasting triglyceride is also a risk factor for CHD in type 1 diabetes (Janka, 1985; Koivisto et al, 1996; Lloyd et al, 1996). This association was independent of HDL-C in the EURODIAB IDDM Complications Study but not in the Pittsburgh Epidemiology of Diabetes Complications (EDC) (Koivisto et al, 1996). An association between CHD and smoking was found in some (Morrish et al, 1991; Lloyd et al, 1996) but not all studies of type 1 diabetes (Jensen et al, 1987; Koivisto et al, 1996). Waist-hip-ratio but not body mass index (BMI) was associated with CHD in the Pittsburgh EDC Study (Koivisto et al, 1996). In the EURODIAB study, women but not men with CHD had higher BMI than those without CHD (Koivisto et al, 1996).

In most studies (Janka, 1985; Morrish et al, 1991), though not all (Krolewski et al, 1987), diabetes duration is an important predictor of CHD independent of age. However, the most important determinant of CHD morbidity among diabetic patients is nephropathy which confers a more than 10-fold risk of CHD (Borch-Johnsen & Kreiner, 1987; Jensen et al, 1987; Krolewski et al, 1987). Nephropathy is associated with hypertension, an atherogenic lipid profile and in some studies, increased fibrinogen concentrations and platelet aggregation (Donahue & Orchard, 1992). It has also been suggested that CHD and nephropathy are strongly associated because they both reflect widespread vascular damage and perhaps a common endothelial dysfunction (Deckert et al, 1989). Consistent with this, microalbuminuria without overt nephropathy was associated with increased cardiovascular mortality in a follow up of 939 type 1 diabetic patients (Rossing et al, 1996). Additionally, in non-diabetic patients with hypertension, microalbuminuria has been associated with evidence of endothelial dysfunction (Yudkin et al, 1988).

In so far as glycaemic control is an important determinant of nephropathy it is also an important determinant of CHD in type 1 diabetes. Poor glycaemic control is also associated with a more atherogenic lipid profile (Abraha et al, 1999). Whether glycaemia per se exerts an independent effect on CHD risk is controversial. In cross-sectional studies such as the EURODIAB type 1 DM Complications Study, glycaemic control was associated with CHD but not independent of other factors (Koivisto et al, 1996). In the EDC study, no association was observed at 4-year follow up (Orchard, 1994). However, glycaemic control was a strong predictor of CHD mortality at 7-year follow up in a Finnish study (Lehto et al, 1999). In the Diabetes Control and Complications Trial

(DCCT), intensive insulin therapy with improved glycaemic control reduced macrovascular disease by 41%. Although the reduction was not statistically significant, this was unsurprising as the total number of macrovascular events was low, in keeping with the age of those studied (The DCCT Research Group, 1995). The lack of clear evidence on the extent to which glycaemia causes CHD stems in part from the small number of macrovascular events in these studies and the difficulty of separating the effects of age and duration from glycaemic control in the analysis.

Although established CHD risk factors are important in determining CHD risk among patients with diabetes, they do not appear to explain the increased risk of CHD in patients with type 1 diabetes compared to the general population. Few studies have addressed this issue in detail but it is clear that even type 1 diabetic patients without nephropathy have an elevated risk of CHD 2-4 times than of the general population (Krolewski et al, 1991; Tuomilehto et al, 1998). In the absence of nephropathy, type 1 diabetic patients usually have a better lipid profile than the general population with increased HDL-C, decreased LDL-C and lower triglycerides (Taskinen, 1993). Whether blood pressure is elevated at all in the absence of albuminuria is controversial (Mathiesen et al, 1990). Thus risk factors yet to be identified must be responsible for the elevated CHD risk. Many candidates have been proposed including more subtle abnormalities of lipids and lipoproteins, increased oxidative stress and cytokine production, endothelial dysfunction and abnormalities of haemostasis and coagulation. A potential role for cardiac autonomic neuropathy has also been proposed. At present, the role of these factors in CHD in type 1

diabetes is under investigation and definitive proof of their importance from prospective studies is lacking.

1.1.3.2 Sex differences in the effect of type 1 diabetes on risk factors

It is unclear to what extent established CHD risk factors explain the attenuation of the sex difference in CHD in type 1 diabetes. Several studies have compared sex differences in risk factors in type 1 diabetic and non-diabetic groups. A comparison was made between the DCCT cohort and lipid values for non-diabetic individuals in the Lipid Research Clinics Program. Diabetic women had a worse lipid profile than non-diabetic women up to their mid-20s. Thereafter lipid profiles were similar or were better in the diabetic group. Diabetic men also had a worse lipid profile at younger age with change to a similar or better profile at older ages. However, the changeover was at an earlier age in men and the adverse differences at the younger age were smaller than in women (The DCCT Research Group, 1992). Walden found that, compared with non-diabetic controls, type 1 diabetic women had a greater elevation of VLDL, LDL and HDL triglycerides than diabetic men (Walden et al, 1984). However, total triglycerides were higher in the diabetic than non-diabetic group in that study whereas in fairly well-controlled type 1 diabetes this is usually not the case (Nikkila, 1984).

In the London cohort of type 1 diabetic patients in the WHO Multicentre Study (mean age 46 years) the prevalence of hypertension at baseline was the same in men and women, despite a higher prevalence in men in the background population at that age (Morrish et al, 1991). Across all centres in the study, the baseline prevalence of hypertension was

higher in women than men (Fuller et al, 1996). In the Pittsburgh EDC Study, the sex differences in baseline risk factors were in the same direction as in the general population, namely higher HDL-C, lower LDL-C, triglycerides and systolic blood pressure in women than men (Lloyd et al, 1996). In the cross-sectional EURODIAB type 1 diabetes Complications Study, none of the sex differences in risk factors were reversed from those expected although the sex difference in hypertension was less than would be expected in the general population of the same age range (Koivisto et al, 1996). The loss of the sex difference in CHD and the attenuation in the sex difference in hypertension cannot be attributed to nephropathy as diabetic men are usually found to have higher (Lloyd et al, 1996; Borch-Johnsen & Kreiner, 1987) or similar rates (Morrish et al, 1991; Koivisto et al, 1996) of nephropathy to those seen in diabetic women. Furthermore, nephropathy was more strongly associated with CHD in men than women in both the EURODIAB and EDC studies. Most studies do not report major sex differences in glycaemic control in adult type 1 diabetic patients (Koivisto et al, 1996; Lloyd et al, 1996; Abraha et al, 1999). Thus the loss of the sex difference in CHD in type 1 diabetes remains unexplained by conventional CHD risk factors. A clear understanding of the basis of this loss of sex difference in CHD in diabetes will give valuable insight into the complex pathophysiology of atherosclerosis. This may lead to effective strategies in future prevention of CHD as well as therapeutic intervention not only in the high-risk groups but also in the general population.

1.1.3.3 Previous work examining sex difference in CHD: The EBCT study

In order to examine the issue of sex difference in CHD further, Dr. Helen Colhoun had already conducted a study examining coronary risk factors and coronary artery calcification as measured by Electron Beam Computed Tomography scanning in type 1 diabetic and non-diabetic men and women (the EBCT study). This study was completed approximately one year before work described in this thesis was commenced. In the EBCT study, coronary calcification was used as a surrogate marker of coronary atherosclerosis. The result of this study showed that in type 1 diabetes, CAC is more markedly increased in women than men and the gender difference in calcification is lost which is unexplained by conventional coronary risk factors (Colhoun HM et al, 2000). Hence the loss of sex difference in CHD in diabetes is at least in part an atherosclerotic process as reflected by coronary calcification. In this study, most risk factors showed similar sex differences in diabetic patients as in control subjects except that:

1. Systolic blood pressure difference between diabetic and control subjects was greater in women (+8 mmHg) than men (+4 mmHg) despite a greater prevalence of albuminuria in diabetic men.
2. HbA1c was worse in women than men ($8.9 \pm 0.2\%$ vs 8.4 ± 0.12, $p<0.001$).
3. Men with diabetes had a better lipid profile than men without diabetes (lower total cholesterol: HDL ratio and triglycerides) but this is not the case in women.

The finding that the effect of diabetes on systolic blood pressure is greater in women than men is intriguing and unexpected since the prevalence of albuminuria is greater in type 1 diabetic men. A plausible explanation for this observation is whether there is a more profound disturbance of endothelial function in diabetic women than men. More specifically, whether the L-arginine: nitric oxide pathway may be defective to greater extent in women than men in diabetes is of considerable interest. This forms the basis of this thesis which examines whether sex difference in the L-arginine: nitric oxide pathway exists in the general population and whether this is attenuated in type 1 diabetes, contributing to the loss of sex difference in CHD in diabetes.

1.2 Background to defective L-arginine: nitric oxide: cGMP pathway as a potential mechanism ?

In this thesis, I sought to explore whether a defect in endothelial function, in particular, in the L-arginine: NO: cGMP pathway could contribute to the excess CHD risk in patients with type 1 diabetes and whether such a defect also accounted for the loss of sex difference in CHD in diabetes. The following sections discuss the anti-atherogenic actions of endothelium-derived NO and the limited evidence that NO production may be greater in females.

1.2.2 From EDRF to Nitric Oxide: Historic perspectives

In 1980, Furchgott and Zawadzki demonstrated that the presence of vascular endothelial cells is essential for acetylcholine (ACh) to induce relaxation of isolated rabbit aorta (Furchgott and Zawadski, 1980). If the vascular endothelium is injured or mechanically removed, the blood vessel fails to relax to ACh but still responds to glyceryl trinitrate (GTN). This endothelial-dependent relaxation of vascular smooth muscle to ACh is mediated by a humoral factor initially named endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadski, 1980). The exact biochemical identity of EDRF was intensely studied and in 1986, Furchgott and Ignarro independently suggested that NO might account for the biological properties of EDRF. This was confirmed a year later by two independent groups (Palmer et al, 1987; Ignarro et al, 1987).

1.2.2 The L-arginine: NO: cGMP pathway

Endothelium-derived NO is synthesised from one of the guanidine-nitrogen atoms of the amino acid L-arginine by the endothelial isoform of NO synthase, yielding L-citrulline as a by-product (Palmer et al, 1988; Schmidt et al, 1988). Nitric oxide is labile and has a short half-life (< 4 seconds in biological solutions) (Knowles et al, 1992). It is rapidly oxidized *in vivo* to nitrite and then nitrate by oxygenated haemoglobin, molecular oxygen, and superoxide anions before being excreted into the urine (Wennmalm et al, 1992; Moncada and Higgs, 1993). The biosynthesis of NO from L-arginine requires several cofactors including nicotinamide-adenine dinucleotide phosphate (NADPH), flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH_4) and calmodulin (Bredt & Snyder, 1990; Lewis et al, 1993; Moncada et al, 1995). Endothelium-derived NO then diffuses across the endothelial cell membrane and enters the vascular smooth muscle cells (VSMC) where it activates guanylate cyclase (GC) leading to an increase in intracellular cyclic guanosine-3',5-monophosphate (cGMP) levels (Gruetter et al, 1981; Ignarro et al, 1984). As a second messenger, cGMP mediates many of the biological effects of NO including the control of vascular tone and blood pressure, platelet activation and neurotransmission. In addition, NO has other molecular targets which include haem proteins, DNA and thiols. These effects may mediate changes in functions of certain enzymes or channels. Interaction of NO with superoxide anion can attenuate physiological responses mediated by NO (Gryglewski et al, 1986) and can produce irreversible inhibitory effects on mitochondrial function as a result of peroxynitrite ($ONOO^-$) formation (Castro et al, 1994; Wolin, 1996).

1.2.3 Nitric Oxide Synthase Isoforms: Expression & Regulation

Three isoforms of nitric oxide synthase (NOS) have been identified, the endothelial isoform (eNOS), neuronal isoform (nNOS) and macrophage or inducible isoform (iNOS) (Forstermann et al, 1994). All three NOS isoforms play distinct roles in the regulation of vascular tone. eNOS and nNOS are constituents of healthy cells. The genes encoding eNOS and nNOS are located on chromosome 7 and 12 respectively. The levels of eNOS and nNOS expression and activity are tightly regulated and may be induced under different physiological conditions (eg. shear stress or nerve injury). Inducible NOS is encoded by a gene located on chromosome 17 (Xu et al, 1994). Under normal physiological conditions, iNOS is not expressed in vascular cells and its expression is seen mainly in conditions of acute infection or inflammation. To classify iNOS as "non-constitutive" is not strictly correct since it is also expressed constitutively in certain epithelial cells (Guo et al, 1995). Similarly, it is clear that the "constitutive" isoforms are transcriptionally regulated and may be induced.

1.2.4 Agonist activation of eNOS

Activation of eNOS can be induced by shear stress as well as hormones (such as catecholamines, vasopressin, oestrogen), autacids (such as bradykinin and ACh) and platelet-derived mediators (such as serotonin and adenine diphosphate). Specific receptors for these stimuli mediate eNOS activation through coupling with G proteins (e.g. serotonin receptors) (Boulanger & Vanhoutte, 1997). Inhibition of eNOS activities may also be induced by the association of eNOS with caveolin-1 and caveolin-3 in endothelial cells and myocytes respectively (Garcia-Cardena et al, 1997). This negative

effect on eNOS activities may be a result of interference with calcium/camodulin binding and electron transfer (Ghosh et al, 1998). Thus the balance between activation and inhibition mechanisms mediated by various receptors and caveolin regulates eNOS activity.

1.2.5 Biological effects of NO on the vasculature

Endothelium-derived NO is a very potent vasodilator in the vasculature. In addition, NO has anti-atherogenic properties including suppression of platelet aggregation, leucocyte migration and cellular adhesion to the endothelium (Radomski et al, 1987; Radomski et al, 1990; Bath et al, 1991; Bode-Boger et al, 1994; Kubes et al, 1991), and inhibition of VSMC mitogenesis, proliferation and migration (Garg et al, 1989; Nakaki et al, 1990; Sarkar et al, 1996). Furthermore, NO inhibits the activation and expression of certain adhesion molecules (De Caterina et al, 1995; Khan et al, 1996), production of superoxide anion (Clancy et al, 1992) and oxidation of low-density lipoprotein (Hogg et al, 1993). Loss of endothelium-derived NO would be expected to promote a vascular phenotype more prone to atherogenesis, a concept supported by animal studies (Carvalho et al, 1987; Chataigneau et al, 1999).

1.2.6 Nitric oxide release from the vascular endothelium

There is a continuous basal release of NO from the vascular endothelium to maintain the resting vascular tone. A number of chemical and physical stimuli may activate eNOS which leads to increased NO production contributing to the control and regulation of the vascular tone (Busse R et al, 1993b).

1.2.6.1 Basal nitric oxide release

The synthesis of NO in vascular endothelial cells in culture and in fresh vascular tissue can be inhibited by N^Gmonomethyl-L-arginine (L-NMMA), an analogue of L-arginine in which one of the guanidino nitrogen atoms is methylated (Palmer et al, 1988). This inhibitory effect of L-NMMA is readily reversed by L-arginine and the inactive stereoisomer (D-NMMA) has no effect on the L-arginine: NO pathway (Vallance et al, 1989a). This NOS-inhibitor has been used to examine the role of NO in various vascular beds *in vitro* and *in vivo* in both human and animal models.

In rings of rabbit aorta, L-NMMA causes significant endothelium-dependent contraction (Rees et al, 1989a). Intravenous infusion of L-NMMA induced a dose-related increase in blood pressure which is reversed by intravenous administration of L-arginine in experimental animals (Aisaka et al, 1989; Rees et al, 1989a). In the human forearm vasculature, infusion of L-NMMA via the brachial artery causes substantial dose-dependent vasoconstriction indicating that continuous generation of NO is crucial in maintaining peripheral vasodilatation (Vallance et al, 1989a). Basal NO production also occurs in every other vascular bed studied including cerebral (White et al, 1997), pulmonary (Stamler et al, 1994), renal (Haynes et al, 1993) and coronary arteries (Lefroy et al, 1993).

In the venous system, however, inhibitors of NOS do not lead to an increase in basal tone in a variety of venous preparations from animals (Ekelund et al, 1990; Martin et al, 1992). Similar findings have also been shown in humans (Vallance et al, 1989b; Yang et al,

1991) suggesting that basal NO production does not have a major role in the maintenance of the resting tone in most veins.

1.2.6.2 Agonist-stimulated NO release

Many chemical substances such as ACh, bradykinin, serotonin and substance P are able to induce endothelium-dependent vasodilatation in experimental settings. In rings of rabbit aorta, endothelium-dependent relaxation induced by ACh, calcium ionophore A23187 or substance P is inhibited by L-NMMA (Rees et al, 1989a), suggesting that vasorelaxation induced by endothelium-dependent agonists is NO-mediated. Additionally, L-NMMA has been shown to inhibit the hypotensive effect of ACh but not that of GTN in rabbits (Rees et al, 1989b) and rats (Whittle et al, 1989). However, the blockade is far from complete and there is now growing evidence for additional mechanisms underlying endothelium-dependent response, particularly in resistance vessels. Similarly in humans, L-NMMA inhibits agonist-stimulated relaxation in both resistance (Vallance et al, 1989a; Lefroy et al, 1993; Cockcroft et al, 1994; Quyyumi et al, 1995) and conduit vessels *in vivo* (Thom et al, 1987; Schoeffter et al, 1988; Yasue et al, 1990; Yang et al, 1991; Collins et al, 1993). However, the degree of inhibition to the agonist varies depending on the specific vascular bed suggesting that mechanisms (eg. prostaglandins and endothelium-derived hyperpolarising factor (EDHF)) other than that mediated by NO may also be involved.

1.2.7 Assessing NO response using acetylcholine and bradykinin

The muscarinic agonist, acetylcholine (ACh), has been conventionally used to assess endothelium-dependent vascular response. Experimental evidence indicates that ACh causes relaxation in human conduit (Thom et al, 1987; Schoeffter et al, 1988; Yasue et al, 1990; Yang et al, 1991; Collins et al, 1993; Jovanovic et al, 1994) and resistance vessels but has only minimal dilator effect on hand veins (Vallance et al, 1989b; Collier et al, 1990) and saphenous veins (Thom et al, 1987; Lawrie et al, 1990; Yang et al, 1991). A biphasic response to ACh has been observed in superficial hand veins (Collier et al, 1989) and the coronary arteries (Angus et al, 1991) in humans with vasodilatation at low doses of ACh and vasoconstriction at high doses. Hence vascular response to ACh varies considerably depending on the blood vessel type as well as the specific vascular bed studied.

The mechanism of ACh-induced vascular response is complex and may involve several components. These include endothelium-dependent relaxation (Bruning et al, 1994), endothelium-independent contraction as a result of a direct effect on muscarinic receptors on VSMCs (Penny et al, 1995), vasorelaxation through inhibition of noradrenaline release as a result of its action on presynaptic muscarinic receptors (Vanhoutte et al, 1974), and vasorelaxation through the release of prostaglandins and/or EDHF (Feletou & Vanhoutte, 1988; Chen & Cheung, 1992). The relative contribution of each mechanism depends on the vascular bed, size of the vessel (Shimokawa et al, 1996) and pathophysiological condition studied (Rosolowsky et al, 1990; Najibi et al, 1994). For this reason,

interpretation of changes in ACh responses may not be solely indicative of changes in the L-arginine: NO pathway.

Similarly, the vasodilator effect of bradykinin is only partially mediated through stimulation of NO generation (O'Kane et al, 1994). Indeed, it has recently been shown that EDHF is the predominant mediator in bradykinin-induced endothelium-dependent vasodilatation in resistance vessels (Honing et al, 2000).

1.2.8 Biochemical detection of NO *in vivo*

Given the difficulty in interpreting NO-mediated vascular responses induced by ACh and other agonists, direct quantification of NO might help resolving the issue. However, there are considerable difficulties in the direct biochemical measurement of NO. Measurement of plasma and urinary nitrate, the stable end product of NO metabolism, has limited value as it does not distinguish exogenous nitrate (present in diet) from endogenous nitrate. Furthermore, plasma nitrate concentration does not differentiate the finer differences in NO production since the rate of NO production, excretion and its volume of distribution are all factors that can influence the plasma concentration. Production of NO can also be quantified by measuring ^{15}N nitrate excretion in urine after intravenous administration of the isotope L-$[^{15}N]_2$-guanidino arginine (Hibbs et al, 1992; Forte et al, 1997). This method has the advantage of ensuring that the measured urinary ^{15}N nitrate is endogenous. However, it does not distinguish the cellular origin of NO production. Even accurate direct measurement of total body nitrate production does not reflect biologically active NO, nor does it provide information regarding the target tissue response to the available

NO. Given these various drawbacks in the measurement and interpretation of NO production and activities, measurement of NO metabolites should be interpreted with caution and ideally in conjunction with functional outcomes (vascular responses).

1.2.9 Loss of NO and predisposition to atherogenesis

Since NO has a number of anti-atherogenic effects, it would be expected that loss of NO or a reduction in its activities would enhance atherogenesis. It has been shown that a reduction in NO activity (as reflected by impaired endothelial-dependent vasodilatation) occurs very early in experimental and human hypercholesterolaemia even before any structural changes in the vascular wall. The impaired endothelial-dependent vasodilatation can be reversed with L-arginine in certain conditions (Cooke et al, 1991; Drexler et al, 1991a). The anti-atherogenic role of NO is further supported by studies of long-term NOS inhibition. Aortic rings from rabbits fed with cholesterol-rich diet had impaired endothelial-dependent vasorelaxation in response to ACh. Furthermore, blockade of NO with nitro-L-arginine methylester (L-NAME) caused structural changes with development of greater lesion surface area in the aorta of hypercholesterolaemic rabbits (Naruse et al, 1994).

1.2.10 NO production and response as a risk factor for CHD and sex difference in the L-arginine: NO pathway

In humans, there is indirect evidence that defective NO production/response or endothelial dysfunction contributes to atherogenesis. Abnormal vasomotor responses to the endothelium-dependent vasodilator and partial-NO synthase agonist, ACh, occur in

coronary atherosclerosis (Ludmer et al, 1986; Zeiher et al, 1991a). Ludmer et al showed that in patients with angiographic evidence of coronary atherosclerosis, intra-coronary infusion of ACh, which normally causes endothelium-dependent vasodilatation, produced a paradoxical vasoconstriction (Ludmer et al, 1986). Zeiher et al demonstrated a progressive impairment in endothelium-mediated modulation of coronary vasomotor tone with different stages of early atherosclerosis in humans characterised by angiography (Zeiher et al, 1991). Since studying coronary endothelial dysfunction by direct intra-coronary infusion of vasoactive drugs is invasive, most studies have chosen to study endothelial function of peripheral vasculature as it has been shown that there is close relationship between endothelial function in coronary and peripheral vasculature (Anderson et al, 1995). Furthermore, there is a large body of evidence that peripheral endothelial dysfunction or defective NO pathway is associated with CHD risk factors (reviewed is section 1.2.11).

Given the pivotal role of NO in the modulation of vascular tone and interactions between the endothelium and circulating blood cells (platelets and leucocytes) and their activities, any potential sex differences in the metabolism of NO could contribute to the sex difference in CHD risk. Understanding of the gender effect on the L-arginine: NO pathway in the general population and how it might be altered by diabetes would help to explain the excess risk of CHD and the loss of sex difference in CHD in diabetes.

There is evidence that basal NO release from endothelium-intact aortic rings was significantly higher in female rabbit than the male (Hayashi et al, 1995). In addition,

acetylcholine, commonly used to infer agonist-stimulated release of NO, evoked significantly greater endothelium-dependent relaxation in aortae from female rats than male (Kauser & Rubanyi, 1994). Moroi et al showed that upon intimal injury, female eNOS mutant mice had less intimal response than male eNOS mutant mice and that pregnancy abolished intimal injury all together (Moroi et al, 1998). The evidence for a gender difference in NO biosynthesis in humans is rather inconsistent. Endothelium-derived NO production in women has been shown to be higher (Chowienczyk et al, 1994; Kharitonov et al, 1994; Forte et al, 1998), lower (Jilma et al, 1996) or the same (Giovannoni et al, 1997) as men. For instance, Chowienczyk and colleagues found that blood flow response to ACh is impaired in hypercholesterolaemic men but not hypercholesterolaemic premenopausal women (Chowienczyk et al, 1994). This provides early evidence that gender may have an important effect in the susceptibility of the L-arginine: NO pathway to defects inflicted by disease states such as hypercholesterolaemia. Kharitonov and associates examined peak exhaled NO and found a significantly higher NO production in women than men (Kharitonov et al, 1994). Similarly, whole-body production of NO, as measured by urinary excretion of ^{15}N nitrate following intravenous administration of radio-labelled L-arginine, was found to be significantly higher in healthy premenopausal women than men (Forte et al, 1998). However, in other studies, exhaled NO and plasma nitrate (Jilma et al, 1996) were found to be significantly higher in men than women.

These discrepancies may be due to the limitations of the methods used for measurement of NO in these studies. For instance, measurement of exhaled NO (Kharitonov et al,

1994; Jilma et al, 1996) may reflect NO generation in the pulmonary vasculature whereas direct measurement of urinary or serum/plasma nitrate is highly affected by dietary intake. Importantly, these quantitative studies do not enable differentiation of the cellular origin of NO. In this respect, functional in vivo studies provide a better insight. Using forearm plethysmography, Kneale et al demonstrated that response to L-NMMA is greater in premenopausal women (n=20) than men (n=20). They also found that noradrenaline response is greater in men than women (Kneale et al. 1997). These findings are consistent with a greater basal NO production in women than men although equally, it could be explained by an increased α-adrenergic response in men than women (Kneale et al, 2000). In another study, Majmudar et al found greater L-NMMA response in premenopausal women than men (Majmudar et al, 2000). Taken together, a clear consensus for a sex difference in endothelium-derived NO production is lacking. Thus the main hypothesis of the work carried out was to establish (1) if there is a sex difference in NO release/response and (2) if NO release/response is attenuated in type 1 diabetes.

1.2.11 The relationships between endothelial dysfunction and coronary risk factors

1.2.11.1 Hyperlipidaemia

Increased total cholesterol/ LDL-C

Impaired endothelium-dependent vasorelaxation to ACh occurs in models of atherosclerosis and hypercholesterolaemic animals (Bossaller, et al, 1987; Shimokawa, et al, 1989). Most human in vivo studies have focused on effects of elevated LDL-C and total cholesterol on vascular responses. Table 1.1 summarizes forearm plethysmography

studies examining effects of atherogenic lipoprotein phenotype on vascular responses. A decrease in agonist-stimulated endothelium-dependent vasodilatation in association with elevated LDL-C was found by nearly all research groups (Creager et al, 1990; Chowienczyk et al, 1992; Casino et al, 1993; Chowienczyk et al, 1994; Gilligan et al, 1994; Stroes et al, 1997a; Stroes et al, 1997b; Garcia et al, 1995; Casino et al, 1995; Lewis et al, 1999; Duffy et al, 1999; Verhaar et al, 1999b) and is independent of coronary artery disease (Creager 1990; Gilligan 1994). Response to the NO donor, SNP however, was found to be unchanged (Chowienczyk et al, 1992; Casino et al, 1993; Chowienczyk et al, 1994; Gilligan et al, 1994; Stroes et al, 1995; Stroes et al, 1997; Lewis et al, 1999; Verhaar et al, 2000) or impaired (Gilligan et al, 1994; Creager et al, 1990; Duffy et al, 1999). This suggests that elevated LDL-C may impair VSMC function in addition to the endothelium. Interestingly, Chowienczyk *et al* found reduced ACh response in patients with elevated LDL-C but unchanged MCh response in these same patients (Chowienczyk et al, 1992). This is in contrast to Creager et al who found impaired MCh response though in the presence of impaired SNP response (Creager et al, 1990). It is possible that the impaired response to MCh in Creager's study may be explained by reduced VSMC sensitivity to NO as suggested by the impaired SNP response. The finding that both muscarinic receptor agonists, ACh and MCh, do not produce the same response in Chowienczyk's study indicates that different muscarinic receptor subtype may be involved in the signal transduction process linked to the activation of eNOS. In addition to defective muscarinic receptors, impaired agonist-stimulated endothelium-dependent vasodilatation in hypercholesterolaemia also involves defective bradykinin B_2, substance-P and serotonin-receptor mediated signal transduction. Overall, these studies of resistance

vessels demonstrated that elevated LDL-C is associated with impaired agonist-stimulated endothelium-dependent vasodilatation and possibly also associated with VSMC defect. Defective response to serotonin, an agonist that is more specific in stimulating NO mediated response than ACh (Bruning et al, 1993), suggests that in hypercholesterolaemia, defective NO pathway accounts for impaired endothelium-dependent vasodilatation. Recent evidence suggests that within the LDL-C species, particle size may be an important determinant of endothelial function. Vakkilainen *et al* showed that men with small dense LDL particles had significantly lower forearm blood flow response to ACh in healthy adults (Vakkilainen et al, 2000) as well as subjects with type 2 diabetes (Makimattila et al, 1999). This may be related to its increased susceptibility to oxidation (Chait et al, 1993) thereby reducing the bioavailability of NO.

Authors (year)	Subjects (M/F)	Mean Age	Dyslipidaemia (mean concentrations)	Agonist-stimulated response	NO donor response	L-NMMA response
Creager et al (1990)	13 (12M/1F)	35	LDL 211 mg/dl	MCh ↓	SNP ↓	-
Chowienczyk et al (1992)	12 (12M)	37	LDL 6.5 mmol/L	ACh ↓ MCh ↔	SNP ↔	-
Casino et al (1993)	33 (17M/16F)	51	T.Chol 292 mg/dl	ACh ↓	SNP ↔	↔
Chowienczyk et al (1994)	26 (12M/14F)	42	LDL 6.6 mmol/L	ACh ↓	SNP ↔	-
Gilligan et al (1994)	12 (5M/7F)	50	LDL 209 mg/dl	ACh ↓ BK ↔	SNP ↓	-
Garcia et al (1995)	20 (11M/9F)	49	T.Chol 293 mg/dl LDL 245 mg/dl	ACh ↓	SNP ↔	-
Casino et al (1995)	16 (8M/8F)	50	T.Chol 316 mg/dl	ACh ↓ Substance-P ↓	SNP ↔	-
Stroes et al (1997)	13 (9M/4F)	32	T.Chol 8.7 mmol/L	5-HT ↓	SNP ↔	↔
Chowienczyk et al (1997)	34 (20M/14F)	37	Triglyceride 147 mg/dl	ACh ↔	SNP ↔	-
Lewis et al (1999)	10 (9M/1F)	44.7	LDL 5.6 mmol/L	ACh ↓	SNP ↔	↔
Duffy et al (1999)	20 (8M/12F)	28.8	LDL 4.9 mmol/L T.Chol 6.9 mmol/L	ACh ↓	SNP ↓	↔
Verhaar et al (1999)	20 (14M/6F)	35	T.Chol 8.0 mmol/L	5-HT ↓	SNP ↔	-
Lewis et al (1999)	11 (10M/1F)	51.7	Triglyceride 6.97 mmol/L	ACh ↓	SNP ↔	↔
de Man et al (2000)	8 (8M)	51	Triglyceride 12.05 mmol/L T.Chol 7.83 mmol/L	5-HT ↓	SNP ↔	-

Hypertriglyceridaemia

Hypertriglyceridaemia has increasingly been recognised as a CHD risk factor (Cullen, 2000) and its atherogenicity may be related to its effect on endothelial dysfunction. In healthy humans, a triglyceride load induced an impaired endothelial (Vogel et al, 1997) and VSMC response within hours (Lundman et al, 1997). Chronic hypertriglyceridaemia has also been found to contribute to impaired ACh (Lewis et al, 1999) and serotonin response (de Man et al, 2000) independent of LDL-C. In contrast, Chowienczyk et al found normal endothelium-dependent responsiveness to ACh in patients with severe hypertriglyceridaemia (1914 mg/dl) (Chowienczyk et al, 1997). The reason for this discrepancy, however, is unclear but may be confounded by associated insulin resistance and obesity. Indeed, 2 out of 17 patients in Chowienczyk's study had type 2 diabetes which could have biased their findings. This highlights the difficulty in studying isolated hypertriglyceridaemia without other co-existing CHD risk factors. In a study examining leg blood flow in subjects normal cholesterol, Steinberg et al found no relationship between triglyceride levels and maximum endothelium-dependent vasodilatation (Steinberg et al, 1997). Several studies have examined the effects of fish oils, which are known to lower triglyceride rich lipoproteins, on endothelial function.

HDL-Cholesterol

Low levels of HDL-C is associated with CHD even when total cholesterol and triglyceride are not elevated (Lien et al, 1996; Jacobs et al, 1990; Barter et al, 1996). Intraultrasound and coronary angiography studies showed a positive correlation between HDL-C and ACh-induced coronary vasoreactivity (Kuhn et al, 1991; Zeiher et al, 1994). Furthermore, low HDL-C is significantly associated with impaired FMD

in subjects with normal triglyceride (Li et al, 2000). Hence the atheroprotective effect of HDL-C may be mediated through its beneficial effect on the vascular endothelium.

1.2.11.2 Hypertension

A large body of evidence from animal studies (Konishi & Su, 1983; Luscher & Vanhoutte, 1986; Carvalho et al, 1987) and *in vivo* human studies indicates that ACh-induced relaxation is impaired in individuals with hypertension (Taddei et al, 1993; Panza et al, 1993a; Panza et al, 1993b; Cardillo et al, 1998). However, at least one study has found no difference in endothelium-dependent vasodilator response to ACh or carbachol between patients with essential hypertension and matched normotensive controls (Cockcroft et al, 1994b). This may reflect differences in methodology and in population subgroup in this heterogeneous condition. Additionally, basal NO release is reduced in essential hypertension (Calver et al, 1992b; Forte et al, 1997). Current data would be most consistent with hypertension causing a decrease in NO-mediated dilatation rather than the loss of NO being causative in essential hypertension (Vallance, 1999). This notion is supported by the observation that impaired endothelium-dependent vasodilatation in essential hypertension can be restored with anti-hypertensive therapy (Hirooka et al, 1992; Panza et al, 1993c; Calver et al, 1994); and that endothelium-dependent vasodilatation is impaired following acute elevation of blood pressure in normotensive subjects (Millgard et al, 1998).

1.2.11.3 Insulin resistance and Type 2 Diabetes

Vascular studies in type 2 diabetes have generally shown impaired muscarinic agonist-stimulated endothelium-dependent response as well as impaired endothelium-independent response although the impaired endothelium-independent response was

not confirmed by other investigators. Interestingly, even normoglycaemic subjects who are prone to develop type 2 diabetes and insulin resistance syndrome, such as those characterised by previous gestational diabetes (Anastasiou et al, 1998) and low birth weight (Leeson et al, 1997), exhibit impaired flow-mediated dilatation. This has led to the suggestion that endothelial dysfunction may precede the development of type 2 diabetes (Tooke & Goh, 1998).

1.2.11.4 Hyperhomocysteinaemia

Elevation of plasma homocysteine has increasingly been implicated as an independent risk factor for CHD (Verhoef et al, 1996; Nygard et al, 1997). Cumulative evidence suggests that this may be at least in part mediated by its adverse effects on endothelial function. Impaired flow-mediated dilatation (FMD) has also been found in homocysteinuric children (Celermajer et al, 1993a) and adults with moderate fasting hyperhomocysteinaemia (Tawakol et al, 1997; Woo et al, 1997). Acute methionine-induced hyperhomocysteinaemia impairs endothelium-dependent FMD in healthy subjects (Tawakol et al, 1997; Woo et al, 1997; Bellamy et al, 1998; Chambers et al, 1998; Usui et al, 1999) which can be reversed by folic acid supplementation (Usui et al, 1999). Even mild physiological increments in plasma homocysteine concentrations was sufficient to induce endothelial dysfunction (Chambers et al, 1999a). The precise mechanism for homocysteine-induced endothelial dysfunction has been subject of controversy. It is thought to be related to oxidative stress since endothelium-dependent vasodilatation is ameliorated following administration of folate (Usui et al, 1999) and vitamin C (Chambers et al, 1999b), both of which are free radical scavangers. Folate has been shown to ameliorate homocysteine–induced endothelial dysfunction. In addition to a homocysteine-lowering effect, folate may also stimulate

generation of endogenous tetrahydrobiopterin, a co-factor for NO synthase, from inactive dihydrobiopterin (Kaufman, 1991). Recent evidence also suggests that endothelial dysfunction in hyperhomocysteinaemia may be due to elevation of ADMA which has been demonstrated in monkeys (Boger et al, 2000) and more recently, in humans (Boger RH, et al, 2001). It is possible that oxidative stress induced by elevated homocysteine decreases the activity of the enzyme, dimethylarginine dimethylaminohydrolase (DDAH) (Vallance P, 2001).

1.2.12 Links between risk factors and atherogenesis

In addition to various disease states, endothelium-dependent vasodilatation is also impaired in old age (Lyons et al, 1997; Celermajer et al, 1994), young healthy subjects with a family history of premature CHD (Clarkson et al, 1997) and cigarette smoking (Celermajer et al, 1993b). The age-related endothelial dysfunction may partially explain the increased cardiovascular risk in the elderly. In asymptomatic young smokers, impairment of endothelium-dependent vasodilatation is reversible with smoking cessation (Celermajer et al, 1993b). It may be that tobacco has a direct toxic effect on the vascular endothelium (Davis et al, 1985; Nagy et al, 1997). Additionally, depletion of the cofactor BH_4 for eNOS in chronic smokers may contribute to decreased NO synthesis (Heitzer et al, 2000). This is supported by the finding that BH_4 supplementation restores endothelial function in chronic smokers (Ueda et al, 2000). Thus various effects on the L-arginine: NO pathway exerted by hypertension, diabetes, hyperlipidaemia, hyperhomocysteinaemia, aging, cigarette smoking, family history of CHD may form a link between risk factors and atherogenesis.

1.3 Background to the conflicting evidence of defective L-arginine: nitric oxide: cGMP pathway in Type 1 diabetes: Literature review

Another important issue is how type 1 diabetes alters the structural and functional aspects of the vascular endothelium and more specifically the L-arginine: NO: cGMP pathway. The current literature provides conflicting evidence regarding whether NO release/response is impaired in type 1 diabetes. The following sections focus on vascular studies in type 1 diabetes and postulate potential factors which may have contributed to the conflicting results.

1.3.1 Morphology of vasculature in diabetes

The vascular endothelium forms the lining of the blood vessel wall separating the lumen from the vascular smooth muscle. Under the electron microscope, normal endothelial cells have a cobble-stone appearance with gap junction formation in between cells. A basement membrane separates this single layer of endothelial cells from the smooth muscle. Changes in morphology of the vasculature in diabetes have been characterised in both animal and human models (Box 1.1). Rats are the animal model commonly used and are rendered diabetic by treatment with streptozotocin (STZ), a nitrosamine which is toxic to the pancreatic β-cell. In other species such as the rabbit which is more resistant to STZ (Kushner et al, 1969), alloxan is used to induce diabetes. In alloxan-induced diabetic rabbits, endothelial alterations occur in the aorta as early as 2 weeks after onset of diabetes, and the changes became more severe by 6 weeks (Hadcock et al, 1991). Using cultured aortic endothelial cells from STZ diabetic minipigs, Grunwald et al showed that endothelial cells derived from diabetic minipigs have a higher rate of proliferation and a higher percentage of large multinucleated cells (Grunwald et al, 1985).

Morphological abnormalities of diabetic vasculature

- Enhanced endothelial adhesion of white blood cells, platelets and fibrin-like material
- Increased endothelial cell proliferation
- Increased numbers of multinucleated endothelial cells
- Increased endothelial metabolism of LDL-C
- Increased basement membrane thickness
- Increased endothelial cell surface area
- Increased fluidity of endothelial cell membrane
- Increased capillary diffusion capacity

Box 1.1. Morphological abnormalities of diabetic vasculature

In humans, an increase in basement membrane (BM) thickness of the microvasculature is the major feature in type 1 diabetes. Using transmission electron microscopy, BM thickening has been shown in capillaries of skin in patients with type 1 diabetes compared to non-diabetic subjects (Aageneas & Moe, 1961). Additionally, it has been shown that intensive glycaemic control in type 1 DM after one year reduces BM width in skeletal muscle capillaries (Rosenstock et al, 1988). Furthermore, diabetic neuropathy has been shown to be associated with an increase in BM thickening (Malik et al, 1993), numbers of endothelial nuclei and endothelial cell area (Malik et al, 1993; Yasuda & Dyck, 1987). These thickened BM in diabetes may be more permeable to molecules such as water and albumin since they undergo qualitative changes as a result of glycation (Cochrane & Robinson, 1995). However, this increased permeability does not necessarily lead to increased diffusion of endothelium-derived NO since it has been shown that thickened BM with accumulation of advanced glycation endproducts (AGEs) may chemically inactivate NO leading to reduced bioavailability (Hogan et al, 1992).

With the advent of transmission electron microscopy and fluorescence anisotropy using specific fluorescent probe anchoring at the endothelial surface membrane, an increase in fluidity of the endothelial membrane, an increase in mitochondrial area and a more fluid phase endocytosis in endothelial cells obtained from umbilical cords had been found in type 1 diabetic pregnant women compared with non-diabetic controls (Cester et al, 1996). The functional relevance of these structural abnormalities remains to be fully elucidated but they may contribute to vasculopathy and premature atherosclerosis in type 1 diabetes.

1.3.2 Defective response to NO or reduced NO availability in type 1 diabetes?

Given the potential anti-atherogenic properties of NO, it is possible that a defect in the L-arginine: NO: cGMP pathway might contribute to the increased cardiovascular risk in type 1 DM. This defect could occur at one or several stages of the pathway. There may be reduced basal and/or stimulated NO release, decreased bioavailability of NO or reduced VSMC responsiveness. Experimental evidence suggests that all these defects are plausible in type 1 diabetes. For instance, it has been shown that the vasodilating effect of insulin in skeletal muscle is mediated via an increase in NO release (Steinberg et al, 1994). Hence in the presence of insulin deficiency in type 1 diabetes, there may be reduced NO release. Even if NO release is normal, its bioavailability may be reduced since advanced glycation endproducts, the non-enzymatic glycation and cross-linking of tissue-structure proteins, as a result of chronic hyperglycaemia has been shown to quench NO *in vitro* (Bucala et al, 1991). Alternatively, there may be defective response to NO since hyperglycaemia interferes with NO-induced guanylate cyclase activation (Weisbrod et al, 1993).

1.3.3 Endothelium-dependent vascular responses in type 1 diabetes

There is considerable controversy regarding the extent of endothelial dysfunction in type 1 diabetes, as studies assessing endothelial function in both animal and human models of type 1 diabetes have produced conflicting results (Chan et al, 2000). Endothelial function may be modulated by a number of factors associated with type 1 diabetes including degree of acute hyperglycaemia, chronicity of hyperglycaemia (disease duration) possibly associated with accumulation of advanced glycosylated end-product, presence or absence of diabetic complications such as autonomic neuropathy and microalbuminuria (Poston & Taylor, 1995; Chan et al, 2000).

Variation in these factors between subjects in different studies may in part account for the conflicting results.

1.3.3.1 Animal studies

Impairment of endothelium-dependent relaxation has mostly been demonstrated in both conduit and resistance arteries of chemically induced diabetic animals. In conduit vessel studies, endothelium-dependent relaxation in diabetic animal models have been reported to be impaired (Oyama et al, 1986; Miyata et al, 1992) or normal (Harris et al, 1988; Mulhern et al, 1989; Brands & Fitzgerald, 1998) compared with controls. Similar disparity has also been observed in resistance vessels with endothelium-dependent relaxation being demonstrated to be impaired (Taylor et al, 1994; Alsip et al, 1996; Keegan et al, 1999) or normal (Gebremedhin et al, 1999). In some animal studies, endothelium-dependent relaxation has even been found to be enhanced (Altan et al, 1989; Heygate et al, 1995). Of note, there is substantial difference in the timing between impairment of endothelial-dependent relaxation and onset of diabetes, ranging from 1 week in intestinal arterioles (Lash & Bohlen, 1991) to 4-6 weeks in mesenteric arteries (Kiff et al, 1991; Diederich et al, 1994). This disparity may be due to difference in disease duration, the vascular bed studied and methods of vessel preparation (e.g. using helical strips) which in turn determines the extent of vascular endothelium preservation. An additional factor to consider is the possible cross-over effects due to multiple drug administration in the same specimen (Head et al, 1987). The importance of disease duration which may partially account for the conflicting results in animal models of diabetes has recently been highlighted. Using aortic rings of STZ-induced diabetic rats pre-contracted with norepinephrine, endothelium-dependent relaxation to ACh was found to be increased at 24 hours following

injection with STZ, normal after 1 and 2 weeks of disease and impaired at 8 weeks of disease compared to controls (Pieper, 1999). In both control and diabetic aortic rings, ACh-induced relaxation was blocked using L-nitroarginine suggesting that the enhanced response was mediated through NO (Pieper, 1999). This study illustrates the triphasic response in relation to disease duration in diabetic animal models.

1.3.3.2 Human *in vitro* studies

There have been two studies on *in vitro* endothelium-dependent relaxation. Using isolated resistance arteries (from biopsy specimen of subcutaneous fat from the gluteal region) from type 1 diabetic patients, McNally *et al* demonstrated impaired relaxation to ACh in precontracted small arteries but not to bradykinin or sodium nitroprusside (McNally et al, 1994). The normal vascular response to bradykinin in their study suggests defective endothelial cell ACh receptor excitation-coupling in type 1 DM rather than a reduction in NO synthesis (McNally et al, 1994). Malik et al recently examined resistance vessels dissected from gluteal fat biopsies in normotensive type 1 diabetic patients with varying degree of microvascular complications. The preliminary data showed no difference in ACh-induced relaxation compared with non-diabetic specimens (Malik et al, 1999).

1.3.3.3 Human *in vivo* studies

(1) Forearm venous occlusion plethysmography

Agonist-stimulated vascular responses

This technique has used to study human resistance arteries (Benjamin et al, 1995). Endothelium-dependent vasodilatation was conventionally assessed by intra-arterial (brachial artery) infusion of muscarinic agonists (ACh, methacholine or carbachol).

Endothelium-dependent relaxation in type 1 diabetic patients have been shown to be impaired (Johnstone et al, 1993; Makimattila et al, 1996; O'Driscoll et al, 1997), unchanged (Halkin et al, 1991; Elliott et al, 1993; Smits et al, 1993; Huvers et al, 1999; Vervoort et al, 1999) and even enhanced (Makimattila et al, 1997) (Table 1.2). In a recent study, Meeking *et al* compared the vascular response between ACh and substance P in a small group of normoalbuminuric type 1 diabetic patients (Meeking et al, 2000a). Response to ACh was unchanged but vasodilator response to substance P was impaired. The authors suggested that substance P may be more suitable in the assessment of NO pathway since it is relatively more NO specific (~70%) (Newby et al, 1997; Cockcroft et al, 1994) in comparison to ACh (40-44%) (Vallance et al, 1989a; Newby et al, 1997).

Vasodilator response to nitric oxide donors

Endothelium-independent vasodilatation in type 1 diabetic patients has been assessed extensively by intra-arterial infusion of sodium nitroprusside (SNP), an NO donor. Most studies found unchanged endothelium-independent response to SNP. Interestingly, Calver *et al* is the only group which observed a diminished response to SNP and this was attributed to reduced VSMC sensitivity to NO (Calver et al, 1992). However, their study was relatively small (n=10) and no female patients were included. In another study, Halkin *et al* found that SNP response correlated inversely with Na^+/Li^+ counter-transport (Halkin et al, 1991), a marker for the development of diabetic vascular complications. Makimattila et al were the only group which studied patients with autonomic neuropathy (Makimattila et al, 1997). They found enhanced vasodilator response to both ACh and SNP in macroalbuminuric type 1 diabetic patients with autonomic neuropathy and the magnitude of response in diabetic patients

was directly related to the severity of autonomic neuropathy (Makimattila et al, 1997). These findings may be due to the progressive withdrawal of sympathetic vasopressor tone which may change the balance between endothelial vasodilator system and sympathetic pressor tone in favour of augmented vasodilatation.

Table 1.2. Summary of *in vivo* endothelial function studies by venous occlusion plethysmography in human type 1 diabetes mellitus.

Authors	Numbers (F/M)	Mean disease duration (years)	HbA1c (%) Fructosamine (mmol/l)*	Euglycaemic-insulin clamp	Diabetic complications	Stimulated NO response	Endothelium-independent response
Venous occlusion plethysmography							
Calver et al (1992)	10 (0/10)	"Recent onset"	6.7 ± 0.5	No	without	unchanged	unchanged (verapamil) impaired (SNP)
Johnstone et al (1993)	15 (11/4)	14 ± 2	11.9 ± 0.6	No	not stated	impaired	unchanged (SNP+verapamil)
O'Driscoll et al (1997)	9 (0/9)	18 ± 2	8.3 ± 0.4	Yes	without	impaired	unchanged (SNP)
Huvers et al (1999)	34 (7/27)	17	8.98	Yes	with and without	unchanged	unchanged (SNP)
Elliott et al (1993)	14 (6/8)	20.7	3.3*	Yes	normoalbuminuria	unchanged	unchanged (SNP)
	14 (3/11)	22.6	3.3*	Yes	microalbuminuria	impaired	unchanged (SNP)
Halkin et al (1991)	18 (2/16)	12.0 ± 8.0	4.074 ± 0.207*	No	without	unchanged	unchanged (SNP)
Smits et al (1993)	11 (0/11)	15.1 ± 8.2	9.2 ± 0.9	No	without	unchanged	unchanged (SNP)
Makimattila et al (1997)	10(0/10)	28 ± 3	8.6 ± 0.3	Yes	macroalbuminuria & autonomic dysf.	enhanced	enhanced (SNP)
	12 (0/12)	18 ± 3	8.6 ± 0.3	Yes	microalbuminuria & autonomic dysf.	unchanged	unchanged (SNP)
Meeking et al (1999)	12 (6/6)	13.4 ± 1.9	8.2 ± 0.3	No	without	unchanged	unchanged (SNP)
Vervoort et al (1999)	50 (25/25)	8.3 ± 0.8	8.4 ± 0.2	No	without	unchanged	not assessed

Vascular response to NO-independent vasodilator

The calcium channel blocker, verapamil, was used in two studies as a NO-independent vasodilator (Johnstone et al, 1993; Calver et al, 1992) to ensure that the capacity of vascular smooth muscle to dilate (unrelated to NO pathways) is intact. In both studies, vascular response to verapamil was unchanged between type 1 diabetic patients and controls.

Vasoconstrictor response to nitric oxide synthase inhibitor

In the assessment of basal NO release using L-NMMA, vasoconstrictor responses are also inconsistent. Vasoconstrictor response to L-NMMA was found to be unchanged (Huvers et al, 1999; Vervoort et al, 1999) or blunted (Calver et al, 1992; Elliott et al, 1993). This blunted response was found to be most pronounced in type 1 diabetic patients with microalbuminuria (Elliott et al, 1993).

(2) Flow-mediated dilatation assessed by vascular ultrasound

Endothelial function of conduit vessels (mainly the brachial artery) has been evaluated using high-resolution vascular doppler ultrasound (Celermajer, 1998) to determine the degree of flow-mediated dilatation (FMD) (Lambert et al, 1996; Zenere et al, 1995; Lekakis et al, 1997; Enderle et al, 1998). The brachial artery is used because it is easily accessable and there is some evidence that endothelial dysfunction in brachial artery parallels that of the coronary artery (Anderson et al, 1995). Although this method of evaluating endothelial function is noninvasive, it has the disadvantage that a highly skilled ultrasonographer is required for imaging to be done accurately and reproducibly. This technique has a relatively low within-person reproducibility (Hardie et al, 1997). Furthermore, differences in the flow velocity profile during

reactive hyperaemia could lead to poor reproducibility of flow-mediated dilatation (Celermajer, 1998). The brachial arteries have mostly been used although one study assessed the common femoral artery (Zenere et al, 1995). In comparison with forearm studies using venous occlusion plethysmography, there are fewer studies of type 1 diabetes involving the use of vascular ultrasound (Table 1.3). The results are again inconsistent with endothelium-dependent vascular responses shown to be impaired (Zenere et al, 1995; Lekakis et al, 1997; Clarkson et al, 1996) or unchanged (Lambert et al, 1996; Enderle et al, 1998). Similarly, endothelium-independent vascular response to GTN was found to be either impaired (Zenere et al, 1995) or unchanged (Enderle et al, 1998; Clarkson et al, 1996). In studies demonstrating reduction in FMD in type 1 diabetic patients, there does not appear to be a relationship with presence or absence of microalbuminuria (Celemajer et al, 1992; Meeking et al, 1999).

Table 1.3 Summary of *in vivo* endothelial function studies using vascular doppler ultrasound in type 1 diabetic patients.

Authors	Numbers (F/M)	Mean disease duration (years)	HbA1c (%)	Euglycaemic-insulin clamp	Diabetic complications	Flow-mediated dilatation	Endothelium-independent response
Flow-mediated vasodilatation assessed by high resolution vascular ultrasound							
Clarkson et al (1996)	80 (40/40)	13 ± 8	9.5 ± 2.2	No	with and without	impaired	impaired (GTN)
Zenere et al (1995)*	10 (5/5)	10 ± 1	7.7 ± 0.2	No	normoalbuminuria	impaired	impaired (GTN)
	8 (6/2)	11 ± 1	7.2 ± 0.5	No	microalbuminuria	impaired	impaired (GTN)
Lambert et al (1996)	52 (22/30)	14.9 ± 8	7.9 ± 1.2	No	retinopathy only	unchanged	unchanged (GTN)
Enderle et al (1998)	17 (10/7)	21.5 ± 10.2	8.0 ± 1.1	No	without	unchanged	unchanged (GTN)
Lekakis et al (1997)	5 (4/1)	20 ± 8.5	7.1 ± 1.0	No	microalbuminuria	impaired	impaired (ISDN)
	26 (17/9)	12.9 ± 8.4	6.5 ± 1.5	No	normoalbuminuria	impaired	unchanged (ISDN)
Meeking et al (1999)	18 (10/8)	27.8 ± 2.4	10.5 ± 2	No	microalbuminuria	impaired	unchanged (GTN)
	18 (10/8)	26.9 ± 2.0	9.6 ± 0.3	No	normoalbuminuria	impaired	unchanged (GTN)
Poredos et al (2000)	28 (14/14)	8.1 (3-15)	8.7 ± 1.4	No	microalbuminuria	impaired	unchanged (GTN)

* The common femoral artery was studied by echo-ultrasound, W/M=women/men

1.3.4 Conflicting data in human *in vivo* studies: potential factors

Sex difference – The sex difference in CHD prevalence is abolished in type 1 diabetes (Krolewski et al, 1987). If there is any differential effect of diabetes on endothelial function between the sexes, then differences between studies could arise if the proportion of women in the studies differs. Furthermore, forearm length (and hence vessel length) is different between men and women. This could result in differences in vascular responses to ACh since ACh is rapidly destroyed by cholinesterase enzymes and the magnitude of response is partially dependent upon forearm length (Chowienczyk et al, 1994). With the exception of one study (Johnstone et al, 1993), all research groups using venous occlusion plethysmography have studied predominantly male diabetic subjects (Elliott et al, 1993; Huvers et al, 1999) with female diabetic subjects not being included at all in some studies (Makimattila et al, 1997; O'Driscoll et al, 1997; Smits et al, 1993; Calver et al, 1992). There is some suggestion from Table 1 that many of the negative studies are those which have included relatively few women. Hence, it is possible that differences in male to female ratio between studies, to a certain extent, contributed to the inconsistent results.

Effect of glycaemic control - There is a large variation in mean HbA1c measured in type 1 diabetic patients used in different research groups. The greatest contrast is between Calver et al (6.7%) compared with Johnstone et al (11.9%), who found stimulated endothelium-dependent vasodilatation to be unchanged and impaired respectively (Calver et al, 1992; Johnstone et al, 1993). Most research groups have studied patients with sub-

optimal glycaemic control with HbA1c ranging from 8.3-9.2% (Makimattila et al, 1997; O'Driscoll et al, 1997; Smits et al, 1993; Huvers et al, 1999).

Degree of hyperglycaemia - There is substantial evidence that acute hyperglycaemia attenuates endothelium-dependent vasodilation (Akbari et al, 1998; Williams et al, 1998). Hence it is possible that the variable degree of hyperglycaemia at the time of measurement in type 1 diabetic patients could have a relevant impact on endothelium-dependent vascular responses. To minimize the acute hyperglycaemic effect during studies, some groups have used the euglycaemic-insulin clamp method to maintain normoglycaemia (O'Driscoll et al, 1997; Smits et al, 1993; Huvers et al, 1999). This aspect will be discussed in greater detail in the "Methods" section.

Effects of diabetic complications - Several groups have used type 1 diabetic patients without microvascular complications in whom endothelium-dependent responses were found to be either unchanged (Calver et al, 1992; Smits et al, 1993) or impaired (Elliott et al, 1993; O'Driscoll et al, 1997). The only human *in vivo* study involving type 1 diabetic patients with documented autonomic dysfunction and macroalbuminuria showed hyperresponsiveness to ACh (as well as to SNP) (Makimattila et al, 1997). It is plausible that this hyperresponsiveness to ACh is a result of increased sensitivity of the VSMC as it would also explain the hyperresponsiveness to SNP (Steinberg & Baron, 1997).

An important limitation in all these *in vivo* human studies using forearm venous occlusion plethysmography is that although patients with clinical macrovascular complications are

excluded, it is possible that some patients have subclinical macrovascular complications (asymptomatic atherosclerosis) which has relevant impact on endothelial function. Furthermore, due to methodological difficulties, the studies are all quite small ($n<34$) and do not allow adequate evaluation of the effects of concomitant diabetic complications. To establish the important determinants within the L-arginine: NO pathway in type 1 diabetes, a large study is needed in which the impact of factors such as sex, disease duration, diabetic complications and glycaemic control can be evaluated.

1.4 Background to methodology

1.4.1 Venous occlusion plethysmography

In this thesis, blood flow in the forearm resistance vessels was assessed using venous occlusion plethysmography. This technique involves intra-brachial arterial cannulation and local administration of vasoactive drugs to the forearm arterial bed. Local infusion of agonists and antagonists of the L-arginine: NO: cGMP pathway allows pharmacological dissection of the NO pathway under normal and pathophysiological conditions. Venous occlusion plethysmography measures total forearm blood flow (FBF) using mercury-in-rubber strain gauge. Changes in blood flow in both arms can be measured and compared during local infusion of vasoactive drugs. This technique is well established worldwide and has the distinct advantage of taking into account of minor changes in arterial pressure or sympathetic activation.

1.4.1.2 Physiological principles

Measurement of FBF response using venous occlusion plethysmography is based on the principle that if venous return from the arm is obstructed and arterial flow continues unimpeded, the forearm expands at a rate proportional to the rate of arterial blood flow (Whitney 1953). Thus the rate of increase in forearm circumference is directly proportional to the arterial inflow (Wilkins & Bradley 1946). Pharmacological agents are delivered into the forearm vasculature to assess vascular response via a 27-guage needle inserted into the brachial artery and the circumference change of both arms is simultaneously measured by mercury-in-rubber strain gauge which is attached to the computer software (MacLab, Hokanson). The experimental set up of the forearm is

shown in Figure 1.1. During each recording period, upper arm cuffs are inflated to 40 mmHg for approximately 10 seconds in every 15 seconds to occlude venous return. The 5-second deflation period during each measurement is often long enough to ensure venous emptying in the forearm veins. This is important since the presence of any residual venous blood will impede the rate of arterial flow. For this reason, both arms are elevated above the level of the heart, supported at the elbow and the wrist. Provided that the subject is comfortable, warm and relaxed, forearm blood flow does not change significantly over several hours and the response to drugs should be consistent.

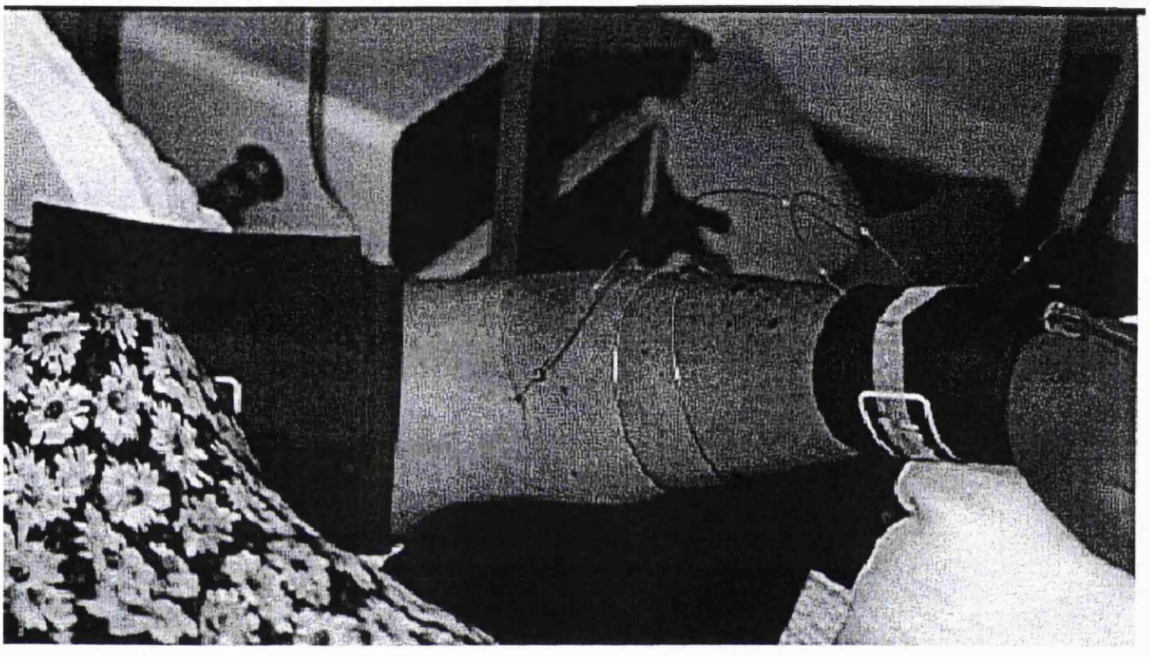


Figure 1.1. Forearm venous occlusion plethysmography.

1.4.1.3 Brachial artery cannulation and effect of arm dominance

Before brachial artery cannulation, the arm is positioned in semi-pronation. This allows the brachial artery to be closest to the overlying skin and be easily palpated. A 27-guage stainless steel needle sealed with wax to an epidural catheter is then inserted into the brachial artery under local anaesthesia. The non-dominant arm is often chosen for cannulation and the dominant (non-infused) arm serves as control. It has been assumed for decades that intra-arterial cannulation and left/right arm dominance has no effects on haemodynamics. However, these assumptions have only recently been formally tested. Kamper and Chang studied the haemodynamic effects of intra-arterial cannulation and arm dominance in response to physiological tests and noradrenaline infusion study in 24 healthy volunteers (Kamper & Chang 1999). They found that intra-arterial cannulation of the dominant or non-dominant arm did not result in differences in FBF responses to physiological tests (Valsalva manoeuvre, mental arithmetic test, lower-body negative pressure and forearm ischaemic response). Furthermore, the reduction in FBF in response to noradrenaline was not significantly different in the dominant or non-dominant arm. It would appear from this small study that either arm can be used for intra-arterial cannulation without affecting the local haemodynamic response. However, further large-scale studies will be required to confirm this and to determine whether this also applies to vasodilator response. In this thesis, the non-dominant arm was chosen in all subjects for intra-arterial cannulation.

1.4.1.4 Exclusion of hand circulation

Throughout the measurement, both hands are excluded from the circulation by inflation of wrist cuffs to suprasystolic pressure (Kerslake 1949). While the forearm

arterial bed primarily supplies the skeletal muscles and thus predominantly reflects mainly muscle blood flow, the blood flow supply to the hand is predominantly through skin with a high proportion of arteriovenous shunts (Roddie & Wallace 1979). These two vascular beds may have different properties in their pharmacological response to drugs. Assuming that the mean ratio of blood flow in both arms approaches unity at rest, the change in ratio of blood flow in the infused arm is the direct result of the infused pharmacological compound (Benjamin et al, 1995).

1.4.1.5 Vascular resistance

Vascular resistance is the degree of obstruction imposed by the blood vessel such that the perfusion pressure must overcome this resistance in order for the blood flow to travel along the blood vessel. In practice, the vascular resistance is determined as follow:

$$\textit{Resistance} = \textit{Perfusion pressure} / \textit{Blood flow}$$

However, this formula is limited by several factors *in vivo* and is an over-simplification of what actually occurs within the blood vessel. This is because blood is driven by a variable and pulsatile pressure through a distensible system. The cardiac output which determines the perfusion pressure is not constant throughout the period of measurement and the resulting blood flow is pulsatile rather than laminar. Furthermore, the blood vessel is not a rigid tube and its distensibility is influenced by neuroendocrine control and autonomic innervation. Hence simple calculation using the above formula does not truly reflect the complex underlying physiological events.

For this reason, results are not expressed in terms of vascular resistance in the present study.

1.4.1.6 Importance of basal flow and arterial pressure

In comparing FBF response to drugs in two groups of subjects, it would be ideal if the basal blood flow is similar although this is sometimes not the case. This is because the concentration of the drug is inversely proportional to the basal blood flow. Thus the higher the basal blood flow, the lower the concentration of drug delivered to the distal circulation and hence the lower the vascular response. The response of a blood vessel to a vasodilator is critically dependent on the pre-contraction state. The greater the degree of pre-contraction, the larger the range over which the blood vessel can dilate. Hence differences in arterial pressure between two groups can lead to different pre-contractile vascular tone resulting in altered responses. Furthermore, differences in arterial pressure directly affects the forearm vascular resistance. Hence, if the results are analysed in terms of changes in vascular resistance, absolute changes may differ but percentage changes should not be influenced.

1.4.1.7 Forearm size

The bioavailability of the infused drug to the vasculature is likely to be dependent on the volume of distribution, which in turn is determined by the forearm length and volume. The forearm geometry is particularly relevant when assessing vascular response to ACh since it is unstable in plasma and is rapidly destroyed by cholinesterase in blood. When administered into the brachial artery, more than 99% of the infused dose of ACh is destroyed before reaching the hand (Duff et al, 1953). Women generally have shorter and thinner arms than men and therefore may have

greater vasodilator response to ACh. Chowienczyk *et al* showed that FBF response to ACh is significantly greater in women than men. However, this sex difference is abolished after correcting for differences in forearm length (Chowienczyk et al, 1994). Hence, it is critical to adjust for these potential confounding factors during data analysis.

1.4.1.8 Dose-response relationships

Incremental doses of vasoactive agents are infused into the forearm vasculature and the response is often expressed as cumulative dose-response curves. The construction of a dose-response curve is essential to assess comparative efficacy, demonstrate agonism/antagonism and compare responses between groups of individuals. The dose-response relationship is conventionally expressed as the *log of the drug dose infused per unit time* (x-axis) against either the *percentage change of FBF ratio (infused arm / control arm)* or *the absolute flow* expressed as ml/100ml forearm volume/min (y-axis). However, the FBF response is determined by the local plasma concentration of the infused drug which is not necessarily the same as the dose of the drug infused. In practical terms, it is not possible to measure the exact plasma concentration of drug delivered and furthermore, this is altered by the response and therefore changes throughout the infusion period. For instance, infusion of a vasodilator drug increases the FBF response resulting in a fall in concentration of the drug delivered to the tissue whereas infusion of a vasoconstrictor will decrease blood flow and cause a rise in local drug concentration. This is further complicated by the fact that basal blood flow varies widely between individuals and consequently FBF response to any given drug will also vary to some extent. Precise calculation of local drug concentration is

possible but does not correlate well with the response (Robinson et al, 1990). In practice, dose-response relationship is best obtained if results are expressed in terms of the drugs infused per unit time (Benjamin et al, 1995; Robinson et al, 1990). The doses of the pharmacological agent chosen in FBF studies are those at the lower linear part of the classical sigmoid dose-response curve since doses which produce near maximal response may produce systemic effects. The duration of each dose of drug infusion is also important. If the effect of a drug dose has not reached a plateau before the next increment drug dose is infused, the progressive dose increments may lead to cumulative local (or even systemic) effects after recording has completed. For a drug such as L-NMMA which has a relatively long half-life, infusing each dose less than 5 minutes may result in underestimation of the true response. On the other hand, for drugs with relatively short half-life such as bradykinin, prolonged infusion of each dose may lead to tachyphylaxis (Benjamin et al, 1989). Vasoactive drugs used in all the experiments carried out in this thesis have previously undergone preliminary investigations and the optimum dose and duration of each drug infusion have been determined.

1.4.1.9 Vascular response in the forearm vasculature

For a given drug infusion, the absolute flow can be expressed as a, b (ml/100ml/min) for basal flow and c, d (ml/100ml/min) for flow during drug infusion.

	Infused arm	Control arm
Basal flow	**a**	**b**
Drug dose	**c**	**d**

The FBF response is then conventionally expressed as the percentage change in the ratio of flow during drug Infusion (infused /control arm) in relation to the ratio of basal flow:

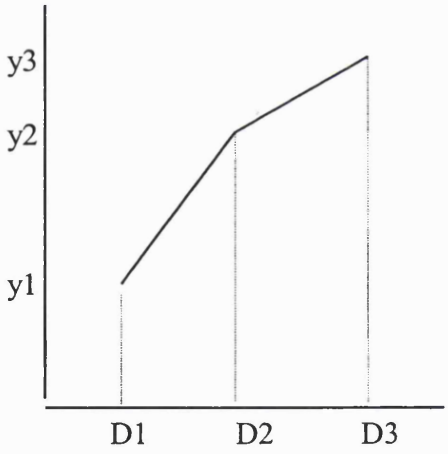
$$\{(c/d) - (a/b)\} / (a/b) \times 100\%$$

During the course of an experiment, minor changes in vascular tone occurs even at rest as a result of systemic alterations in the level of arousal or due to sympathetic activation. These systemic alterations in vascular tone will affect both arms equally and thus the ratio of blood flow in the infused arm compared to that in the control arm remains constant (Greenfield & Patterson 1954). Hence any change in the FBF ratio will be the direct effect of the locally infused drug. There has been considerable discussion in the literature on how to best to adjust for external factors leading to sympathetic activation {Benjamin, Calver, et al. 1995 7621 /id}; Chin-Dusting et al, 1999b; Petrie et al, 2000). It has been suggested that one way to control for background systemic changes in flow is to express the response to changes in terms of the percentage change in the ratio of flow in the infused arm / flow in the control arm

to ratio of flow at baseline. This assumes that any differences in basal flow are controlled for. However, this percentage change is not independent of basal flow.

1.4.1.10 Expressing and analysing data

Since forearm blood flow response is measured across 3 doses of drugs, some ways of summarising the data across 3 doses is required is data analysis. Conventionally, the dose-response curves of two groups of individuals are analysed and compared using analysis of variance for repeated measures (ANOVA) (Matthews et al, 1990). Alternatively, a single summary measure can be used to compare two groups such as the area under the dose-response curve (AUC). The calculation of AUC is as below:



$$AUC = [(D2-D1) \times (y1+y2)/2] + [(D3-D2) \times (y2+y3)/2]$$

Figure 1.2 A hypothetical dose-response curve demonstrating the calculating of area under the curve (AUC).

Using AUC as a single summary measure, however, has the disadvantage of putting a disproportionate amount of weight on the response to the second dose (D2) since this response (y2) is used twice in the calculation to determine the AUC. Some researchers use the maximum response to a given drug dose for comparison between groups

(Meeking et al, 2000b). However, this does not take into account the full data set. Furthermore, the maximum response to a drug dose is dependent on the previous dose infused and hence all the responses need to be taken into account. However, none of the above methods of analysis truly takes into the account of the effect of basal flow, which is increased in diabetes (Halkin ezt al, 1991; Vervoort et al, 1999). The biological response of the infused arm vasculature is critically dependent on the vascular tone at rest which controls the basal flow. In other words, basal flow in the infused arm (a) has a significant effect on the ratio (c/a) and using the formula {(c/a)/(b/d)} does not fully adjust for basal flow in the infused arm (a).

Given the importance of basal flow, it is imperative that any analysis should adjust for basal flow. With a relatively large sample size such as this study, it is possible to take advantage of regression techniques to more fully adjust for basal flow in the infused arm and concomitant change in flow in the non-infused arm. Basal blood flow is greater in men than women, greater in diabetic patients than non-diabetic healthy volunteers. We used analysis of covariance to define how flow under drug infusion differed by diabetes and sex adjusted for basal flow between groups. Further adjustment was made for forearm volume and flow in the control arm (detail in 2.6 Statistical analysis section).

1.4.1.11 Reproducibility

The reproducibility of venous occlusion plethysmography is not very well established. Earlier studies examining the reproducibility of this technique have only focused on unilateral FBF measurement. Roberts et al found reasonable reproducibility of baseline unilateral FBF in six subjects studied on six occasions with a mean

coefficient of variance (CV) of 10.5% (Roberts 1986) whereas Altenkirch *et al* reported poor reproducibility of baseline unilateral FBF in twelve subjects studied on three occasions (mean CV 25%) (Altenkirch et al, 1990). In the only study which examined reproducibility in bilateral measurement, Petrie *et al* found that FBF expressed as ratio of infused arm over control arm is more reproducible than unilateral measurement (Petrie et al, 1998) since it takes into account small changes in arterial pressure and sympathetic activation. In this study, it was shown that using the non-infused arm as a concurrent control with expression of data as forearm blood flow ratios resulted in an improvement in between-day, intra-subject variability from 31% to 19% (Petrie et al, 1998). Yki-Jarvinen reported a CV of 13% but this is based on a within-day reproducibility study for unilateral plethysmography (Yki-Jarvinen & Utriamen, 1999).

1.4.1.12 Methodological considerations

(1). Euglycaemic-insulin clamp

There is some evidence that acute hyperglycaemia attenuates endothelium-dependent vasodilatation (Akbari et al, 1998; Williams et al, 1998). It is possible that the variable degree of hyperglycaemia in diabetic subjects at the time of study influenced forearm blood flow. For this reason, many research groups used the insulin clamp method to render diabetic subjects euglycaemic during forearm blood flow studies (Elliott et al, 1993; O'Driscoll er al, 1997; Makimattila et al, 1997; Huvers et al, 1999). Whilst this technique has the advantage of removing one (of many) potential confounders of blood flow, there are several disadvantages. Firstly, diabetic patients are by definition hyperglycaemic. Rendering diabetic patients euglycaemic with the clamp technique does not allow them to be studied in their usual physiological environment. Secondly, to maintain euglycaemia, a greater amount of insulin (compare to the usual dosage of insulin) would be required. Insulin is a vasodilator mediated by at least in part by NO (Steinberg et al, 1994) and hence this would lead to increased vasodilatation. Thirdly, the introduction of clamping would introduce an experimental condition for the patient generates additional anxiety which subsequently influences the sympathetic control of forearm blood flow. To obtain a crude estimate of changes of glucose, we measured plasma glucose concentrations before and after the study were measured and adjust for them during data analysis.

(2). Effects of phases of the menstrual cycle

The female sex hormone, oestrogen, has direct effect on the vasculature both at the level of endothelium and vascular smooth muscle cells (detail discussion in chapter 4). Endogenous oestrogen level fluctuates during the menstrual cycle and hence vascular reactivity would be altered accordingly. Ideally in our study, the female subjects should be controlled for phases of the menstrual cycle so that this potential confounder can be taken into account. However, in practice, this proved to be difficult and restricting women (many of whom had a job) to be studied on certain days of the month would significantly reduce our sample size. To take into the account the influence of the menstrual phases, we recorded the menstrual phase of each female subject during which forearm blood flow was performed. Additionally, in order to evaluate how changes in phases of the menstrual cycle alter forearm blood flow response to specific drugs, we conducted a small study in 15 healthy young women utilising the same drugs (all except ACh) as in the main study (Chapter 4). We were then able to interpret our main findings taking into account the results from this menstrual cycle study.

1.4.2 Electron beam computed tomography measurement of coronary artery calcification

We chose coronary artery calcification since it is the most direct measurement of atheroma burden within the coronary vasculature and electron beam computed tomography (EBCT) is a well-validated method to quantify the volume or area of coronary calcium. The following section reviews the basis of using EBCT-defined calcification score as a surrogate marker for CHD.

1.4.2.1 The biological role of calcium in atheromatous plaque

Calcium is one of the key components of atherosclerotic plaques and calcification of coronary arteries has been associated with CHD (Frink et al, 1970; McCarthy & Palmer, 1974; Wexler et al, 1996). In atherosclerotic plaques, calcium is found as calcium hydroxyapatite (the form found in bone) rather than amorphous calcium phosphate (Schmid et al, 1980). Far from being a degenerative process, the process of calcification in atherosclerotic plaque development is active and regulated in a fashion similar to bone mineralization (Fitzpatrick et al, 1994; Wexler et al, 1996). It has been shown that inflammatory cells such as macrophages and mast cells co-localise with intimal calcification (Jeziorska et al, 1998). This has led to the suggestion that hydroxyapatite forms in vesicles in necrotic or apoptotic cells in the atheromatous lesion. A lipid-rich environment may promote the nucleation of the hydroxyapatite (Proudfoot et al, 1998). Some vascular wall cells and inflammatory cells can produce bone-associated proteins, e.g. bone morphogenetic protein-2 and Matrix Gla protein which have been found in atherosclerotic plaques (Proudfoot et al, 1998). Their role in the initiation or remodeling of calcification is unclear. Matrix Gla protein knockout

mice develop extensive vascular calcification suggesting that it may have an inhibitory role in calcification (Luo et al, 1997), whereas lipid oxidation products may upregulate vascular calcification (Parhami & Demer, 1997; Parhami et al, 1997) possibly by increasing intracellular free calcium concentrations in vascular smooth muscle cells (Weisser et al, 1992). The signaling pathway for vascular calcification has not been fully elucidated although it may involve cAMP which has been shown to mediate osteoblast-like differentiation of calcifying vascular cells (Tintut et al, 1998).

1.4.2.2 EBCT as a method to measure coronary artery calcification

Electron Beam ("ultrafast") Computed Tomography (EBCT) is a relatively recent radiological innovation developed only since the 1980s. It allows non-invasive quantification of CAC (Agatston et al, 1990). In conventional CT scanning, the X-ray source is rotated around the patient and thus scan times are slow (1 to 2 seconds). Spiral CT, introduced in the early 1990s, is faster and can be used to measure calcification but takes about 500ms per scan (Ohnesorge et al, 2000). With EBCT, a rotating electron beam is directed towards four tungsten targets to generate X-rays that pass through the patient to the detectors. Since the X-ray source does not move around the patient, rapid scans (100 ms) are possible. These are programmed to trigger by electrocardiography to capture during late diastole, thus avoiding motion artefact. The end result is a full series of images with excellent spatial resolution obtained at relatively low radiation dose (<1 mSv). The entire procedure takes approximately 10 minutes and requires minimal patient effort (only breathholding).

1.4.2.3 Standard protocol & CAC calculation

The most common scanning protocol used is to take two sets of 20 transverse tomograms of 3 mm thickness from the lower margin of the bifurcation of the right branch of the pulmonary artery to the apex of the heart with the subject breath-holding. A radiologist then places a region of interest around each potentially calcific lesion (i.e. all those with a peak density > 130 Hounsfield units) (Agatston et al, 1990) within the four coronary arteries, right coronary, circumflex, left anterior descending and left main. The area and peak density in Hounsfield units of each lesion is then measured automatically by the software in the CT workstation. A calcification score (termed the Agatston score) is then calculated by multiplying the area by a weighting coefficient based on the peak density within each focus. The weighting coefficient ranges from 1 (peak density 131 – 199 HU) to 4 (≥400 HU). The scans are taken using a 26 cm^2 field view and a 512 x 512 reconstruction matrix so that the area of 1 pixel is 0.26 mm^2. The focus must have an area of at least 2 pixels in order to be included in the score. A total score for each artery and for the entire heart is calculated by summing the lesion scores.

Protocols for EBCT scanning can vary in terms of the number of slices taken and their thickness, the minimum area threshold included in the score, whether area or volume is calculated and the methods of incorporating density in to the score. Some protocols involve taking 20x 6mm tomograms or in some cases 20 x 3 mm tomograms. The 6-mm protocol may have higher within person repeatability (Wang et al, 1996) but is a less sensitive measure of atheroma burden. In terms of predictive ability, both 3-mm and 6-mm slices yield equivalent results (Secci et al, 1997). Others calculate the volume of calcification rather than the area and have found it to have better

repeatability (Callister et al, 1998). Another variation is to use the average density of a lesion rather than the peak density. Kaufmann demonstrated that using average density had similar test characteristics for detecting angiographic stenosis as the peak density (Kaufmann et al, 1995). Omitting density from the score altogether also improves repeatability but whether it alters the correlation with atheroma burden is unclear (Callister et al, 1998). In this research project, the standard protocol as described above and standard scoring system was chosen as these are the most widely used allowing comparability with other studies. They are also the most widely validated against atheroma burden. A low threshold for area of the lesion was chosen ($0.52mm^2$) since our concern was with maximising the sensitivity to detect atheroma.

1.4.2.4 Repeatability of EBCT

(1) Interobserver agreement

High levels of interobserver agreement have been reported for the same EBCT scans read by two or more observers. In one study, of 88 scans two observers reported the same score for 80% of scans. Most of the disagreement was due to a computer error on the early software (Agatston et al, 1990). Similarly, Kaufmann demonstrated almost perfect agreement between three observers reading the same scans (r=0.998) (Kaufmann et al, 1994). The agreement was similar using calcification area rather than score. The sources of disagreement were observers denoting slightly different areas as the region of interest to be scored, observers omitting very small distal lesions and scoring the same area twice in error. For defining calcification as present or absent, Sheilds *et al* found a high level of agreement between two observers (kappa statistic of 0.83) (Shields et al, 1996). The correlation between the scores from two observers

was 0.99 for the total heart score and interobserver variability was low (3.5%) (Hernigou et al, 1996).

(2) Within-person / interscan repeatability

The within-person repeatability has been assessed by scanning subjects twice over a short period of time and with the scan read by a single observer on both occasions. This encompasses both the within-observer repeatability and the repeatability of the actual scanning technique. Biological variation is negligible since the actual amount of calcium is unlikely to change within a short space of time. Several parameters has been used to measure repeatability. The agreement in the proportion classified above a particular score on two occasions can be used, ideally adjusted for chance using the kappa statistic. The correlation coefficient (r) between the two scores or the variability, defined as the difference in the two scores / mean of the two scores, may be used.

Studies have generally shown extremely high within–observer repeatability when the same scans were read on two different occasions by the same observer (Kaufmann et al, 1994; Hernigue et al, 1996). Most of the within-person variability in scores is due to the actual scanning technique. Using the standard protocol (40 x 3mm slices) in 91 subjects the agreement for classifying patients as having a score of zero or not was 89% (Devries et al, 1995). All those classified as having calcification in one scan but not the other had scores approaching zero. The variability did not differ with age, sex or BMI and was higher at low scores than for patients with high scores (Devries et al, 1995). Callister et al also found higher repeatability at higher calcification scores. They also demonstrated that the use of volume estimates rather than area leads to a

better repeatability (Callister et al, 1998). Using the 20 x 3mm-slice protocol the repeatability coefficient was 0.99 for the total heart score and was lowest for the left main score at 0.83 (Shields et al, 1995). For classifying patients as having a score above zero or not Hernigou found agreement between scans on two occasions in 89% (kappa not reported) and the correlation between the two scores to be >0.99 (Hernigou et al, 1996). For the 30 x 3 mm scanning protocol variability was 29% (Wang et al, 1996). However, Yoon *et al* found considerable interscan variation in 951 asymptomatic subjects (Yoon et al, 2000). In this study, in order to minimize the positional difference between scans every subject underwent two consecutive cardiac scans at one imaging session without being moved. The average percentage difference in calcium score was 43% for men and 28.4% for women (Yoon et al, 2000). This interscan variability may represent an important limitation of EBCT.

In summary, the EBCT score of an individual is likely to vary minimally when the same scan is read by two different people or read by the same person twice. However, CAC score will vary to a more important extent if the same individual's scan is performed on two different occasions. In general, the closer ones score is to zero the greater the proportional variation. The level of variation is such that in a cohort where about half have been defined as having calcification about 10% might be reclassified on the second occasion. Nonetheless there is a high degree of consistency in the ranking of subjects if scanned twice. In the present study, this misclassification may reduce the strength of associations between endothelial function and calcification but it will not lead to false positive results. The main implication of these data for the present study is that the robustness of comparisons on the prevalence of any calcification (score >0) between groups should be confirmed by repeating the analyses

with a slightly higher threshold score (say five or over) as denoting the presence of calcification.

1.4.2.5 Coronary artery calcification: an index of atheroma burden

Autopsy studies in the 1950's and 1960's demonstrated that intimal calcification is pathognomonic of atherosclerosis (Blankenhorn & Stern, 1959; Eggen et al, 1965). Although not every atherosclerotic plaque had calcification, the amount of calcification increased with the amount of atherosclerosis (Blankenhorn & Stern, 1959; Eggen et al, 1965; Warburton et al, 1968). More recent autopsy studies have confirmed the very high correlation between the amount of atheroma and calcification. Sangiorgi and coworkers carried out detailed segment by segment analysis of 723 coronary artery segments from 13 hearts (one female) obtained from consecutive autopsies (mean age 54) (Sangiorgi et al, 1998). They found that 92% of 723 coronary segments had some calcification and the calcium volume was highly correlated with plaque volume at segmental level ($r=0.52$, $p<0.0001$), vessel level ($r=0.89$, $p=<0.0001$) and, most importantly for our purposes, at the individual heart level ($r=0.87$, $p<0.0001$). Rumberger *et al* examined 13 hearts from 8 men and 5 women (mean age 43) who were consecutively referred for autopsy (Rumberger et al, 1995a). Two had a previous diagnosis of CHD. The hearts were excised and scanned whole and then the coronary arteries were dissected out and scanned again. The calcium score for the whole heart scan was almost identical and was highly correlated with the scan of the excised vessels ($r=0.99$). The correlation between EBCT-defined CAC score and histologically defined plaque area was high at 0.93 at heart level and 0.90 at vessel level. At segment level correlation with CAC score was lower at $0.56 \leq r \geq 0.76$

(Rumberger et al, 1995a). Thus these studies indicate that measurement of CAC is a good indicator of atheromatous plaque burden at the total heart level especially.

1.4.2.6 Coronary calcification and luminal disease severity

Findings from autopsy studies suggest that EBCT-defined calcification does not correlate well with actual luminal narrowing. Simons *et al* studied 522 paired coronary computed tomographic/histologic sections from 13 autopsy hearts. They found that while coronary artery calcium burden and quantitative extent of coronary artery atheromatous plaque are directly correlated, the range for plaque area or percent lumen stenosis, or both, associated with a given calcium burden was wide on a segment-by-segment basis (Simons et al, 1992). In concert with this observation, it has been shown that some high-grade lesions and many smaller lesions lack detectable calcium (Mautner et al, 1994; Rumberger et al, 1995a), and that the total area of calcification is approximately 20% of the total plaque area (Rumberger et al, 1995a). The lower correlation between calcification and lumen size at autopsy partially reflects the difficulty in accurately quantifying the *in vivo* coronary lumen using histologic specimens (Rumberger et al, 1995a). In the preparation of histological specimens the shrinkage of the lumen area and wall varies with the amount of atheroma present (Siegel et al, 1985). The low correlation with lumen size also reflects the fact that plaque burden and stenosis are themselves poorly correlated (Clarkson et al, 1994). Sangiorgi and co-workers examined quantitative histologic calcification, plaque, and lumen narrowing and suggested that these differences may be largely due to atherosclerotic coronary remodeling through vessel enlargement as a compensatory process (Sangiorgi et al, 1998). Haberl et al recently showed in 1764 patients with suspected CHD that exclusion of coronary calcium defines a substantial

subgroup of patients with a very low probability of significant stenoses (Haberl et al, 2001).

1.4.2.7 EBCT for detection of subclinical CHD

The currently available non-invasive modalities such as exercise threadmill testing and myocardial single photon emission computed tomography (SPECT) can only identify subjects with advanced CHD. Hence asymptomatic subjects with subclinical CHD who are at risk of sudden death (Chambless et al, 1997) are often not identified by exercise threadmill testing and perfusion scans (Fleg et al, 1990; Blumenthal et al, 1996). In this context, EBCT may have a role as a screening tool in high-risk asymptomatic individuals. In a prospective study of 3895 asymptomatic subjects with coronary risk factors, He and coworkers showed that EBCT is useful to predict silent myocardial ischaemia as detected by SPECT (He et al, 2000). In this study, a significant proportion (46%) of subjects with CAC score ≥ 400 has SPECT evidence of myocardial ischaemia and conversely, only 6.6% of those with CAC scores ≤ 400 had an ischaemic defect by SPECT and virtually all (99.3%) were small. Thus EBCT in combination SPECT with may greatly improve the efficacy in identifying asymptomatic subjects with subclinical CHD.

1.4.2.8 Comparison of EBCT with angiography

Unlike autopsy studies, studies examining the association between angiographically defined stenosis and calcification score replicate real scanning conditions (Budoff et al, 1996; Rumberger et al, 1997; Schmermund et al, 1998). On the other hand, these studies are necessarily carried out on symptomatic high risk and usually much older

subjects than in this research project. As such the results are not directly applicable to the present study population. Furthermore, the purpose of EBCT in the present study is to estimate plaque burden rather than stenosis. The studies found that on an arterial segment-by-segment basis the association between calcification and stenosis is not strong enough to allow EBCT to be used as a diagnostic and localising tool for stenosis. Not surprisingly the specificity of calcification for angiographic stenosis is quite low i.e. a high proportion of people who do not have any angiographic stenosis do have some vascular calcification. However, a fairly high sensitivity of the calcification score for the whole heart for detecting stenosis was consistently found supporting the results from autopsy studies. Some investigators reported that the sensitivity was lower in studies of younger patients (Budoff et al, 1996; Fallavollita et al, 1994), who are active smokers (Schmermund et al, 1997). In other words, particularly at younger ages considerable stenosis and plaque may be present without any calcification. This would be consistent with the contention that plaque calcification may increase with age as well as plaque area.

1.4.2.9 Prognostic value of coronary artery calcification

Several studies examined the value of CAC as a prognostic indicator of clinical events. In 800 symptomatic patients who also underwent angiography, the 5-year event free survival was 58% in those with fluoroscopically defined CAC compared to 87% in those without CAC (Margolis et al, 1980). The prognostic significance of calcification was independent of extent of angiographic disease or of haemodynamic abnormalities. In the South Bay Heart Watch Cohort 1,461 asymptomatic subjects with at least one CHD risk factors were followed up for 55 months after fluoroscopic examination (Detrano et al, 1997). The incidence of CHD events and CHD death were

significantly associated with the number of calcified vessels present independently of other risk factors. Diabetes and cholesterol were associated with CHD independent of the number of calcified vessels. Having at least one calcified artery had a sensitivity of 76%, and a specificity 43% for prediction of either coronary death or myocardial infarction at 3 years follow up. A subset of these patients who were asymptomatic at follow up had an EBCT scan (n=1196) and were followed up for a further 3 years (Detrano et al, 1999). Subjects with a CAC score above 44 (median) was 2.3 times as likely to have coronary events than subjects with lower scores. The CAC score was independently predictive of clinical events but did not add significantly to the ability of the Framingham risk estimate to predict coronary events (Detrano et al, 1999). In that study, having an above median score had a sensitivity of 71% and a specificity of 51% for coronary death or myocardial infarction. A score above 0 had a sensitivity of 87% and specificity of 32%. A score in the top tertile (above 151) had a sensitivity of 52% and a specificity of 67% for detecting either coronary death or myocardial infarction at 3 years. An important limitation of this study is the lack of information on what interventions took place or were adopted post examination and whether these differed according to calcification score. It has been shown that risk-reducing behaviours may be reinforced by the knowledge of a positive scan result (Wong et al, 1996). It is of note that in this follow-up study, 13% of those with coronary events had no detectable calcium (Detrano et al, 1999).

Arad and coworkers followed up 1173 asymptomatic patients (mean age 53 years) for an average of 19 months following a screening EBCT scan (Arad et al, 1996). During this period, there were 26 cardiovascular events in 18 patients (1 death, 7 nonfatal myocardial infarctions, 8 coronary bypass procedures, 9 angioplasties and 1 non-

haemorrhagic stroke). Odds ratios ranged from 20:1 for a calcium score of 100 to 35:1 for a calcium score of 160. The magnitude of the coronary calcium score was highly predictive of subsequent clinical events although conventional risk factors were not measured (Arad et al, 1996). The same cohort of subjects were further followed up for an average period of 3.6 years. Thirty-nine subjects sustained coronary events: 3 coronary deaths, 15 nonfatal myocardial infarctions and 21 coronary revascularisation procedures. A CAC score ≥160 was associated with odds ratios of 15.8 and 22.2, respectively (Arad et al, 2000).

Secci *et al* followed up 326 elderly (mean age 66 ± 8 years) but initially asymptomatic subjects who underwent both 3- and 6-mm image-slice thickness EBCT scanning over a period of 32 months. They demonstrated that for calcium scores above the median, especially when the baseline score was at or above the 75th percentile, there was a significant trend for more subsequent revascularizations and total cardiac events, although the trend for cardiac death and documented myocardial infarction alone failed to reach statistical significance. Quantification of CAC score with either protocol was equally accurate (Secci et al, 1997). This study, however, is significantly underpowered (n=326) for hard cardiac events which accounts for the lack of statistical significance for hard events (death and infarction). Only by combining hard and soft (revascularisations) events were they able to demonstrate a correlation to higher CAC scores. Furthermore, the follow-up may be too short (3 years) to examine cardiac events. Their findings are nevertheless consistent with previous studies in younger adults (Detrano et al, 1996; Arad et al, 1996) and supports the notion that the EBCT-defined CAC score has the potential to become a useful non-invasive predictor of cardiac mortality and morbidity in adults at risk, irrespective of age and gender.

In another study published in abstract form, Agatston and colleagues described 367 originally asymptomatic men and women with a mean age of 52 years who underwent an initial EBCT scan and were then followed up for 36-72 months (Agatston et al, 1996). A total of 26 coronary events (angina, infarction, revascularisation) occurred. The mean baseline CAC score for patients with a cardiac event was 399 whereas it was 76 for those without events ($p<0.01$).

In the only published prognostic study of CAC in diabetes (type 2), CAC score predicted events but interestingly, patients experienced most events were those in the second tertile of CAC scores though caution should be exercised as other conventional risk factors were not adjusted for (Le et al, 1999). To date, there are no data in the prognostic value of CAC in type 1 diabetes.

In the present study, EBCT was used to define plaque burden rather than clinical event risk. The determinants of plaque rupture and thus clinical events are not well understood at present but some determinants such as cap thickness are poorly correlated with plaque size (Mann & Davies, 1996). Many plaques that give rise to clinical events are thought to be relatively small and non-stenosing (Ambrose et al, 1988). Furthermore, the relationship of plaque calcium content to its propensity to rupture is unknown but there are theoretical arguments for both increased and decreased rupture risk (Wexler et al, 1996). All these factors might serve to reduce the predictive value of calcification score for clinical events without implying a lack of correlation between calcification score and atheroma burden. This will be especially true where the prevalence of significant atheroma is high. The fact that CAC is a

strong predictor of event risk, despite these factors, is supportive of its validity as a measure of atheroma burden.

1.4.2.10 Detection of CAC progression by EBCT

Several studies have examined the progression of EBCT-quantified coronary calcification. In a retrospecive study of 149 asymptomatic subjects, Callister *et al* found a significant reduction in calcium-volume score in those treated with HMG-CoA reductase inhibitors (with reduced LDL-C) for a period of 12-15 months (Callister et al, 1998). Similarly, Budoff et al studied 299 asymptomatic individuals with a variety of coronary risk factors over a period of 1-6.5 years. A reduction in calcium-volume score was observed only in those with a final LDL-C < 120 mg/dl (Budoff et al, 2000). It is of note that very few subjects with initial CAC score of zero had any progression of calcification (Budoff et al, 2000). Sutton-Tyrrell et al studied a subgroup of premenopausal women (n=80) from the Pittsburgh Healthy Women Study cohort for a period of 8 years until they reach menopause with EBCT scan performed at 2, 5 and 8 years. There was clear progression with the median CAC score zero at baseline and mean change over follow-up was +11($p<0.001$ for change). An increase in coronary calcium in this study was significantly associated with lower HDL-C, higher triglycerides and cigarette smoking (Sutton-Tyrrell et al, 2001). The progression of coronary calcification has also been studied recently in 97 type 1 diabetic patients. Preliminary results showed that over a period of 2-3 years, there is measurable progression of CAC in those over 30 years of age which parallels an increase in total cholesterol and less so an increase in LDL-C and blood pressure (Snell-Bergeon et al, 2001). This may indicate that aggressive lipid and blood pressure lowering may slow the progression of coronary calcification. Collectively, these

studies demonstrate the potential use of EBCT in monitoring of atheroma progression and its role in assessing efficacy of intervention.

1.4.2.11 Calcification and plaque rupture

Rupture of the atheromatous plaque directly causes acute coronary thrombosis. The role of calcium in plaque rupture is controversial. It has been suggested that a calcified plaque itself is less (not more) likely to rupture (Cheng et al, 1993). However, the presence of calcified plaque may imply the likely association of lipid-rich and possibly unstable plaque which maybe more likely to rupture (Wexler et al, 1996). In addition to calcification, inflammation may play an important role in plaque rupture (van der Wal et al,1994). The interface of hard calcium deposits with plaque components weakened by inflammation may represent an unstable structure that is more likely to rupture (Demer et al, 1994).

1.4.2.12 Coronary calcification: relationship with age and gender

The clinical interpretation of EBCT-defined coronary calcification should take into account age and gender. Both incidence and magnitude of CAC has been clearly shown to increase with advanced age (Janowitz et al, 1993; Kajinami et al, 1997) which reflect the age-related increase in coronary atherosclerosis (Rumberger et al, 1996; Grundy, 1999). The effect of gender on calcification or other compositions for a given volume of atheroma for the same age remains unclear. With regards to gender, *in vitro* studies have shown that oestrogen has an important role in bone mineralisation and differentiation of calcifying vascular cells (Balica et al, 1997) and inhibition of bone associated protein expression in arterial plaques. However, few studies have examined sex differences in the composition of atheroma. Mautner found

that atheroma in women contained less dense fibrous tissue but this was in keeping with younger atheroma in women than men (Mautner et al, 1993). With regards to EBCT-detected calcification, the sex difference in calcification is consistent with the sex difference in clinical event rates (Janowitz et al, 1993; Kung & Detrano, 1996). Two studies failed to find any difference in the sensitivity of calcification score for obstructive disease amongst men and women (Budoff et al, 1996; Kajinami et al, 1997). Evidence from histopathologic and clinical studies suggests that when matched for severity of luminal disease, EBCT coronary calcium area or score has similar predictive values for atheroma in men and women (Rumberger et al, 1994; Rumberger et al, 1995b). Thus in the general population, similar calcium scores are diagnostic of similar overall atherosclerotic plaque burdens regardless of age and gender. In type 1 diabetes, however, there is some evidence that EBCT-defined coronary calcification correlates stronger with coronary artery disease (defined as history of myocardial infarction, occlusion ≥ 50% by angiography and ischaemic ECG) in men than women (Olson et al, 2000). Further studies are required to examine this issue in type 1 diabetes.

1.4.2.13 Coronary artery calcification in diabetes

It has been shown that in diabetes, level of transforming growth factor beta 1 (TGF-β 1) which stimulates vascular cells to calcify *in vitro* (Watson et al, 1994) is increased (Fagerudd et al, 1997). There is some evidence to suggest that for a given volume of atheroma, the amount of intimal calcification maybe greater in those with diabetes than those without. However, there is limited evidence to support this in human studies. Robertson and Strong found increased CAC in diabetic patients in an autopsy study (Robertson & Strong, 1968) but this is expected given the greater degree of

atheroma. Lundbaeck reported that the calcium content of coronary artery plaques was lower in diabetic than non-diabetic patients at autopsy (Lundbaeck & Petersen, 1953) although the reverse has also been reported (Lehnherr, 1933). In another study, the intimal plaques in type 1 diabetic autopsy subjects and non-diabetics were found to have a similar calcium content (Dziedzic Goclawska et al, 1984). In all these studies, there were important differences between the selection methods for diabetic and non-diabetic subjects which are likely to bias the findings and make the interpretation of the data difficult. In most studies, the majority of diabetic patients had type 2 diabetes. Mautner reported lower amounts of calcification in plaques in younger type 1 diabetic patients compared with older non-diabetic patients (Mautner et al, 1992). Since calcification is highly correlated with age as well as plaque volume this is not unexpected. Thus it remains unclear whether the amount of calcium deposition in type 1 diabetic patients is any different compared to non-diabetic subjects for a given plaque area.

1.4.2.14 Intimal and medial vascular calcification

Arterial calcification may occur at two sites in the vessel wall – in the tunica media (also known as Monckeberg's sclerosis), and in the tunica intima where it is invariably associated with atherosclerosis. Calcification at these two sites has distinct morphology and pathology which suggests that different molecular mechanisms are involved. Intimal calcification develops at the fatty streak stage and is associated with the presence of macrophages and mast cells (Jeziorska et al, 1998). Medial calcification occurs between smooth muscle cells in the absence of inflammatory calls and lipid deposition and is not necessary associated with atherosclerosis (Jeziorska et al, 1998; Shanahan et al, 1999). EBCT cannot distinguish these two types of vascular

calcification and it is therefore at least plausible that some of the CAC observed in the present study may represent medial calcification. However, non-atherosclerotic medial calcification is rare in the coronary vasculature (Blankenhorn, 1961). Extensive medial calcification has mainly been reported in patients with renal failure (Lachman et al, 1977), the type of patients that were excluded from this study. With regards to diabetes, medial calcification has been described in peripheral vessels of type 2 diabetic patients and was found to be associated with autonomic neuropathy and nephropathy (Bevan & Tsuru, 1979; Edmonds et al, 1982; Gilbey et al, 1989). It is thought that denervation of the vascular smooth muscle cells in subjects autonomic neuropathy may play an aetiological role. Although medial arterial calcification is not directly associated with atherosclerosis, there is increasing evidence to suggest that medial calcification is a strong marker of future cardiovascular mortality and diabetic complications independent of other risk factors (Everhart et al, 1988; Lehto et al, 1996; Edmonds, 2000). Overall, it is likely that the CAC in the present cohort of relatively young type 1 diabetic patients without renal failure is intimal and indicative of atherosclerosis.

1.4.2.15 Summary

Substantial evidence has shown that coronary calcium is specific for atherosclerotic plaque and that it can be sensitively detected and accurately quantified by EBCT. The extent of coronary calcification directly correlates with severity and extent of coronary disease. With the use of EBCT, it is possible to study large number of young patients with subclinical CHD. Since the prevalence of CAC is substantial even at younger ages, the disease process can be studied at an age where the difference between diabetic and non-diabetic subjects is greatest i.e. when the relative risk of CHD is

highest. An additional advantage is that, by studying younger subjects, the prevalence of CHD risk factors and their relationship to CAC can be studied before they have become secondarily disrupted by the other complications of diabetes, e.g. nephropathy. Finally in using calcification rather than clinical events the epidemiology of atherosclerosis itself rather than, for example, determinants of plaque rupture are being measured. Thus more specific hypotheses can be tested. In comparison with prospective cohort studies, cross-sectional studies of CHD are more likely to be biased by differential survival rates in those with and those without a risk factor, or by reverse causation leading to changes in risk factor levels following diagnosis of disease. However, the use of EBCT allowed us to study a relatively young group of patients in whom survival bias is unlikely. The prevalence of a clinical diagnosis of angina and nephropathy is fairly low at this age so that reverse causation is unlikely to have affected our results.

1.5 Rationale and aims for this thesis

In summary, studies in experimental animals and *in vitro* have shown that loss of NO is associated with increased vascular reactivity to constrictors, enhanced platelet adhesion and aggregation, increased adhesion of leucocytes, promotion of VSMC growth and accelerated atherogenesis (Busse et al, 1993). Type 1 diabetes may affect NO production through generalised actions on the endothelium, through specific effects on signal transduction mechanisms linked to NO synthase (receptors, ion channels). The effects on NO synthase activity, expression of post-translational modification or destruction of the NO once it has been synthesized. In addition, it is possible that down regulation of guanylate cyclase or the other effector mechanisms of NO may be affected. Whilst many studies in animal models have indicated that type 1

diabetes is associated with functional defects in parts of the NO pathway, in human the data are conflicting and there is no clear consensus about the level at which the disease might alter NO signaling. The variable results of endothelial function obtained in different studies can be partially explained by differences in methodology, blood vessel size and the presence of diabetic complications. A large definitive study of type 1 diabetes is required to clarify the conflicting evidence regarding vascular dysfunction.

Furthermore, as discussed previously, the sex difference in CHD in diabetes is lost which is unexplained by conventional CHD risk factors. Whether defects in the L-arginine: NO pathway contributes to this loss of sex difference in CHD in diabetes remains undetermined. Hence it provides the rationale behind this MD thesis.

With the above background in mind, I conducted this large-scale definitive study in type 1 diabetic and non-diabetic subjects to test the following hypotheses:

1. Nitric oxide release/response is reduced in type 1 diabetic patients compared to non-diabetic controls, thus contributing to the excess CHD risk unexplained by the conventional risk factors.

2. There is a sex difference in NO release/response that is lost in type 1 diabetes or the effect of diabetes on diminished NO release/response is stronger in women than men. Such an effect would help to explain the loss of sex difference in CHD in diabetes.

3. Defective NO release/response is associated with coronary artery calcification.

4. We also examined the relationship between NO release/response and conventional CHD risk factors to determine the importance of each risk factor as a determinant of vascular function.

Chapter 2. Methods

2.1 Subjects

Participants were recruited from a cohort of 400 subjects including 199 type 1 diabetic patients and 201 non-diabetic controls, who already had coronary artery calcification measured by electron bean computed tomography in the preceding 12 months. Type 1 diabetes was defined as diabetes with age of onset ≤ 25 years with continuous use of insulin within one year of diagnosis. The sampling frame for the diabetic patients was the diabetes registers of 5 London Hospitals. The non-diabetic participants were a random sample from the patient registers of two London general practices, stratified to have the same age and sex distribution as the diabetic patients. All were aged between 30-53 years and 50% were female. Of these 400 participants, all were invited by letter to participate in the forearm blood flow study. Of these, 157 took part (39%), 44 had moved address and were uncontactable, 15 were ineligible (1 had Raynaud's disease, 1 had arm deformity due to previous poliomyelitis, 3 were pregnant and 10 had co-existing infections) and 184 refused (Figure 2.1). In total, there were 88 (56.1%) type 1 diabetic (54 men and 34 women) and 69 (43.9%) non-diabetic subjects (34 men and 35 women). Of the 400 original participants, the response rates for this forearm blood flow study were slightly higher amongst diabetic men (52%) than non-diabetic men (36%), and also slightly higher in diabetic women (36%) than non-diabetic women (33%). Those who were found to have calcification at the original study were as likely to participate in this forearm blood flow study (response rate 40%) as those who had no calcification (39%). This was also the case within each of the four diabetes-sex groups. Subjects were recruited without regard to their history of CHD or diabetic

complications. Twenty-five diabetic and 5 non-diabetic subjects were on anti-hypertensive drugs. Two diabetic patients and none of the non-diabetic subjects were on lipid-lowering drugs. Seven non-diabetic women and 5 diabetic women were on the oral contraceptive pill. Five non-diabetic women and one diabetic woman were on anti-epileptic medications. One diabetic woman had hypothyroidism and was replaced with thyroxine. None of the participants agreeing to take part were on nitrate therapy and none had clinical macrovascular complications (such as peripheral vascular disease). Diabetic patients who had hypoglycaemia (plasma glucose $\leq$ 2.5 mmol/L or with symptoms) within 24 hours prior to the study were re-scheduled for the vascular study. Pregnant women and those with cancer, psychiatric illness, renal impairment or acute infection were excluded. All participants gave their informed consent. The study had the approval of the local ethics committee and was conducted over a period of 14 months.

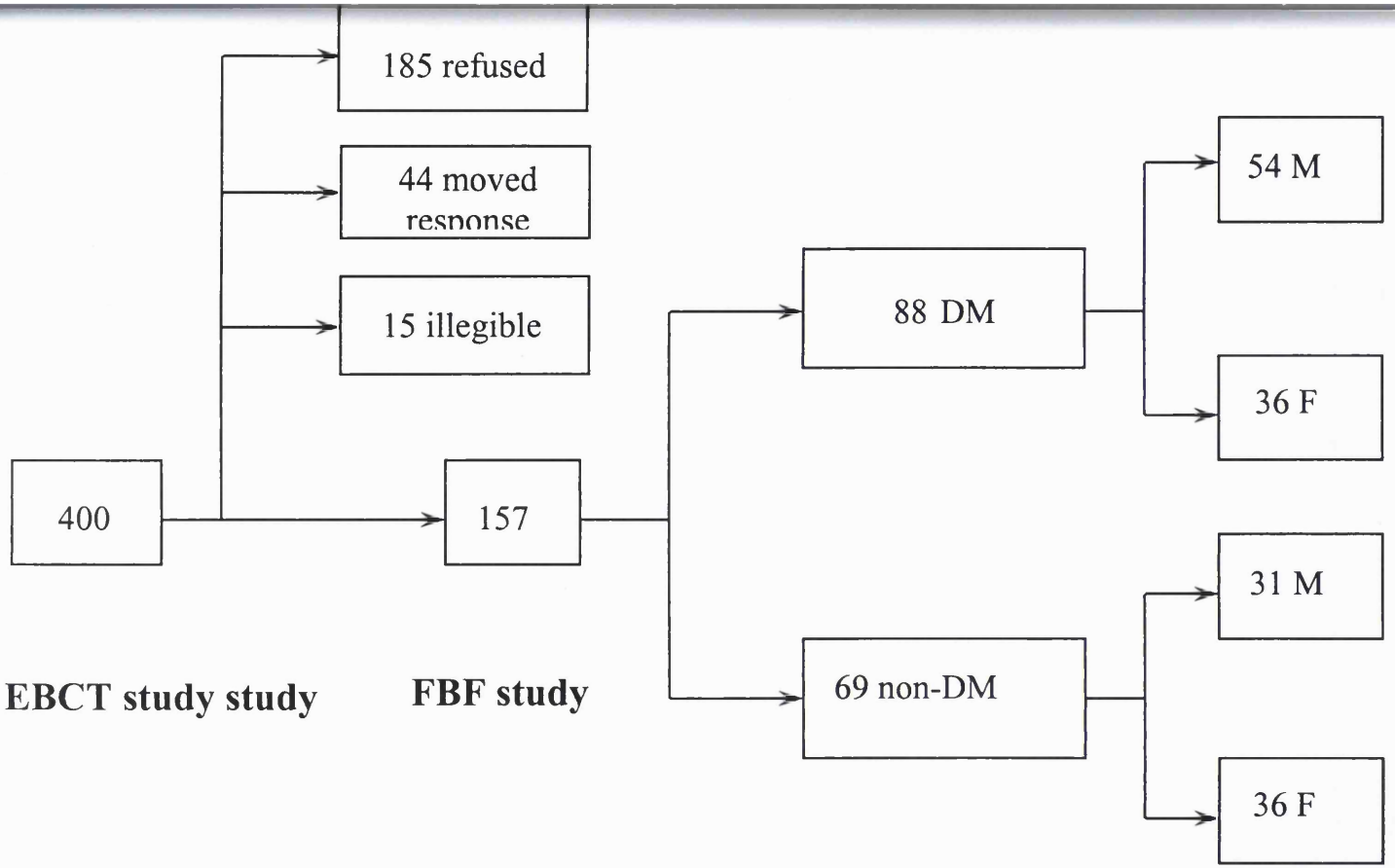


Figure 2.1. Flow chart showing proportion of volunteers recruited.

2.2 Electron beam computed tomography

The EBCT data were derived from a previous study performed by Dr.Helen Colhoun and were not repeated. The study was done fasting. An Ultrafast CT scanner (IMATRON C-150XL) was used to quantify coronary calcification. Two sets of 20 transverse tomograms of 3-mm thickness were obtained from the lower margin of the bifurcation of the right branch of the pulmonary artery to the apex of the heart with the subject breathholding. A radiologist placed a region of interest around each potentially calcific lesion (peak density > 130 Hounsfield units) within the right coronary, circumflex, left anterior descending and left main coronary arteries. The area and peak density of each lesion was measured. A density score of 1 to 4 was defined based on the peak density of the lesion and calcification score was then calculated as the product of the area of the lesion and its density score as described (Agatston et al, 1990). To be included in the calcification score a lesion had to have an area of at least 0.51mm^2, i.e. two contiguous pixels, and a peak density of at least 130 Hounsfield units. A total score for each artery and for the entire heart was calculated by summing the lesion scores. The radiation exposure was < 1 mSv. All scans were scored by the same radiologist who was blinded to the sex and the diabetes status of the subject. Based on a small repeatability study (n=20) the within-observer agreement for the presence of any calcification was high (kappa = 0.84).

2.3 Questionaires and assessment of risk factors

A standardised questionnaire was filled in by each participant before the fore arm blood flow study (questionaire samples for diabetic and non-diabetic subjects are shown in Appendix 1 and 2 respectively). The average weekly consumption of alcohol units was calculated and cigarette smoking exposure was quantified as pack years. The phase of menstrual cycle for each female participant was recorded and was classified as follow: menses as day 1 – 5; follicular phase as day 6-10; midcycle as day 10-13; luteal phase as day 14 – 28. Obesity was defined as a body mass index (BMI) $\geq 30 kg/m^2$. A score combining duration and intensity of physical activity was calculated from items on walking, cycling, sporting and occupational activity, and dichotomized as low (weekly score ≤ 10) or high (quantified at the EBCT study) (Appendix 3). Prior to the vascular study, blood pressure in the right arm was measured 3 times using automated digital monitor (Omron 705CP, OMRON Healthcare Europe B.V., the Netherlands) with the subject seated after a 5-minute rest. The mean of the two measurements is presented in our data.

2.4 Laboratory methods

Serum samples from the EBCT study were used for lipoprotein measurement. Total cholesterol, HDL-C and triglyceride concentrations were measured using enzymatic colorimetric methods (Allain et al, 1974; Fossati et al, 1982) . HDL-C was measured directly after stabilisation of other lipoproteins (Sugiuchi et al, 1995). LDL-C was calculated using the Friedewald formula (Friedewald et al, 1972). Plasma glucose concentrations immediately before and after the forearm blood flow study were measured using the Integra method (Palmer et al, 1995). HbA1c was measured using a latex enhanced immunoassay (intra-assay CV 1.7%). Participants completed two-timed overnight urine collections. Urinary albumin was measured using an immunoturbidimetric method (intra-assay CV 2.3%) and menstruating women were excluded from analyses.

2.5 Venous occlusion plethysmography and experimental protocol

All the studies in this thesis were carried out in a laboratory which was temperature-controlled (25-27°C) and equipped with cardiopulmonary resuscitation facilities, centrifuge and refrigerator for storage of drugs as well as blood samples. Studies were conducted at various times (mornings, afternoons or evenings) depends on subject availability.

All participants were asked to keep to their usual dietary and insulin regimes. They were also advised to avoid consuming alcohol and drinks containing caffeine including coffee and tea 24 hours prior to the study. Before the study, blood pressure in the right arm was measured 3 times using automated digital monitor (Omron 705CP, OMRON Healthcare Europe B.V., the Netherlands) with the subject seated. Venous non-fasting blood was taken from the dominant arm, centrifuged immediately for 15 minutes and 1 ml samples of serum and plasma were stored at –70°C. The infusion forearm length and volume were measured. The forearm length was measured from the medial epicondyle to the ulnar head and forearm volume was measured by water displacement method.

With the participant supine, a 27-gauge stainless steel needle (Cooper's Needle Works, Birmingham, UK) sealed with wax to an epidural cannula was inserted into the brachial artery of the non-dominant arm (left arm in 140 cases) under local anaesthesia (1-2 mls 1% lignocaine). Drugs were dissolved in 0.9% sodium chloride (normal saline) solution and were infused at 0.5 ml per minute using a constant infusion pump (Harvard Apparatus, USA). Forearm blood flow was recorded simultaneously in both arms by

venous occlusion plethysmography (Whitney 1953) calibrated to measure absolute blood flow with electrically temperature-compensated strain-gauges attached to the upper part of the forearms. During measurements, upper arm collecting cuffs were inflated to 40 mmHg for 10 out of every 15 seconds and circulation to the hands was excluded by inflating the wrist cuffs to 200 mmHg as described previously.

After insertion of arterial cannula, saline solution was infused prior to drug infusion. FBF measurements were made at 5 minutes intervals twice with each period of measurements lasting 5 minutes. Basal blood flow was then measured for 2 minutes before drug infusion. For the main study of type 1 diabetes in this thesis, the following vasoactive drugs were used in the protocol (Figure 2.2): acetylcholine (Sigma Diagnostics, USA; doses of 25, 50, 100 nmol/L, each dose for 3 minutes), bradykinin (Clinalfa, Laufelfingen, Switzerland; doses of 10, 30 and 100 pmol/min, each dose for 3 minutes), glycerine trinitrate (David Bull Laboratories, Warwick, UK; of 4, 8, 16 nmol/min, each dose for 5 minutes), noradrenaline (Levophed; Sanofi Winthrop Ltd, Guildford, UK; 60, 120, 240 pmol/min, each dose for 5 minutes) and L-NMMA (Clinalfa, Laufelfingen, Switzerland; 1, 2, 4 μmol/min, each dose for 5 minutes). Each drug infusion separated by a 10-minute saline washout period to avoid crossover effects. The order of administration of ACh, bradykinin and GTN was randomised whereas noradrenaline and L-NMMA administration was not randomised and was given following the dilators, since L-NMMA has a long half-life.

The following orders were allocated and recorded on questionaire for each participant:

I	ACh - BK – GTN – NA - L-NMMA
II	ACh - GTN – BK – NA - L-NMMA
III	BK - ACh – GTN – NA - L-NMMA
IV	BK - GTN – ACh – NA - L-NMMA
V	GTN - BK - ACh – NA - L-NMMA
VI	GTN - ACh - BK – NA - L-NMMA

Flow was recorded for approximately 10 s in every 15 s and the mean of the last four measurements of each recording period was used for data analysis. Blood flow was expressed as ml of blood per 100 ml of forearm volume per minute (ml/100ml/min). The ratio of blood flow in the infused arm compared with that in the control arm was calculated for each measurement period.

Figure 2. Protocol for forearm blood flow study. Shaded areas ▨ represents recording period during saline infusion.

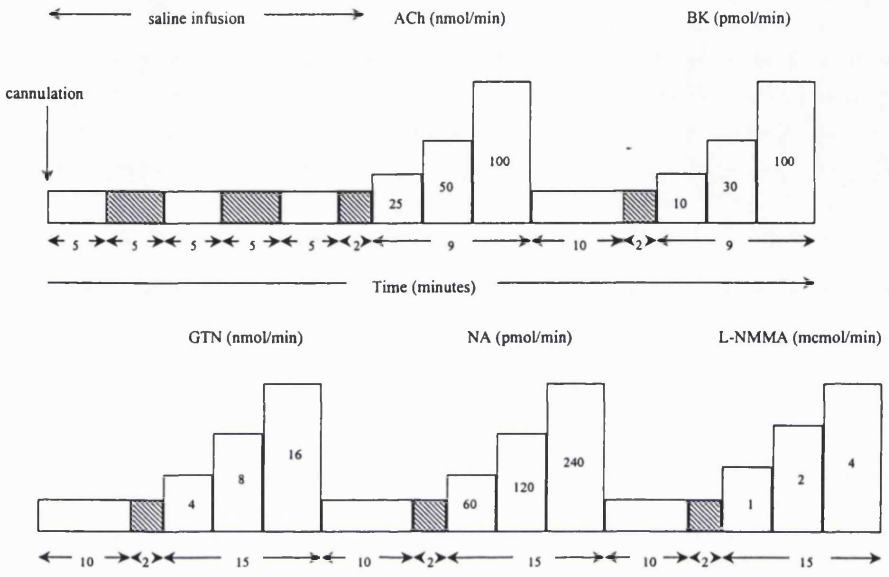


Figure 2.2. Protocol for forearm blood flow study in Type 1 diabetes. Shaded areas indicate each recording periods.

2.6 Statistical analysis

All the data were analysed using STATA 6.0. Comparison of background characteristics between the sexes and between diabetic and control groups was assessed using univariate ANOVA adjusting for age. In comparing the response to vasoactive agents between groups, adjustment must be made for differences in basal flow. It is also important to adjust for concomitant changes in blood flow in the non-infused arm so that the effects of external influences such as state of arousal, sympathetic activation are taken into account. There has been considerable discussion in the literature on how to best to adjust for basal flow and external / systemic effects (Benjamin et al, 1995; Chin-Dusting et al, 1999; Petrie et al, 2000). Conventionally this is addressed by expressing the response to a vasodilator at a given dose as the proportional or percentage increase in the ratio of flow in the infused to flow in the control arm during drug infusion compared with this ratio during preceding saline infusion. A dose-response curve is then constructed with the response plotted against the log of drug dose and the area under the curve calculated. Similarly, vasoconstrictor responses are expressed as percentage decrease in the infused:control forearm blood flow ratio relative to the immediately preceding baseline flow ratio.

This method has some limitations. The ratio may not be independent of basal flow. This is particularly important where a focus of the analysis is comparison of groups (males vs females, diabetic vs non-diabetic subjects) with different basal flow. An alternative approach is to use a modeling procedure known as repeated measures ANOVA. This allows the outcome measure of drug response to be adjusted for basal flow and any other

important covariates e.g. forearm volume. Because time-dependent covariates can be entered into the model this allows the flow in the control arm throughout the experiment to be adjusted for in the model. Accordingly as well as the conventional calculation of ratio of flow of infused / control during drug infusion to flow of infused / control at baseline, we also used repeated measures ANOVA where the outcome (dependent) variable was the log (flow in the infused arm during drug – flow at baseline) which back transformed i.e. the ratio of flow during drug / flow at baseline. This allowed basal flow, flow in the control arm to be included as covariates. Repeated measures ANOVA utilizes the flow data across all three doses and so a term for drug dose was included in the model. In these models, the effects of adjusting for potential effect-mediators (e.g. systolic blood pressure) on the observed differences between groups was examined by including these as covariates in the model. The association between CAC and NO release/respone was examined by including CAC as a covariate in the models.

Chapter 3. Results of Forearm blood flow study in Type 1 diabetic and non-diabetic subjects

3.1 Subject Characteristics

All subjects were Caucasians except one diabetic woman and one non-diabetic woman who were Afro-Caribbean. Of the 34 diabetic women and 35 non-diabetic women, the majority (71% and 77% respectively) were studied during the follicular phase of the menstrual cycle. Other background characteristics and prevalence of complications are shown in Table 3.1. Consistent with other studies of type 1 diabetic patients without renal failure (Nikkila, 1984), diabetic subjects in our study had higher HDL-cholesterol (HDL-C), lower LDL-cholesterol (LDL-C) and, amongst men, lower triglyceride than non-diabetic subjects. Few diabetic patients had previous retinopathy or albuminuria (Table 3.1) and although not selected on the basis of their cardiovascular disease history none had a previous diagnosis of angina or myocardial infarction. None had any ischaemic changes on resting ECG.

3.2 Basal Forearm Blood Flow

Basal flow (flow during saline infusion preceding the first drug infusion) was higher in men than in women (Table 3.1, p=0.001, adjusted for diabetes). Diabetic subjects had slightly higher basal flow than non-diabetic subjects (Table 3.1, p=0.1 adjusted for age and sex).

3.3 Response to endothelium-dependent and endothelium-independent vasodilators

The mean blood flow during pre-drug saline and during drug infusion is shown in Table 3.2. ACh, BK and GTN produced a dose-dependent increase in flow in all 4 groups (Table 3.2 & Figures 3.1-3.3). In all subjects combined, ACh infusion (at 25, 50 and 100 nmol/min) was associated with a 2, 2.2 and 2.9-fold increase in flow respectively. BK infusion (at 10, 30 and 100 pmol/min) caused a 1.6, 2 and 2.5-fold increase in flow and GTN infusion (at 4, 8, 16 nmol/min) caused a 1.7, 2 and 2.3-fold increase.

There was a 2.66-fold increase in flow with ACh (averaged over the three doses) in non-diabetic subjects and a 2.18-fold increase in diabetic subjects. Thus the response was 18% ($p=0.0008$) lower in the diabetic group adjusted for age, sex, basal flow and changes in flow in the control arm (Table 3.3). Similarly, the diabetic group also had an 8% ($p=0.042$) lower response to BK and a 17% ($p=0.0001$) lower response to GTN. The degree of reduction in drug response associated with diabetes did not differ significantly by sex for ACh (16% lower in diabetic men, 21% lower in diabetic women, $p=0.5$) or BK (10% and 5% lower for men and women respectively, $p=0.5$). Neither was there any sex difference in the effect of diabetes on GTN response (19% lower in men, 15% lower in women, $p=0.5$).

Although pre- and post-study plasma glucose concentrations were significantly higher in the diabetic than non-diabetic patients, there was no association between pre- or post-study plasma glucose concentrations and any of the drug responses. Accordingly,

adjusting for (i.e. adding to the ANOVA model) pre- and post-study plasma glucose concentrations did not alter the difference between diabetic and non-diabetic subjects for ACh, BK or GTN. Adjusting for BMI, systolic blood pressure, HDL-C, LDL-C and triglyceride did not alter the difference in response to vasodilators between diabetic and non-diabetic subjects. Other models including forearm volume had been tried but did not result in statistical improvement.

The overall results were not altered after removing patients receiving drug therapy.

3.4 Relationships between vasodilator responses

The response to GTN was positively associated with the response to ACh (p=0.001) and the response to BK (p=0.001). We examined whether the lower response to the NO donor (GTN) in diabetic patients could account for the lower response to agonists (BK and ACh). On adjustment for GTN response, the 18% difference between diabetic and non-diabetic subjects in ACh response was reduced to a 10% difference (p=0.06). Adjusting for GTN response completely abolished the difference in BK response between diabetic and non-diabetic subjects.

3.5 Vascular responses to vasoconstrictors

Both L-NMMA and noradrenaline produced a dose-dependent decrease in blood flow in all four groups (Table 3.2). Overall NA infusion (at 60, 120, 240 pmol/min) caused flow to reduce to 0.78, 0.73 and 0.67 of basal flow. L-NMMA infusion (at 1, 2, 4 μmol/min) caused flow to reduce to 0.8, 0.71 and 0.63 of basal flow. Neither the vasoconstrictor

response to noradrenaline nor to L-NMMA differed significantly between diabetic and non-diabetic subjects when adjusted for age, sex, basal flow and flow in the control arm (Table 3.3). This was also the case within each sex examined separately. Adjusting for pre- and post-study plasma glucose concentrations did not alter this result. As the infusion doses for L-NMMA and noradrenaline were chosen to give the same degree of vasoconstriction we examined the responses to the drugs within the diabetic and non-diabetic groups. There was no significant difference in response to the two drugs within either group.

3.6 Diabetes duration & control

Among diabetic subjects, a 1% increment in HbA1c was significantly associated with a 5% reduction in ACh response (p=0.02) and a 5% increase in noradrenaline response (p=0.01) but was not associated with response to other drugs. Diabetes duration was not associated with vasodilator response. The shortest diabetes duration was 7 years so we were not able to assess the association of very short-term diabetes with drug responses. Only 14 diabetic subjects had micro or macroalbuminuria. Response to vasodilators was not associated with albuminuria or with a self-reported history of retinopathy (present in 10 subjects) or neuropathy. Diabetic subjects with any of these complications (n=25) did not have lower drug responses than those with none.

3.7 Sex differences in vascular responses

Averaged across the three doses ACh was associated with a 2.1 fold increase in (basal) flow in men and a 2.7 fold increase in women (a 22% difference in response). Adjusting

for age, basal flow and flow in the control arm reduced this to a 12% difference (a 2.26-fold and 2.58-fold increase in flow with drug respectively, p=0.035, Table 4). The sex difference in ACh response was not significantly greater in non-diabetic subjects (17% lower in men) than diabetic subjects (10% lower in men, p=0.5 for the diabetes by sex interaction). On further adjustment for forearm volume this difference of 12% in ACh response in men and women was reduced to non-significance (7% lower in men p=0.42). In contrast, response to BK was similar in men and women before adjusting for basal flow and flow in the control arm. On adjustment for these covariates response to BK was 10% higher in men than in women (p=0.03). Further adjustment for forearm volume increased this difference 21% (p=0.001). No sex differences were apparent in the response to any other drugs (Table 3.4) either with or without adjustment for forearm volume.

3.8 Alternative Method of Analysis

For comparison with other studies, data analysis was also carried out comparing the area under the curve for the ratio of flow in the two arms during infusion compared to baseline (Table 5). This method of analysis also showed that diabetic subjects had a significantly reduced response to ACh (p<0.001), BK (p=0.02) and GTN (p<0.001). The L-NMMA response was slightly lower in diabetic than non-diabetic subjects using this approach (p=0.03). However, on further adjustment for basal flow and control arm flow this difference was not apparent (p=0.2). Using this approach women had a significantly higher ACh response than men (p=0.002) and there was no sex difference in response to the other drugs. On adjustment for basal flow and control arm flow and forearm volume

the results were the sex difference in ACh response was no longer significant and men had a slightly higher BK response than women.

3.9 Association between established CHD risk factors and vascular responses in the general population

Table 3.6 shows the association between established CHD risk factors and drug response in type 1 diabetic and non-diabetic subjects. In addition to the risk factors shown, alcohol intake and physical activity were also examined and they showed no association with any of the drug responses.

3.10 Agonist-stimulated endothelium-dependent vasodilatation

A similar pattern of risk factor associations was found for BK and ACh response (Table 3.6) with responses being lower in those with higher BMI, total cholesterol to HDL-C ratio, lower HDL-C and those who smoked. LDL-C was associated with BK response only. Table 3.6 shows how much difference in drug response was associated with a given difference in the risk factor. For example, for every 1 kg/m^2 higher BMI, there was a 3% lower response to ACh and BK. The association of BMI with drug response was independent of forearm volume for BK ($p=0.04$) but not ACh. Overall, BMI, total cholesterol: HDL-C ratio, HDL-C, LDL-C and smoking accounted for 5%, 7%, 7%, 4% and 7% of variation in ACh response respectively, and 9%, 11%, 9%, 10% and 4% of variation in BK response when each factor was examined separately. Because these risk factors cluster with each other, the total variance explained by the entire risk factor profile

is less than the sum of the variance explained by each risk factor examined separated. Taken altogether, they accounted for 13% of ACh and 19% of BK response respectively.

3.11 Endothelium-independent vasodilatation

Endothelium-independent vasodilatation, as assessed by GTN response, was associated with BMI and total cholesterol: HDL-C ratio (Table 3.6). When both sexes were examined separately the relationship between BMI and GTN response was only present in women. Among women for every for every 1 kg/m^2 increase in BMI, there was a 3% reduction in GTN response (p=0.006). Adjusting for forearm volume did not reduce this association between BMI and GTN response (p=0.005 after adjustment). The association between total cholesterol: HDL-C ratio and GTN response (Table 3.6) was of similar magnitude in both sexes (a 5% and 6% lower response for every one unit difference in this ratio in men and women respectively). Overall, total cholesterol: HDL-C ratio explained about 6% of the variation in GTN response. BMI accounted for 18% of the between-subject variation in GTN response in women and the association was independent of total cholesterol: HDL-C ratio (p=0.03 on adjustment).

3.12 Basal NO release

The only risk factors that showed a significant association with basal NO release, as assessed by L-NMMA response, was smoking. Subjects who had at least 10 pack-years of smoking had a 13% lower L-NMMA response than non-smokers (Table 3.6). Overall, smoking accounted for about 9% of the inter-individual variation in response to L-NMMA. The relationship was apparent in both sexes. The relationship was not

attributable to a generalised increased vasoconstrictor response in smokers because there was no difference in noradrenaline response with smoking (Table 3.6).

3.13 Framingham risk score

The relationship between Framingham risk score and vascular response in the general population is shown in Table 3.7. A higher Framingham score was associated with a reduced response to BK and L-NMMA. These relationships were stronger for women than men and were non-significant in men when examined separately (data not shown). A higher Framingham score was also associated with a reduced response to ACh in women. There was no relationship between Framingham risk score and response to GTN.

3.14 The effect of diabetes on the relationship between CHD risk factors and vascular responses

As shown in Table 3.6, type 1 diabetes dilutes the strengths of most of the associations between risk factors and vascular response. The only exception was observed for noradrenaline response in association with BMI. A higher BMI was significantly associated with increased noradrenaline response in diabetic subjects but not non-diabetic subjects.

3.15 The relationship between diabetes-related factors and vascular responses

Amongst several diabetes-related factors, a relationship was only observed between HbA1c and response to ACh and noradrenaline (Table 3.8). A higher HbA1c was significantly associated with diminished ACh response and also diminished adrenaline

response in type 1 diabetic subjects. No association was observed between disease duration, albuminuria, retinopathy with all drug responses.

3.16 Coronary artery calcification and vascular responses

In both diabetic and non-diabetic group, the presence of coronary artery calcification was not significantly associated with vascular responses to any of the infused drugs (Table 3.9). The same relationship was observed in both men and women.

Table 3.1. Subject characteristics by diabetes and sex expressed as mean (SEM).

	Non-Diabetic Males (N=34)	Diabetic Males (N=54)	Non-Diabetic Females (N=35)	Diabetic Females (N=34)
Age (Years)	38.26 (0.81)	38.82 (0.54)	37.57 (0.60)	38.44 (0.75)
BMI	24.62 (0.45)	25.19 (0.39)	25.22 (0.73)	24.88 (0.55)
Disease duration (years)	N/A	23.62 (1.05)	N/A	25.34 (1.18)
Total daily insulin (IU/day)	N/A	57.39 (2.44)	N/A	42.03 (2.21)
SBP (mmHg)	127.52 (1.89)	128.63 (1.60)	118.57 (2.09)	124.10 (2.23)*
DBP (mmHg)	81.96 (1.64)	77.55 (1.24)	74.57 (1.70)	75.84 (1.45)
HbA1c (%)	5.34 (0.07)	8.32 (0.18)***	5.27 (0.06)	9.29 (0.31)***
LDL Cholesterol (mmol/L)	3.36 (0.20)	3.13 (0.15)	2.88 (0.15)	2.75 (0.12)
Triglyceride (mmol/L) (#)	1.60 (0.18)	1.11 (0.08)*	0.99 (0.09)	1.02 (0.11)
HDL Cholesterol (mmol/L)	1.53 (0.06)	1.74 (0.06)*	1.81 (0.07)	1.93 (0.09)
Pre-study glucose (mmol/L)	5.51 (0.19)	8.79 (0.67)**	5.01 (0.11)	11.01 (1.15)***
Post-study glucose (mmol/L)	5.27 (0.10)	7.74 (0.60)*	4.97 (0.10)	8.04 (1.01)**
Forearm volume (ml)	1172 (29)	1160 (24)	862 (27)	882 (27)
Basal flow (ml/100ml/min)	2.98 (0.3)	3.0 (0.2)	2.1 (0.2)	2.2 (0.1)
Albuminuria (no. of subjects)	1	11	0	3
Retinopathy (no. of subjects)	-	7	-	7
Neuropathy (no. of subjects)	-	5	-	2
Hypertension (SE)	26% (8%)	28% (6%)	3% (3%)	15% (6%)

(#) For triglyceride the geometric mean is summarized. Hypertension= SBP≥140mmHg or DBP ≥90mmHg or on BP lowering drugs

*p<0.05, **p<0.005, ***p<0.0005 for within sex comparison

Table 3.2. Mean (SEM) Blood flow before and during infusion drugs in ml/100ml/min

	Non-diabetic men	Diabetic men	Non-diabetic women	Diabetic women
Blood flow in infusion arm (ml/100ml/min)				
Baseline	3.28 (0.34)	3.39 (0.22)	2.18 (0.16)	2.38 (0.15)
ACh 25	6.32 (0.62)	5.72 (0.38)	6.03 (0.54)	5.35 (0.49)
ACh 50	7.18 (0.68)	6.78 (0.49)	7.36 (0.56)	5.53 (0.50)
ACh 100	9.26 (0.90)	7.98 (0.54)	9.65 (0.88)	7.67 (0.81)
Baseline	3.28 (0.33)	3.50 (0.23)	2.40 (0.16)	2.43 (0.16)
BK10	5.06 (0.40)	5.48 (0.31)	4.02 (0.24)	3.78 (0.26)
BK30	6.88 (0.52)	6.64 (0.34)	5.08 (0.37)	4.83 (0.35)
BK100	8.64 (0.73)	8.37 (0.41)	7.01 (0.50)	6.20 (0.51)
Baseline	3.22 (0.33)	3.24 (0.19)	2.31 (0.16)	2.18 (0.15)
GTN4	5.84 (0.42)	5.16 (0.27)	4.75 (0.38)	3.90 (0.28)
GTN8	7.46 (0.55)	6.24 (0.30)	5.87 (0.48)	4.46 (0.29)
GTN16	8.57 (0.63)	7.29 (0.36)	6.87 (0.51)	5.20 (0.32)
Baseline	3.65 (0.30)	3.78 (0.23)	2.96 (0.18)	2.78 (0.23)
NA60	3.01 (0.25)	3.15 (0.19)	2.30 (0.15)	2.40 (0.18)
NA120	2.84 (0.26)	2.99 (0.19)	2.28 (0.16)	2.24 (0.16)
NA240	2.79 (0.31)	2.74 (0.16)	2.08 (0.14)	2.10 (0.13)
Baseline	3.85 (0.40)	3.70 (0.22)	3.23 (0.23)	2.78 (0.19)
L-NMMA1	3.30 (0.32)	3.19 (0.19)	2.53 (0.15)	2.34 (0.16)
L-NMMA2	2.98 (0.26)	2.91 (0.17)	2.40 (0.16)	2.22 (0.15)
L-NMMA4	2.78 (0.23)	2.66 (0.16)	2.25 (0.14)	2.04 (0.14)

Figure 3.1. Mean ± SEM of blood flow with acetylcholine (ACh) infusion. P-value indicates the significance level for the difference between diabetic and non-diabetic subjects adjusted for age, basal flow and flow in the control arm.

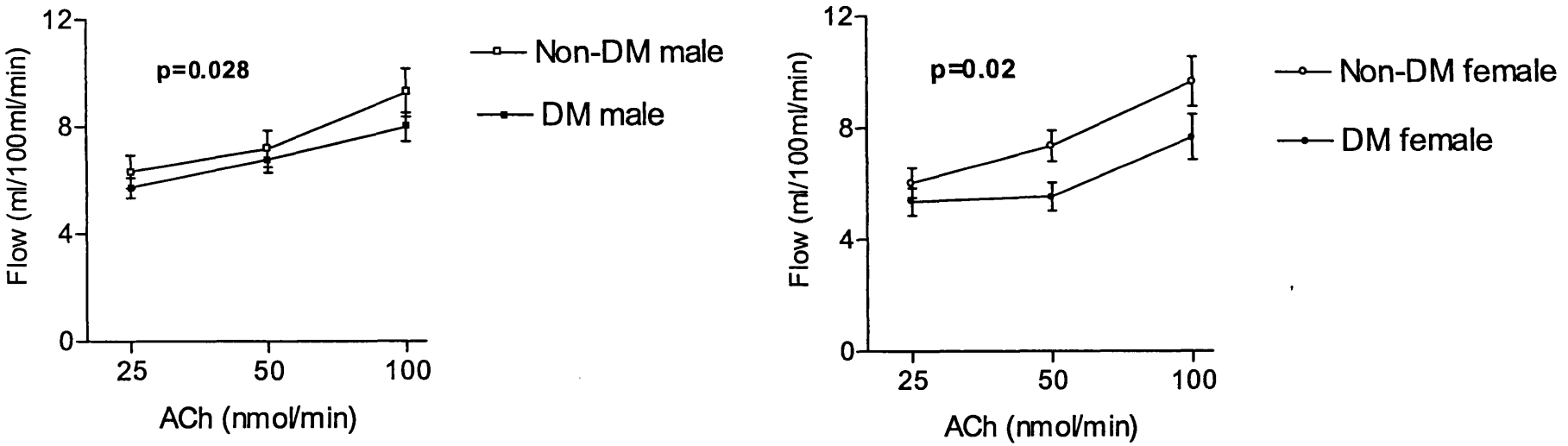


Figure 3.2. Mean ± SEM of blood flow with bradykinin (BK) infusion. P-value indicates the significance level for the difference between diabetic and non-diabetic subjects adjusted for age, basal flow and flow in the control arm.

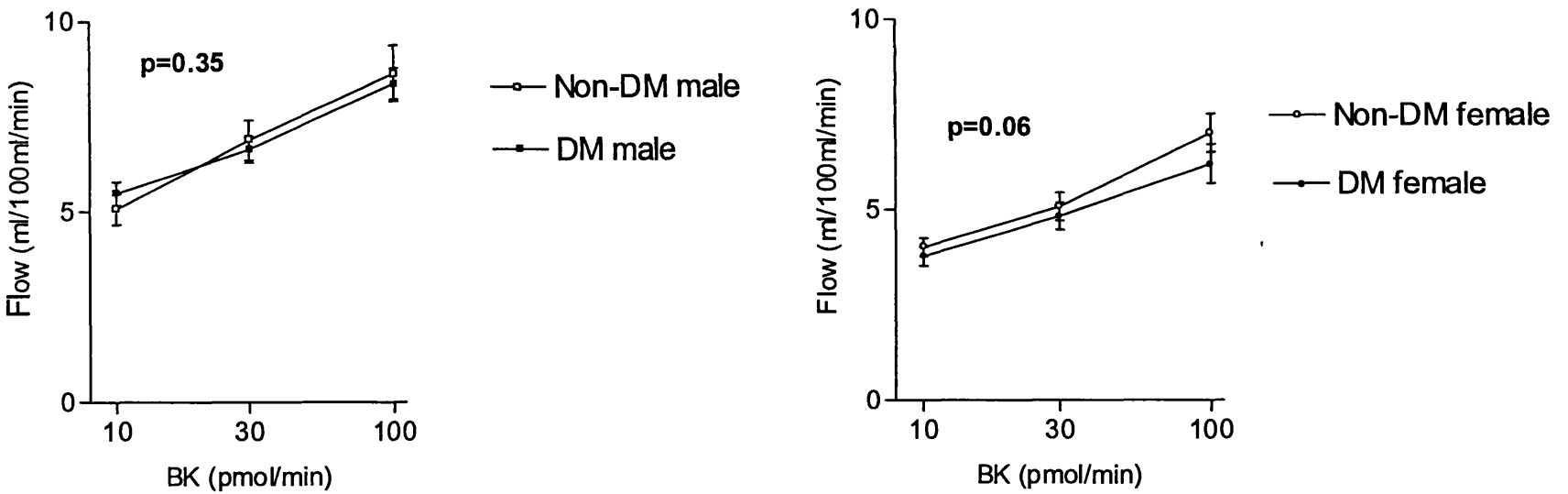


Figure 3.3. Mean ± SEM of blood flow with glyceryl trinitrate (GTN) infusion. P-value indicates the significance level for the difference between diabetic and non-diabetic subjects adjusted for age, basal flow and flow in the control arm.

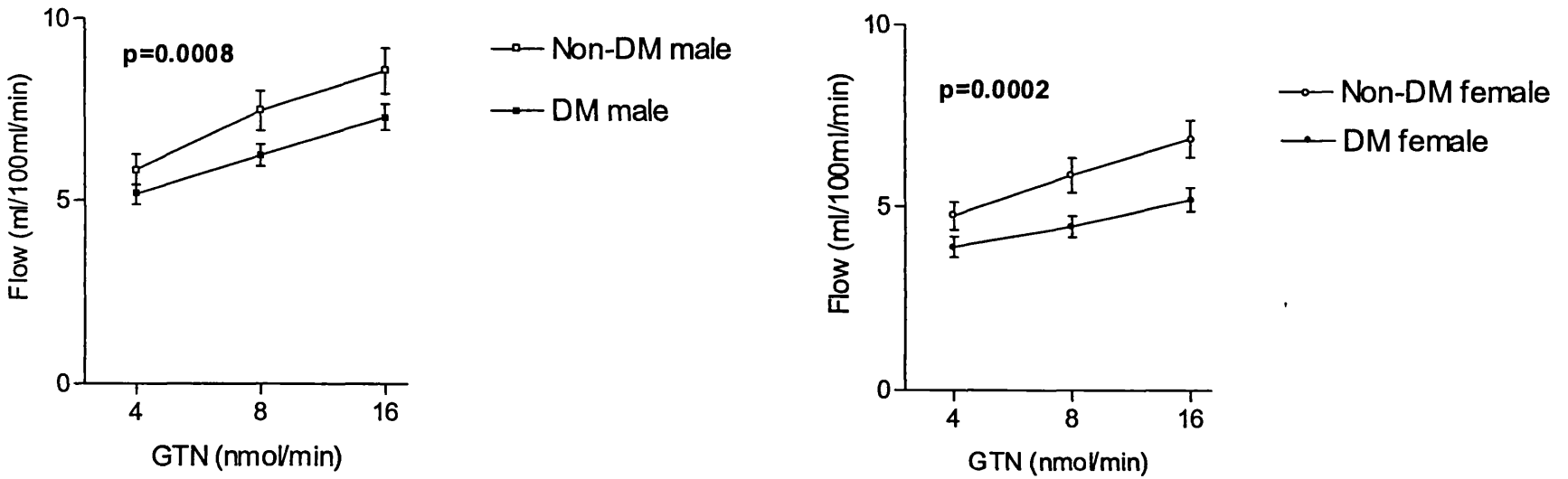


Figure 3.4. Mean ± SEM of blood flow with noradrenaline (NA) infusion. P-value indicates the significance level for the difference between diabetic and non-diabetic subjects adjusted for age, basal flow and flow in the control arm.

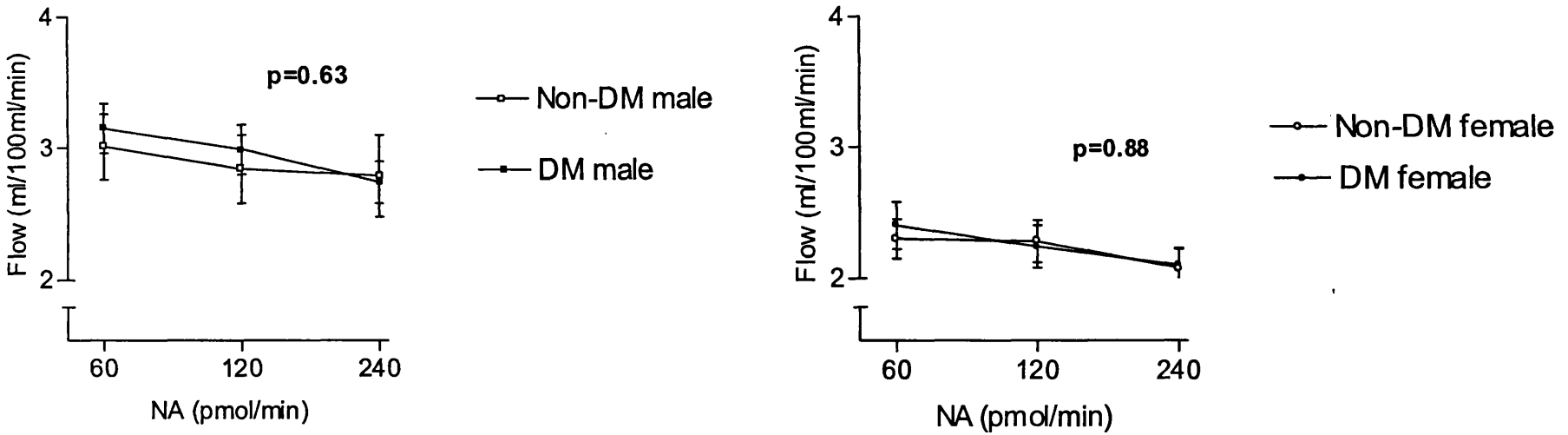


Figure 3.5. Mean ± SEM of blood flow with N^G-monomethyl-L-arginine (L-NMMA) infusion. P-value indicates the significance level for the difference between diabetic and non-diabetic subjects adjusted for age, basal flow and flow in the control arm.

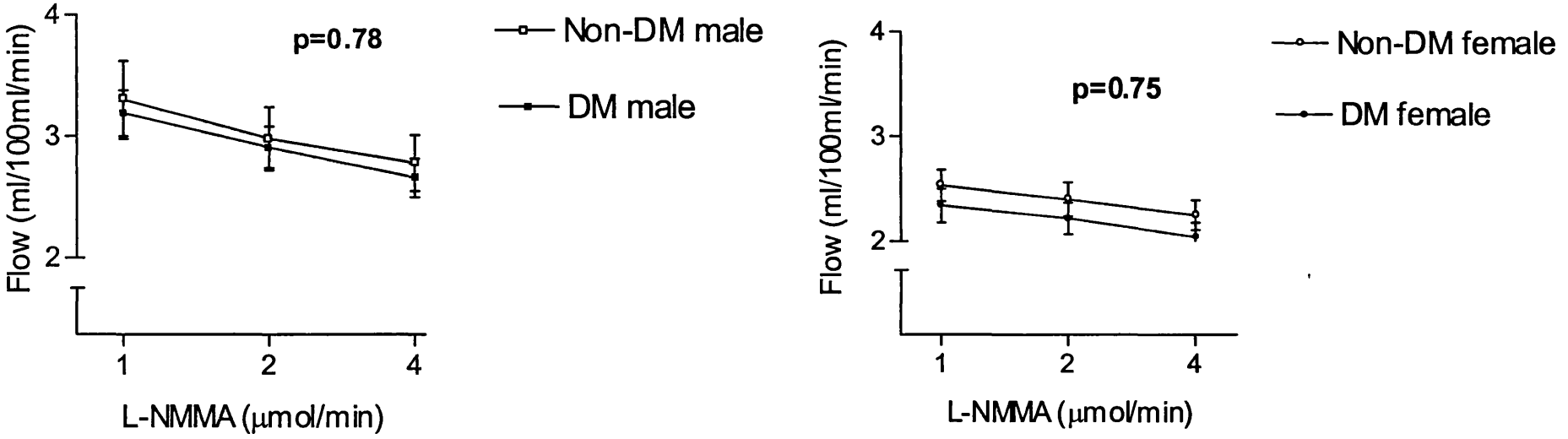


Table 3.3. Response to drugs by diabetes, adjusting for age, sex, basal flow and flow in the control arm.

	Ratio of flow during drug infusion to flow at baseline		% difference (95% CI) in response to drugs	p-value for diabetes difference in drug response
	Diabetes	Non-diabetes	(Diabetes vs Non-diabetes)	
Ach	2.18	2.66	-18% (-27%, -8%)	0.0008
BK	1.96	2.13	-8% (-15%, 0%)	0.042
GTN	1.98	2.38	-17% (-22%, -11%)	0.0001
L-NMMA	0.79	0.78	-2% (-6%, 3%)	0.49
NA	0.78	0.76	-3% (-10%, 6%)	0.55

Table 3.4. Response to drugs by sex, adjusting for age, diabetes, basal flow and flow in the control arm.

	Ratio of flow during drug infusion to flow at baseline		% difference (95% CI) in response to drugs	p-value for sex difference in drug response
	Male	Female	(male vs female)	
ACh	2.26	2.58	-12% (-22%, -0.01%)	0.035
BK	2.14	1.95	10% (1%, 19%)	0.032
GTN	2.19	2.15	2% (-6%, 10%)	0.64
NA	0.76	0.78	2% (-6%, 12%)	0.59
L-NMMA	0.79	0.77	-2% (-7%, 2%)	0.31

Table 3.5. Area under the curve for the response to drugs by diabetes and sex.

	Non-diabetic men	Diabetic men	Non-diabetic women	Diabetic women	Non- diabetic men and women	Diabetic men and women
			Mean Area Under the Curve in Arbitrary units[#] (95% CI)			
ACH	147 (113-189)	95(75-121)	223 (171-290)	140 (108-180)	181 (150-218)	111(93-132)
BK	216 (159-294)	180 (149-217)	225 (181-280)	180 (149-217)	220 (184-265)	180 (157-206)
GTN	146 (122-174)	100 (83-120)	166 (133-206)	113 (95-134)	156 (136-179)	105 (92-119)
NA	38 (30-45)	36 (30-43)	41 (32-49)	39 (33-46)	39 (34-45)	37 (32-42)
L-NMMA	42 (35-48)	35 (32-40)	45 (40-49)	41 (35-46)	43 (39-47)	37 (34-41)

[#] for vasodilators the geometric mean is shown as the distribution was skewed

~response to drug was defined as the % change in the ratio of flow in the infused / control arm during drug infusion

Table 3.6. Percentage change of each drug response for per unit change of risk factors in type 1 diabetic and non-diabetic subjects, with 95% confidence interval.

	ACh		*BK*		*GTN*		*NA*		*L-NMMA*	
	DM	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***
SBP	-4%	0.8%	-3%	-0.4%	-0.1%	-2%	-4%	-3%	0%	0%
(10 mmHg)	(-7,3)	(-8,10)	(-6,2)	(-1,0.1)	(-4,4)	(-7,4)	(-6,1)	(-9,3)	(-3,4)	(-3,3)
DBP	-4%	0.3%	-4%*	-0.1%	-2%	-2%	-1%	-4%	-0.2%	0.4%
(10 mmHg)	(-6,2)	(-1,0.6)	(-6,1)	(-1,0.6)	(-5,1)	(-8,5)	(-5,6)	(-11,3)	(-3,3)	(-3,4)
BMI	-1%	-3%*	-1%	-3%**	-2%*	-3%*	-2%*	1%	0%	-1%
(kg/m^2)	(-3,1)	(-5,0)	(-3,1)	(-4,-1)	(-3,0)	(-6,-1)	(-4,0)	(-1,3)	(-1,1)	(-2,0)
LDL	-2%	-3%	-5%	-9%**	-2%	-4%	-3%	6%	-0.3%	-2%
(mmol/L)	(-10,6)	(-12,7)	(-11,0)	(-14,-4)	(-7,3)	(-10,2)	(-8,3)	(-2,14)	(-4,4)	(-5,1)
HDL	12%	36%*	7%	30%**	·4%	11%	-11%	-13%	-0.4%	-0.2%
(mmol/L)	(-4,30)	(7,73)	(-5,19)	(11,51)	(-5,15)	(-4,30)	(-20,0)	(-18,4)	(-7,6)	(-8,9)
T.Chol/HDL	-5.6%	-9%*	-7.4%*	-9%***	-3%	-5%*	1%	-4%	2%	2%
	(-9, 1)	(-16,-2)	(-15, 0)	(-13,-5)	(-8, 2)	(-10,-1)	(-4, 5)	(-9,1)	(-1, 5)	(-0.5,5)
Triglyceride	-3%	-4%	-7%**	-3%	-4%	-3%	1%	1%	-2%	-1%
(mmol/L)	(-10,4)	(-9,2)	(-11,2)	(-7,0)	(-8,0)	(-6,0.3)	(-4,7)	(-3,6)	(-5,1)	(-3,0)
Smoking#	13%	-23%*	0%	-17%*	-0.2%	-1%	-5%	-8%	3%	-13%
	(-1,31)	(-40,-2)	(-9,11)	(-29,-3)	(-8,9)	(-16,15)	(-15,5)	(-22,9)	(-3,9)	(-5,-22)

at least 10 pack/year vs no pack/year

*p<0.05, **p<0.005, ***p<0.0005 adjusted for basal flow, flow in control arm, age, sex and where appropriate diabetes.

Table 3.7. Difference in drug response per quartile increase in Framingham risk score 2000 (95% C.I.) in men and women.

ACh	BK	GTN	L-NMMA
-10%, p=0.07 (-2%, 1%)	-11%, p=0.003 (-17%, -4%)	0.01%, p=0.9 (-7%, 7%)	-6%, p=0.003 (-10%, -2%)

Percentage variance in drug response explained by Framingham risk score beyond that explained by age and sex.

ACh	BK	GTN	L-NMMA
4%	7%	0%	7%

Table 3.8. Percentage change of each drug response for per unit change of diabetes-related factors in type 1 diabetic and non-diabetic subjects, with 95% confidence interval.

	ACh		*BK*		*GTN*		*NA*		*L-NMMA*	
	DM	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***
HbA1c (%)	-5%* (-9,-1)	-3% (-25,24)	-2% (-5,1)	-5% (-20,11)	1% (-2,4)	2% (-13,18)	5%* (1,8)	3% (-14,23)	1% (-1,3)	5%
Diab duration (year)	-0.1% (-1,1)	-	0% (-1,1)	-	0.4% (0,1)	-	-9% (-22,5)	-	0% (-0.5,0)	-
Albuminuria[#]	2% (-15,24)	-	-2% (-5,13)	-	7% (-5,2)	-	-10% (-22,5)	-	0% (-8,8)	-
Retinopathy[$]	-7% (-23,12)	-	-3% (-16,11)	-	1% (-11,15)	-	-3% (-17,12)	-	-2% (-9,7)	-

*p<0.05, **p<0.005, ***p<0.0005 adjusted for basal flow, flow in control arm, age, sex and where appropriate diabetes.

[#]Any albuminuria vs no albuminuria. [$]Any degree of retinopathy vs no retinopathy.

Table 3.9. Percentage change of each drug response for the presence or absence of coronary calcification in type 1 diabetic and non-diabetic subjects, with 95% confidence interval.

	ACh		*BK*		*GTN*		*NA*		*L-NMMA*	
	DM	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***	***DM***	***Non-DM***
Coronary calcification#	-8% (-19,5)	6% (-15,32)	-5% (-14,5)	-5% (-18,9)	-3% (-9,3)	-1% (-9,8)	-10% (-22,3)	-4% (-14,6)	-5% (-11,23)	-4% (-12,3)

#Any calcification vs no calcification.

Chapter 4. Discussion

4.1 Summary of results

This large study utilising dose-response relationships to multiple drugs demonstrates that the primary defect in forearm resistance vessels in type 1 diabetes is impaired vascular response to exogenous NO donor. Furthermore, this study showed that in the general population, the response to endothelium-dependent dilators is not demonstrably higher in women than men once sex differences in forearm volume have been taken into account. Thirdly, the impairment in the L-arginine: NO: cGMP pathway seen in diabetes is of similar magnitude in men and women and is therefore unlikely to underlie the loss of the sex difference in CHD in diabetic patients.

4.2 Vascular response to NO donors is impaired in Type 1 diabetes

Glyceryl trinitrate is metabolised to yield NO and causes endothelium-independent vasodilatation through the same mechanism as endogenous (endothelium-derived) NO. Previous studies in resistance and conduit vessels have produced conflicting results on vascular smooth muscle responsiveness to NO donors in type 1 diabetes (Chapter 1, Table 1.1). In plethysmography studies of type 1 diabetic patients, the response to NO donor was impaired in one study (Calver et al, 1992) but unchanged in others (Smits et al, 1993; Johnstone et al, 1993). One study showed enhanced response to NO donor in type 1 diabetic patients with macroalbuminuria and autonomic neuropathy (Makimattila et al, 1997) and has been attributed to either reduced sympathetic vasoconstrictor tone or increased vascular smooth muscle sensitivity (Steinberg et al, 1997). However, it is of note that in the vast majority of

resistance vessel studies reporting "normal" responses to NO donors in diabetes, the response to exogenous NO was slightly lower in the diabetic group. Similarly, in the majority of studies on conduit arteries, the GTN response appears to be depressed, and in some studies this reached statistical significance (Zenere et al, 1995; Clarkson et al, 1996). In general, the sample sizes in studies of flow-mediated dilatation of conduit arteries (usually brachial) have been larger (n=17-80) than those performed in resistance vessel studies using forearm plethysmography (n=9-34) (Chan et al, 2000). The magnitude of the difference in GTN response between diabetic and non-diabetic subjects that was detected (17%) is such that a sample size of about 120 subjects would be required to have 90% power to detect it. This may explain previous negative studies. Thus, the present study of 157 subjects has now provided compelling evidence that the resistance vessel response to NO donors (at least GTN) is impaired in type 1 diabetes. Clearly it will be important to determine the nature of the defective response to NO in diabetes and explore whether it is also seen in other important target cells for NO such as platelets and leukocytes or beyond the cardiovascular system. It will also be important to determine whether this defective response to GTN is also shared by other NO donors (such as sodium nitroprusside), although a previous study suggests that it is (Calver et al, 1992).

4.3 Defective NO Response in Diabetes: Potential Mechanisms

Defective GTN response could represent impaired vascular smooth muscle responsiveness to NO or enhanced inactivation/destruction of NO. For GTN-induced vasodilatation to occur, the GTN or exogenous NO will need to diffuse across the basement membrane and reach the vascular smooth muscle cells where it activates soluble guanylate cyclase to generate cGMP which subsequently causes

vasorelaxation. In type 1 diabetes, one or more of the above processes may be defective. For instance, the AGEs accumulated in the basement membrane may inactivate NO (Hogan et al, 1992). Alternatively, cGMP production may be diminished (Vesely et al, 1977) as a result of reduced guanylate cyclase expression or activity in diabetes (Michimata et al, 1996). Even if cGMP production is normal, there may be structural defects within the vascular smooth muscle cells in diabetes restricting vasorelaxation. However, this is unlikely since if it is the case, vasoconstrictor response to noradrenaline would be expected to be impaired and the present study found no evidence of this. Defects in one or all of these complex processes may account for impaired NO response. Excessive superoxide anion generation in diabetes could lead to enhanced NO destruction (Diederich et al, 1994). In addition, NO may be quenched by advanced glycation endproducts (Bucala et al, 1991). Further studies will be required to investigate these mechanisms. Overall, current evidence and the results of the present study support a functional rather than a structural defect within the diabetic vasculature.

4.4 Agonist-Stimulated Endothelium-Dependent Vasodilatation

Acetylcholine and BK were used as agonists to stimulate NO release. Response to ACh was reduced in individuals with diabetes as reported in some (Johnstone et al, 1993) but not all previous small studies (Calver et al, 1992; Smits et al, 1993), whilst response to BK has not been studied previously in type 1 diabetic patients. Although responses to ACh, BK and shear stress (induced flow-mediated dilatation) are often taken as an indicator of the ability of endothelium to release NO, results from this study showed that the impaired response to NO donor is sufficient to account for a large part of the impaired response to ACh seen in diabetic subjects and for all of the

impaired response to BK. Flow-mediated dilatation of the brachial artery also appears to be impaired in type 1 diabetes (Zenere et al, 1995; Clarkson et al, 1996), but again, in view of the diminished response to exogenous NO, it is unclear how much of the deficit lies at the level of endothelial NO generation and how much at the smooth muscle level. The present analysis suggests that the defective response to ACh cannot be completely explained by reduced sensitivity to NO so that there may be additional specific defects in the muscarinic transduction pathways. However, it is important to note that only part of the response to ACh and BK is mediated by NO in the human forearm (O'Kane et al, 1994). Hence, mechanisms other than the NO pathway may be defective in diabetes.

4.5 Vascular Response to NO Synthase Inhibition

There was no evidence of any decrease in basal NO-mediated dilatation in diabetic subjects as assessed by L-NMMA response. Two previous studies have shown blunted L-NMMA response in type 1 diabetes. Calver et al demonstrated this in a small group of male type 1 diabetic patients and also reported impaired response to SNP (Calver et al, 1992). Elliott *et al* showed blunted response to L-NMMA (with normal SNP response) and this effect was more pronounced in diabetic patients with microalbuminuria (Elliott et al, 1993). The number of microalbuminuric patients included in the present study (n=12) was comparable to that in Elliott's study (n=14) and no significant difference in L-NMMA response was found compared to normoalbuminuric patients. Overall, the results of the present study are not consistent with a significantly abnormal response to L-NMMA in type 1 diabetes although a small decrease in the response before full adjustment for forearm flow and volume was detected. This may account for the observations seen previously. A largely

preserved L-NMMA response in the presence of reduced sensitivity to NO would be consistent with enhanced basal NO generation in type 1 diabetes. Indeed, in a recent study total-body NO synthesis (quantified by urinary excretion of ^{15}N-nitrate) was increased in normoalbuminuric type 1 diabetic patients (O'Byrne et al, 2000) although the origin of the excess NO was uncertain. Thus we conclude that basal NO production in type 1 diabetes is not decreased and may even be increased.

4.6 Sex differences in vascular reactivity

We found no sex difference in forearm blood flow response to the drugs, other than BK. Women had higher response to ACh than men but this was not independent of forearm size. This is consistent with a previous study where adjusting forearm length abolished a sex difference in ACh response (Chowienczyk et al, 1994). These data highlight the importance of adjusting basal flow and forearm size in studies involving male and female subjects. In a recent study, there was a higher response to methacholine and L-NMMA in leg vessels in women than men stratified by obesity but no adjustment for limb volume was made (Steinberg et al, 2000). Adjusting for obesity in the current study made no difference to the conclusions. A reduced response to BK in women was found and this unexpected observation will require further investigation. There was no sex difference in noradrenaline or L-NMMA response. This is in contrast to Kneale *et al* who reported a greater vasoconstrictor response to noradrenaline in men than women (Kneale et al, 1997). However, data from these studies were expressed as percentage increase in blood flow compared to baseline so some residual confounding effects by sex differences in basal flow was possible. Forte *et al* found that total-body production of NO is greater in healthy premenopausal

women than in men (Forte et al, 1998) although the cellular origin of the increased NO is uncertain.

4.7 Effect of diabetes on vascular responses is the same in men and women

Steinberg *et al* reported a greater effect of type 2 diabetes on response to methacholine and L-NMMA in women than men with type 2 diabetes such that a sex difference in these responses was abolished (Steinberg et al, 2000). In contrast, the magnitude of the reduction in response to ACh, BK and GTN associated with diabetes in the present study was not significantly different in men and women. Thus there was no evidence to support the hypothesis that the loss of sex difference in CHD risk in type 1 diabetes might be attributable to a greater effect of diabetes on the L-arginine: NO: cGMP pathway in women than men.

4.8 The relationship between conventional risk factors and vascular responses in the general population

Studies that compare vascular function between extremes of a distribution e.g. obese or hypercholesterolemic patients and controls provide useful information at relatively small sample sizes about the pathophysiological mechanisms that may be involved in vascular dysfunction. However, such studies do not give insight into the likely quantitative effect of risk factors in the general population. In contrast, the present study, in a sample representative of the range of factors and vascular function in the general population, provides a different type of information that allows the role of a given risk factor in variation in vascular response in the population to be quantified. The results show that lipid profile is the major determinant of responsiveness to a NO

donor and possibly stimulated NO release in the general population. Cigarette smoking also makes a contribution. It is clear that HDL–C and the total cholesterol: HDL-C ratio is as important as LDL-C, a finding in keeping with the importance of both these measures as risks factors for cardiovascular events. Most of the explicable variation in basal NO-mediated dilatation, as assessed by L-NMMA response, is attributable to smoking. Overall however, the present study also shows that, although these factors are clearly important, they explain relatively little of the variation in the measure of vascular function we have observed (13% for ACh, 19% for BK, 18% for GTN and 9% for L-NMMA). About 30-45% of the variation between subjects in the change in flow in response to drugs was accounted for by age, sex, differences in resting basal flow and the concomittant changes in flow in the control arm. The rest of the variation is either due to measurement error in this type of study or there are as yet unidentified major determinants of variation in vascular function that are worth identifying.

4.8.1 Body mass index

A higher BMI is an important predictor of CHD mortality, in part reflecting its effects on blood pressure and lipids (Jousilahti et al, 1996). Several studies have suggested that one atherogenic consequence of obesity may be impairment of endothelium-dependent vasodilatation. The results of our study indicate that BMI is an important determinant of both endothelium-dependent (BK and ACh response) and endothelium–independent vasodilatation (GTN response), although for BK and GTN the relationship was seen only in women. This effect of BMI has been reported previously (Steinberg et al, 1996; Perticone et al, 2001) and the results of the present study indicate that it is independent of other conventional risk factors. The

mechanisms may relate to the inflammatory effect of obesity (Yudkin et al, 2000) but further studies would be required to test this directly.

4.8.2 High-density lipoproteins

High HDL-C (and low total cholesterol: HDL-C ratio) was associated with greater response to ACh, bradykinin and GTN in forearm resistance vessels independent of other lipoprotein concentrations. This is consistent with a recent study in which Li *et al* studied the relationship between lipoproteins and conduit vessel response in 63 subjects with CHD and 45 controls (Li et al, 2000). They found that only elevated HDL-C was significantly related to flow-mediated vasodilatation of brachial arteries (Li et al, 2000). It has become clear that HDL-C is a major determinant of cardiovascular risk and it is interesting to note that it is a prime determinant of vascular reactivity in a general asymptomatic population.

4.8.3 Low-density lipoproteins

High LDL-C levels were associated with impaired bradykinin response but responses to other agents were unaffected. This is in contrast to studies in which individuals with high or normal LDL-C were compared; these showed that increased LDL-C is associated with impaired ACh-stimulated endothelium-dependent vasodilatation (Chowienczyk et al, 1992; Casino et al, 1993; Gilligan et al, 1994) . It remains to be determined whether the differences are due to the selection of subjects in those studies with high cholesterol who also have other risk factors (eg. low HDL-C) that affect vascular responses, or whether the relatively narrow range of LDL-C levels in the present study has suggested that a weak effect of LDL-C has not discernible.

4.9 Relationship between other risk factors and vascular responses in the general population

With respect to blood pressure, defective endothelium-dependent vasodilatation has been demonstrated in subjects with established essential hypertension (Calver et al, 1994; Panza et al, 1993a; Panza et al, 1993b) although this has been debated (Cockcroft et al, 1994). In our study, systolic, diastolic blood pressure (measured at the beginning of each study) were not associated with changes in any drug responses. This may be due to the relatively low blood pressure range (SBP: 96-155mmHg) seen in our cohort of participants, though it is notable that even when the top and bottom quartiles for blood pressure were compared there was no difference in vascular response to any of the drugs..

Consistent with other published studies (Heitzer et al, 1996; Heitzer et al, 2000), an inverse association was found between ACh and BK-stimulated endothelium-dependent vasodilatation with cigarette smoking. Additionally, smokers were found have lower response to L-NMMA than non-smokers. This suggests that either basal NO production is diminished in smoking or there is diminished NO bioavailability, or a combination of these two factors. It has been shown that smoking is associated with depletion of co-factors for NO synthase such as tetrahydrobiopterin (BH_4) (Heitzer et al, 2000; Ueda et al, 2000) and increased oxidative stress (Reilly et al, 1996). The present finding that there is no association between smoking and noradrenaline response indicates that defective L-NMMA response in smoking is not due to enhanced noradrenaline-mediated vasoconstriction. This loss of basal NO-mediated dilatation in smokers may be of particular importance since loss of basal NO in animal models predisposes to enhanced atherogenesis.

The association between physical activity and decreased risk of CHD is well defined (Berlin et al, 1990), and physical activity leads to an increase in basal levels of NO metabolite, nitrite and nitrate (Poveda et al, 1998). However, in the present study, physical activity per se was not an important determinant of vascular function. Most previous studies were of trained and non-trained individuals (Poveda et al, 1997) which makes direct comparison with our study difficult. It remains possible that physical activity is not in itself a direct determinant of vascular function but rather prevents age-related decline in vascular function (DeSouza et al, 2000).

4.10 Framingham risk score and vascular responses

Interestingly, the most widely used cardiovascular risk prediction tool, the Framingham risk score, is clearly predictive of both agonist-stimulated endothelial-dependent vasodilatation and basal NO release in women though less so in men. This may be because the female Framingham risk score is a more accurate CHD risk predictor than the male score. Alternatively, it is possible that NO release is more important to CHD risk in women than men. Whatever the explanation, it is of importance that forearm responses are significantly influenced by the score and this strengthens the use of forearm responses as a surrogate marker in clinical studies of new therapies.

4.11 The effect of type 1 diabetes on the relationship between risk factors and vascular responses

As shown in Table 5.4, the presence of diabetes generally weakens the association between coronary risk factors and vascular responses. The reason for this observation

is not entirely clear but may be due to various diabete-related factors contributing to "noise" in forearm blood flow measurement.

4.12 The relationship between diabetes-related factors and vascular responses

Individual diabetes-related factors including HbA1c, disease duration, presence or absence of albuminuria and retinopathy were examined independently in relation to vascular responses. Only high HbA1c was found to be associated with reduced ACh response and increased noradrenaline response (Table 3.7). None of the other factors appeared to be important determinants of vascular responses. However, this study was not specifically designed to assess the relationship of diabetes-related factors and vascular responses. Furthermore, the number of patients with albuminuria or retinopathy was too small to assess the relationship with vascular responses. Larger studies of patients with specific diabetic complications will be required in future.

4.13 The lack of relationship between coronary calcification and vascular responses

In this study of middle-aged type 1 diabetic and non-diabetic subjects, coronary calcification was found to be unrelated to any of the drug responses in both subject groups (Table 3.9). This observation is somewhat unexpected and indicates that NO-mediated vascular responses and calcification of the coronary arteries may reflect different pathological processes in atherogenesis. Alternatively, a potential association between vascular function and coronary calcification may be diluted by measurement errors using the forearm plethysmography technique. It should be noted that there is an approximately 1 year delay between the EBCT study and the vascular study. This time lap may further weaken any potential association between CAC and vascular function.

4.14 Study limitations

The sample of diabetic patients recruited was representative of the spectrum of complication risk in type 1 diabetes and hence the present study does not have sufficient power to examine the impact of various complications (such as retinopathy) on vascular response (as discussed above). This could account for the lack of association between drug responses and diabetes-related factors such as retinopathy, micro or macroalbuminuria and disease duration. It is possible that diabetic patients with very short disease duration may have a very different vascular response profile. Glyceryl trinitrate, an organic nitrate, was used as a NO donor to assess vascular smooth muscle response since it produces similar dose-response curves as direct NO donors (such as sodium nitroprusside) (MacAllister et al, 1995). The vasorelaxant effect of organic nitrate occurs through several undefined biochemical reactions. It is plausible that in type 1 diabetes there may be deficient thiol-containing molecules as has been shown in type 2 diabetes (Murakami et al, 1989) which may account for the difference in GTN response we observed between diabetic and non-diabetic subjects. Furthermore, subjects were not studied at the same time of the day which may result in non-current lipid determination and detailed information regarding retinopathy were not provided (only by history from participants). Finally, it is possible that results in the forearm vasculature may not be representative of vascular function elsewhere in the body.

4.15 Conclusions

Following completion of the forearm blood flow study, the following conculsions are reached:

- The main defect in resistance vessel function in type 1 diabetes lies beyond endothelial NO generation and is due to either impaired vascular smooth muscle responsiveness to NO or enhanced destruction/inactivation of NO. Together with studies showing enhanced NO generation in diabetes, the present study clearly highlights the need to study NO quenching or destruction as a pathogenic mechanism in diabetes.

- Vascular smooth muscle responsiveness to NO is impaired to a similar degree in diabetic men and women, hence it does not contribute to the loss of the sex difference in CHD in type 1 diabetic patients.

- The lack of association between vascular responses to drugs and coronary artery calcification suggests that vascular function may not be a useful measure of coronary atheroma although a potential association may be obscured by measurement error in the forearm blood flow study of length of time from measurement of coronary artery calcification to study of vascular function.

- Amongst conventional risk factors, BMI, lipids and smoking but not blood pressure were associated with demonstrable variation in the NO pathway in early middle age. Much of the changes occur in responsiveness to NO, rather than just in

endothelium-dependent responses, although for smoking it appears as though there is a true defect in basal NO generation.

- A high Framingham risk score is predictive of reduced agonist-stimulated and basal endothelium-dependent vasodilatation in the general population. The vast majority of the inter-individual variation in vascular function in the general population cannot be explained by classical cardiovascular risk factors.

Chapter 5. The effect of phases of the menstrual cycle on vascular reactivity in healthy women

5.1 Potential effects of changes in endogenous female sex hormone levels on the vascular reactivity in women

As discussed in Chapter 1, one of the methodological concerns was the potential effect of fluctuation of the female sex hormone during the menstrual cycle on the vascular reactivity of forearm resistance vessels in premenopausal women. Restricting to particular phases of the menstrual cycle for the forearm blood flow study would limit the number of female participants. Hence, the phase of menstrual cycle was recorded for each female participant so that this potential confounder could be taken into account during data interpretation. In the main study, adjusting for phases of the menstrual cycle did not affect results as the vast majority of women happened to be in the follicular phase. In order to understand better how vascular reactivity alters with phases of the menstrual cycle, I conducted a small study examining the effect of menstrual phases on vascular reactivity in healthy non-diabetic women. This chapter provides details of this study.

5.2 Vascular effect of oestrogens

Experimental evidence suggests that oestrogen may alter vascular reactivity both acutely and chronically via modulation of NO production by the vascular endothelium (Mendelsohn and Karas, 1999b). Physiological concentrations of oestrogen cause a rapid release of NO in cultured bovine and human endothelial cells (Arnal et al, 1996) (Caulin-Glaser et al, 1997) whilst long-term administration of oestrogen in women enhances

endothelium-dependent vasodilatation (Williams et al, 1997) (Pinto et al, 1997). There is evidence that acute effects of oestrogens may be mediated in part by activation of preformed eNOS protein (Russell et al, 2000), and chronic effects through increased transcription of endothelial NO synthase (eNOS) (Kleinert et al, 1998). Oestrogens also exert a number of other effects including effects on cyclooxygenase, prostaglandins and adrenoreceptors, all of which may contribute to the maintenance of vascular tone (Reimann et al, 1987; Mikkola et al, 1995; Ferrer et al, 1998).

5.3 Vascular effects of progesterone

In contrast to the vast numbers of studies on the vascular effects of oestrogens, few studies have examined the potential vascular effect of progesterone. Nevertheless, there is evidence that progesterone also has direct vascular effects independently of oestrogens. For instance, administration of progesterone lowers blood pressure in humans (Rylance et al, 1985; Regensteiner et al, 1991) and blunts the pressor response to angiotensin II in human pregnancy (Gant et al, 1973). It remains unclear whether progesterone has an effect on the L-arginine: NO pathway. Overall, it would appear that progesterone has vasodilatory properties. A recent study showed that this vasodilatory action may, at least in part, be mediated by modulation of the L-type calcium channel current activity and, consequently, of cytosolic-free calcium content (Barbagallo et al, 2001).

5.4 Changes of the female sex hormones during the menstrual cycle

The female sex hormones fluctuate during the menstrual cycle. During early menstrual phase and early follicular phase, oestrogen level is low but rises during late follicular phase reaching a peak just before day 14. Thereafter, there is gradual reduction in luteal phase with another slight rise just before menses. In contrast, progesterone level is low during early menstrual phase and during follicular phase. Its level rises sharply from day 14 onwards. During the luteal phase, progesterone level declines slightly (Figure 5.1). Hence, comparison of vascular response between phases of menstrual cycle that differ in oestrogen levels but where progesterone levels are fairly similar e.g. during early menstrual phase vs mid-cycle should enable a physiological assessment of effects of endogenous oestrogen. I chose to study healthy women during early menstrual phase (day 1-4, low oestrogen and low progesterone) and midcycle (day 10-13, high oestrogen and low progesterone). The reason that these 2 phases were chosen was to minimise the influence of progesterone (low in both phases) on vascular reactivity.

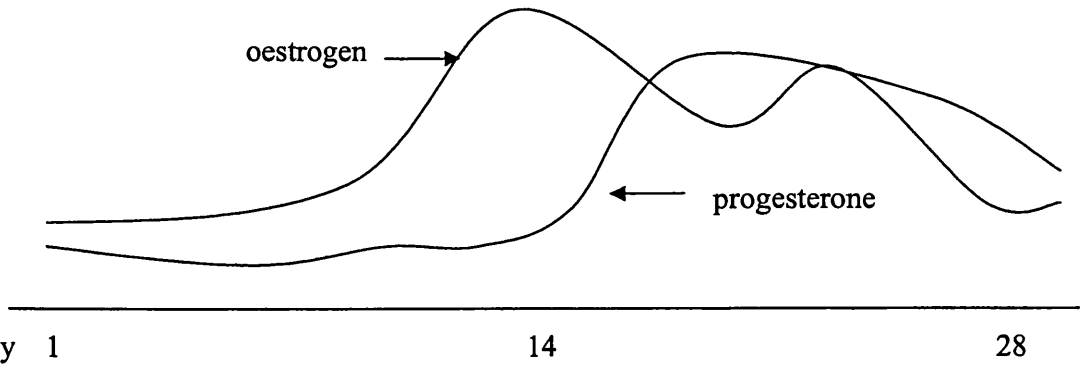


Figure 5.1 Cyclic changes of the female sex hormones during the menstrual cycle.

Previous studies of women have demonstrated variability in cardiovascular responses including flow-mediated dilatation (Hashimoto et al, 1995; Kawano et al, 1996; English et al, 1998), arterial distensibility (Giannattasio et al, 1999), sympathetic outflow (Minson et al, 2000) and α_2-adrenergic responses to agonists (Freedman and Girgis, 2000b) between different phases of the menstrual cycle. The aim of the present study was to test the hypothesis that agonist-stimulated and basal endothelium-dependent NO-mediated vascular responses in resistance vessels differ between the phase with peak estrogen level (mid-cycle) and the phase with lowest estrogen level (early menstrual phase) of the menstrual cycle.

5.5 Methods

5.5.1 Subjects

Fifteen healthy female volunteers were recruited including 10 Caucasians, 2 Afro-Caribbeans, 2 Indians and 1 Chinese. All subjects were aged between 19 to 47 years and had regular menstrual cycle (26-34 days) for at least 3 months before the study. All were non-smokers, had no past medical history of note and were not on any form of medication. No subject had been on the oral contraceptive pill in the 6 months prior to the study. Apart from one subject who previously had a termination of pregnancy, all other subjects were nulliparous. Other subject characteristics are summarized in Table 5.1. Each volunteer was given detailed written information sheet prior to enrolment into the study. Informed consent was obtained from each participant before the study. The study was approved by the local ethics committee.

5.5.2 Study design

Forearm blood flow studies were performed on two occasions in each volunteer. One study after the onset of menses (EMP) on day 1-4 and the other at mid-cycle on day 10-13. Studies were performed either in the morning (3 EMP and 4 mid-cycle studies) or in the afternoon (12 EMP and 10 mid-cycle studies) with the exception of one subject who was studied in the evening during mid-cycle. All subjects were advised to avoid drinks containing caffeine 24 hours prior to the study. After the subjects had relaxed for 5 minutes, blood pressure was taken in right arm using an automated device (Omron 705CP, OMRON Health Europe B.V., The Netherlands) with subject seated. Non-fasting blood samples were taken, centrifuged immediately for 15 minutes and serum stored at -70°C. Sex hormone levels and the lipid profile of all subjects were measured following completion of the study. Serum estradiol and progesterone concentrations were measured by a sensitive radioimmunoassay (Dia Sorin Ltd, Woking, Berks) and serum lipids were measured enzymatically. In 12 subjects the EMP study was performed first.

5.5.3 Study protocol

Studies were performed in a quiet temperature-controlled (24-27°C) laboratory. With participants supine, a 27-guage stainless steel needle (Cooper's Needle Works, Birmingham, UK) was inserted into the brachial artery of the non-dominant arm under local anaesthesia with 1 ml of 1% lignocaine. Drugs were dissolved in 0.9% sodium chloride solution (normal saline) and were infused at 0.5 ml per minute. Forearm blood flow was recorded simultaneously in both arms by venous occlusion plethysmography[20] calibrated to measure absolute blood flow with temperature-compensated strain-gauges

attached to the forearms. During measurements, upper arm congesting cuffs were inflated to 40 mmHg for 10 out of every 15 seconds and circulation to the hands was excluded by inflating the wrist cuffs to 200 mmHg.

After 15 minutes of normal saline infusion basal blood flow was measured for 2 minutes. Infusions of bradykinin (Clinalfa, Laufelfingen, Switzerland; 10, 30 and 100 pmol/min, each dose for 3 minutes), GTN (David Bull Laboratories, Warwick, UK; 4, 8, 16 nmol/min, each dose for 5 minutes), noradrenaline (Levophed; Sanofi Winthrop Ltd, Guildford, UK; 60, 120, 240 pmol/min, each dose for 5 minutes) and L-NMMA (Clinalfa, Laufelfingen, Switzerland; 1, 2, 4 μmol/min, each dose for 5 minutes) were performed, with responses to each drug infusion separated by a 10 minute saline washout period. Forearm blood flow was measured for the final minute of each infusion period for each dose of each drug. Flow was recorded for approximately 10 s in every 15 s and the mean of the four measurements of each recording period was used for data analysis. Baseline blood flow was expressed as ml of blood per 100 ml of forearm volume per minute (ml/100ml/min) and dose-response curves were constructed for all 4 vasoactive agents.

5.5.4 Statistical analysis

The ratio of blood flow in the infused forearm to that in the non-infused forearm was calculated for each measurement period. Responses to drugs were expressed as percentage change in the forearm blood flow ratio (infused arm / control arm) relative to the immediately preceding baseline flow ratio (infused arm / control arm). The area under the dose-response curve (AUC) was calculated for each drug as a summary measure of

drug response to allow quantitative comparison between the two phases of menstrual cycle. The differences in AUC and subject characteristics at mid-cycle and early menstrual phase were compared using the paired *t*-test. A *P*-value of less than 0.05 was considered statistically significant. In this study, I chose to use a different statistical analysis using the AUC rather than the method employed in the main study using ANOVA adjusting for different variables because study has a much smaller sample size compared to the main study. Furthermore, using this method to analyse and express results allows comparison with similar studies.

5.6 Results

5.6.1 Baseline variables

There was no significant difference in basal blood flow, blood pressure, glucose, lipid profile between early menstrual phase and mid-cycle (Table 5.1).

5.6.2 Dilator studies

Both bradykinin and GTN caused dose-dependent increases in blood flow in both phases of menstrual cycle. The increase in FBF in response to bradykinin during mid-cycle was significantly greater than that during early menstrual phase as determined by AUC (1349.7±180.2 vs 863.1±112.6) (Figure. 5.1). The mean within-person difference in AUC between phases was 486.5±165.0, p=0.01 (Table 5.2). The response to GTN during mid-cycle, however, was not significantly different from that during early menstrual phase (AUC 1565.5±195.27 vs 1379.7±235.35) (Fig. 2) and the mean within-person difference in AUC was 185.8±239.0, p=0.45.

5.6.3 Constrictor studies

A decrease in blood flow in response to both noradrenaline and L-NMMA was observed in both phases of menstrual cycle. The reduction in FBF in response to noradrenaline during mid-cycle was significantly greater than that during early menstrual phase (AUC 307.9±37.9 vs 210.9±25.4) (Figure.5.3). The mean within-person difference in AUC was 97.1±39.4, (p=0.027) (Table 2). No significant difference in the response to L-NMMA was detected (AUC 348.89±36.6 vs 366.4±26.2) (Figure.5.4). The mean within-person difference in AUC was 17.7±35.2, (p=0.63). As shown in Table 5.2, serum oestradiol was higher at mid-cycle than early menstrual phase (376.1±62.2 pmol/L, range 166-1082 pmol/L vs 123.8±18.2 pmol/L, range 58-324 pmol/L) with a mean within-person difference in AUC of 252.3±56.0 (p=0.0005) but progesterone concentrations were not significantly different (1.01±0.12 nmol/L, range 0.45-2.1 nmol/L vs 1.15±0.13 nmol/L, range 0.35-2.0 nmol/L) with a mean within-person difference in AUC of –0.11±0.1 nmol/L, p=0.3. Nor was oestrogen level a predictor of inter-individual variation in response to drugs between phases of the menstrual cycle.

The difference in oestrogen level between the two phases for each individual did not predict the magnitude of the difference in bradykinin or noradrenaline responses. However, this study was not designed or powered to detect an association between estrogen level and magnitude of response to drugs.

Table 5.1. Clinical characteristics of subjects (n=15) during early menstrual phase (EMP) and mid-cycle (MC).

Phase	EMP	MC	P value
Age (years)	28.07 ± 2.1	-	-
SBP (mmHg)	106.6 ± 2.4	105.9 ± 3.1	NS
DBP (mmHg)	68.5 ± 1.5	67.8 ± 1.8	NS
Total cholesterol /HDL (mmol/L)	5.19 ± 0.2	4.61 ± 0.1	NS
Triglyceride (mmol/L)	1.05 ± 0.3	1.12 ± 0.1	NS
HDL (mmol/L)	1.63 ± 0.1	1.71 ± 0.12	NS
LDL (mmol/L)	2.07 ± 0.13	2.38 ± 0.15	NS
Baseline blood flow (ml/min/100ml)	2.27 ± 0.21	2.33 ± 0.36	NS

Data are expressed as mean ± SEM.

Table 5.2. The difference in area under the drug response curve (AUC) and sex hormone levels between mid-cycle and early menstrual phase.

	Mean difference ± SEM	**p-value**
Area under the curve (AUC)		
Bradykinin	486.5 ± 165.0	0.01
GTN	185.8 ± 239.0	0.45
Noradrenaline	97.1 ± 39.4	0.027
L-NMMA	17.5 ± 35.2	0.63
Sex hormones		
Estradiol	252.3 ± 56.0	0.0005
Progesterone	-0.11 ± 0.1	0.28

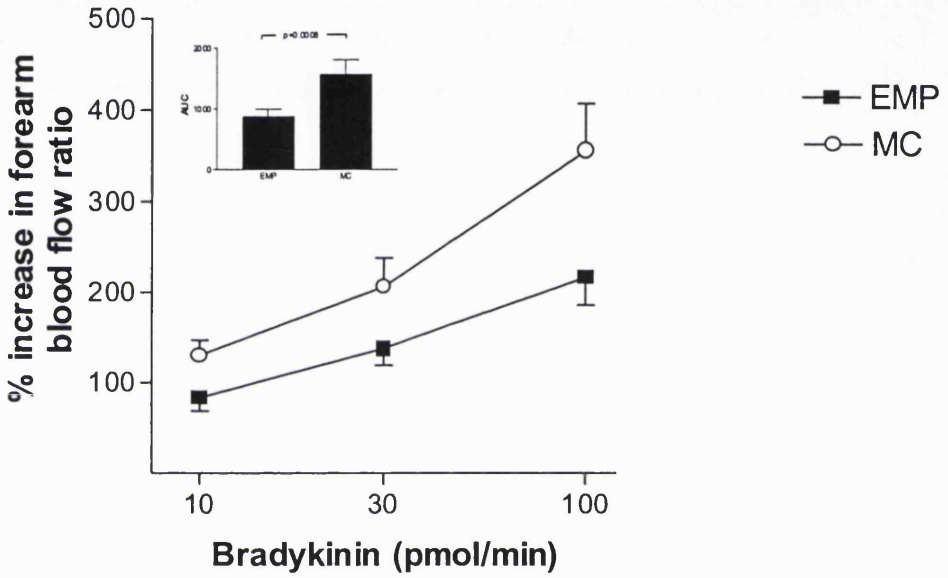


Figure 5.2. Forearm blood flow in response to bradykinin expressed as dose-response curves and area under the curve (AUC).

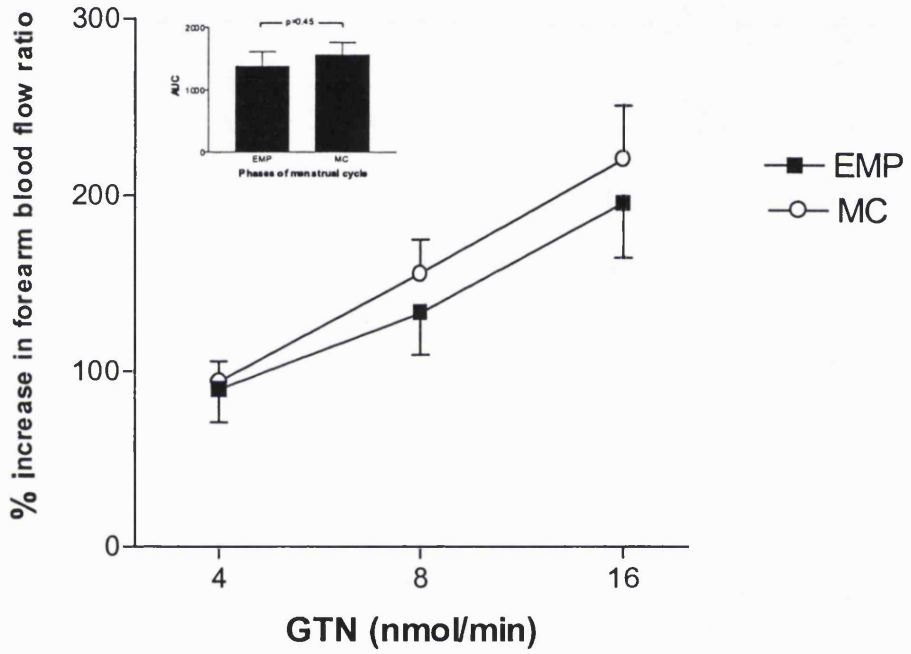


Figure 5.3. Forearm blood flow in response to glyceryl trinitrate expressed as dose-response curves and area under the curve (AUC).

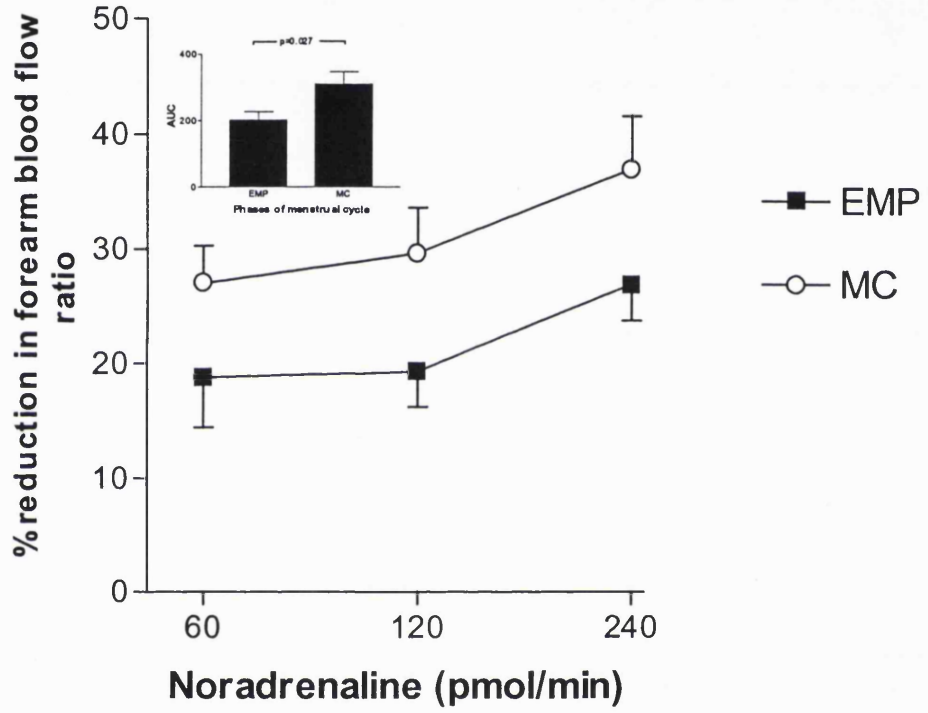


Figure 5.4. Forearm blood flow in response to noradrenaline expressed as dose-response curves and area under the curve (AUC).

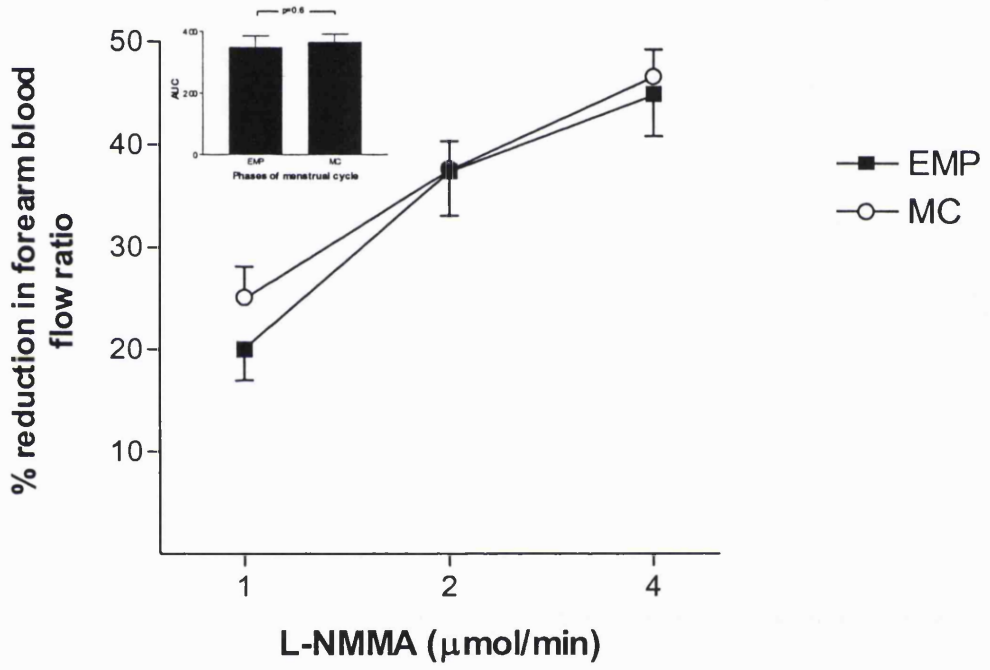


Figure 5.5. Flow arm blood flow in response to L-NMMA expressed as dose-response curve and area under the curve (AUC).

5.7 Discussion

The results of this study indicate that vascular responses in forearm resistance vessels alter during the menstrual cycle in women. Specifically, at a time of high-oestrogen levels, the responses to the endothelium-dependent dilator bradykinin were enhanced with no change in basal NO-mediated dilatation or vascular smooth muscle responsiveness to an exogenous NO donor. However, changes in vascular reactivity were not confined to endothelium-dependent responses and noradrenaline-induced vasoconstriction was also enhanced during high-oestrogen phase of the cycle. Progesterone, blood pressure and lipid profile did not change during the two menstrual phases in our study. Since progesterone may attenuate actions of oestradiol on endothelium-dependent vasodilatation (Teoh and Man, 1999b), we chose to study our subjects during phases of the menstrual cycle where progesterone levels are lowest to minimise its effect on vascular reactivity. Thus it is likely that changes in endogenous oestrogen level contribute to this vascular variability in forearm resistance vessels. However, we were unable to demonstrate a relationship between the degree of change in oestrogen with phases of cycle with the degree of change in vascular reactivity. This may be because a one-off measurement of oestrogen is a poor measure of exposure at the vascular level. Thus although oestrogen is the most likely factor to account for changes in vascular reactivity, it remains possible that changes in vascular reactivity may also be due to cyclical changes in factors not measured in this study. Nevertheless, these findings have important implications for and indicate a need for greater understanding of the physiology of vascular changes during phases of the menstrual cycle.

5.7.1

5.7.2 Agonist-stimulated endothelium-dependent vasodilatation

Bradykinin, one of the major endogenous regulators of vascular tone, produces endothelium-dependent vasodilatation via the bradykinin B_2 receptor. Its effects in the forearm circulation are mediated in part through stimulation of NO release (Taddei et al, 1999). In the present study in 15 healthy women, we found that bradykinin-induced vasodilatation was significantly enhanced during mid-cycle in comparison to early menstrual phase, whilst vasodilatation to GTN (endothelium-independent vasodilatation) was not significantly different between the two phases. This is consistent with previous findings in conduit vessels that vasodilatation in response to increased flow, another endothelium-dependent dilator stimulus, was greatest during mid-cycle compared to follicular and luteal phases (English et al, 1998) and that responses in the follicular phase were greater than those in luteal phase (Kawano et al, 1996). Similarly, sex hormone deprivation following ovariectomy (for uterine leiomyoma) in women has been associated with a significant reduction in acetylcholine-induced vasodilatation in resistance vessels which was restored 3 months after estrogen replacement therapy (Pinto et al, 1997). Together these studies and ours indicate that endogenous estrogen significantly enhances agonist-stimulated endothelium-dependent vasodilatation. Indeed the degree of variation in bradykinin response throughout the cycle was comparable to the difference reported between health and cardiovascular disease in previous studies of endothelial function (Taddei et al, 1999). With regards to the lack of difference in GTN response between the two menstrual phases, the present study had 75% power to detect a 30% change in the AUC of GTN dose-response curve at a significance level with $p=0.05$. Hence this

negative result could reflect a type II error. Future studies of larger sample size would be required to assess whether vascular responses to exogenous NO donors are different in different menstrual phases.

5.7.3 Effect of NO synthase inhibition

Despite the clear changes in endothelium-dependent vasodilatation, we found no difference in degree of vasoconstriction to L-NMMA between the two phases of menstrual cycle suggesting that basal NO-mediated dilatation in the forearm circulation is not altered by different menstrual phases. Previously, it has been found that peak expiratory NO in healthy women is markedly increased at mid-cycle (Kharitonov et al, 1994) and that total NO production, as assessed by nitrite/nitrate concentrations, peak at mid-cycle in premenstrual women (Cicinelli et al, 1996) and are significantly increased in postmenopausal women following estrogen replacement therapy (Cicinelli et al, 1999). The reasons for the differences between these studies and ours are not clear. However, the response to L-NMMA in our study offers a ***functional*** assessment of the basal NO: cGMP pathway whereas measures of NO or nitrite/nitrate in other studies (Cicinelli et al, 1996; Cicinelli et al, 1999) reflect purely ***quantitative*** NO synthesis. Furthermore, NO may arise from any of the three NO synthase isoforms and from multiple cellular/tissue sites and therefore changes in total body NO production may not give clear insight into endothelial NO generation. The present study had 80% power to detect a 30% change in the AUC of L-NMMA dose-response curve at a significance level with $p=0.05$. While it is possible that smaller differences in L-NMMA response occur during the menstrual

cycle the simplest explanation of our finding is that basal NO-mediated dilatation does not significantly alter with physiological changes in oestrogen level, at least in the forearm resistance vessels.

In many situations, alterations in basal NO release (as assessed by L-NMMA constriction) and stimulated NO release (as assessed by dilatation to ACh or bradykinin) occur together. However, this is not always the case (Kiowski et al, 1998). In the present study where enhanced bradykinin-stimulated vasodilatation was not accompanied by any changes in the L-NMMA response, it is possible that oestrogen upregulates signal transduction mechanisms that link B_2 receptor activation to NO synthesis without altering basal NO synthesis. An alternative explanation is that since the vascular effect of bradykinin is only partially NO-mediated, the enhanced vasodilatation may be due to augmentation of other endothelium-derived mediators during the high-oestrogen phase. It has been shown that endothelium-derived hyperpolarising factor (EDHF) might be the predominant mediator in bradykinin-induced vasodilatation in human forearm resistance vessels (Honing et al, 2000). The findings of enhanced bradykinin-induced vasodilatation in our study may reflect modulation of EDHF release/activity during the menstrual cycle. Further studies would be required to test this hypothesis directly.

5.7.4 Changes in α-adrenergic receptor-mediated vasoconstriction

While much recent interest has focused on effects of oestrogen on endothelial function, there is also evidence that hormonal changes during the menstrual cycle may modulate

the sympathetic nervous system, with effects on noradrenaline synthesis (Davidson et al, 1985), and α-adrenergic receptor number (Jacobson et al, 1987) and sensitivity (Kneale et al, 1997). Increased urinary and plasma noradrenaline (Davidson et al, 1985) (Wasilewska et al, 1980) have been reported during the luteal phase of the cycle and free plasma noradrenaline concentration is higher in women than in men, and correlates with plasma oestradiol concentration (Davidson et al, 1985). Oestrogen also upregulates α_2-adrenergic receptor in myometrium in animals and humans (Jacobson et al, 1987) (Bottari et al, 1983) and several lines of evidence suggest that oestrogen may modulate α-adrenergic receptor sensitivity in the vasculature. In ovariectomized rats, oestrogen enhances vasoconstriction induced by vascular smooth muscle cell α_2-adrenergic receptor activation in isolated mesenteric arteries (Ferrer et al, 1998). In humans, vasoconstriction in response to noradrenaline is greater in men than premenstrual women (studied during the first 14 days of the menstrual cycle) (Kneale et al, 1997) and α_1-adrenergic vasoconstriction in forearm resistance vessels is significantly greater during the luteal phase than the follicular phase of the menstrual cycle (Freedman and Girgis, 2000b). In contrast, α_2-adrenergic vasoconstriction was significantly increased during the follicular phase in white (but not black) women (Freedman and Girgis, 2000b). Thus, there is a growing body of evidence to support the concept that estrogens modulate sympathetic activity but the effects are complex and the magnitude and direction of these changes have differed from study to study. In this study, the effects of changes in oestradiol concentrations within the physiological range were examined. A relatively shallow dose-response to noradrenaline was found as reported previously in women (Kneale et al, 1997) and I found that vasoconstriction in response to noradrenaline was significantly

enhanced during mid-cycle in comparison to the early menstrual phase. These findings are consistent with data from animal studies and may reflect estrogen-mediated alterations in α-adrenergic receptor sensitivity. Although the mechanisms involved in the menstrual phase-dependent change in α-adrenergic receptor sensitivity are unclear, the changes in α-adrenergic response has important implications. Firstly, this oestrogen-mediated increase in α-adrenergic vasoconstriction may counteract the enhanced vasodilatory effects of other mediators. Secondly, noradrenaline has been widely used as a comparative control agent for L-NMMA in vascular studies, and it is now clear that the menstrual phase of women should be taken into account during data interpretation.

5.7.4 Study limitations

Although the simplest interpretation of our findings is that high concentrations of oestrogens alter vascular reactivity to bradykinin and noradrenaline, there are limitations that warrant discussion. Firstly, all subjects were studied in the non-fasting state (1-2 hours after breakfast or lunch), hence there may have been variations in the lipid concentrations. Further analysis showed that within person difference in triglyceride concentrations between menstrual phases does not account for changes in bradykinin and noradrenaline responses ($p=0.434$ and $p=0.644$ respectively). Secondly, other factors such as body weight, exercise diet and physical activity were not measured but the fact that each subject serves as her own control should minimize these differences. Thirdly, studies were performed at different times of day rather than the same time of day which may increase the biological variability of vascular responses. Lastly, in 12 out of the 15

participants, the EMP study was performed first and therefore it is possible that there might be an order effect.

5.8 Conclusions for the menstrual cycle study

In conclusion, there is enhanced endothelium-dependent vasodilatation and enhanced α-adrenergic vasoconstriction of resistance vessels in women at mid-cycle compared to the early menstrual phase. Differences in oestradiol levels between cycle phases may underlie changes in bradykinin and noradrenaline responses.

Chapter 6. Conclusions

6.1 Overall conclusions for this thesis

Following completion of the main forearm blood flow study in type 1 diabetic men and women, and the general population, as well as young healthy women during two different phases of the menstrual cycle, the following conclusions are reached:

- The main defect in resistance vessel function in type 1 diabetes is impaired response to exogenous NO donor. This may be due to either impaired vascular smooth muscle responsiveness to NO or enhanced destruction/inactivation of NO. Together with studies showing enhanced NO generation in diabetes, the present study clearly highlights the need to study NO quenching or destruction as a pathogenic mechanism in diabetes.

- Impaired responsiveness to exogenous NO donor is impaired to a similar degree in diabetic men and women, hence it does not contribute to the loss of the sex difference in CHD in type 1 diabetic patients.

- The lack of association between vascular responses to drugs and coronary artery calcification suggests that vascular function may not be a useful measure of coronary atheroma although a potential association may be obscured by measurement error in the forearm blood flow study as well as the time lap between measurement of CAC and the vascular study.

- Amongst conventional risk factors, BMI, lipids and smoking but not blood pressure or lipoproteins were associated with demonstrable variation in the NO pathway in early middle age. Much of the changes occur in responsiveness to NO, rather than just in endothelium-dependent responses, although for smoking it appears as though there is a true defect in basal NO generation.

- A high Framingham risk score is predictive of reduced agonist-stimulated and basal endothelium-dependent vasodilatation in the general population. The vast majority of the inter-individual variation in vascular function in the general population cannot be explained by classical cardiovascular risk factors.

- Vascular reactivity is altered by different phases of the menstrual cycle which is likely to be mediated through oestrogens. This, however, does not alter the result of the main study as the vast majority participants were studied in the follicular phase.

6.2 Future research and directions

The research in this thesis examined NO-mediated vasodilatation and found that the primary defect in type 1 diabetic patients is defective NO responsiveness. The underlying mechanism for defective NO responsiveness is not clear and warrants further investigations. An important question is whether there is defective generation of its secondary messenger, cGMP. Further vascular studies will need to address this important issue perhaps using the cGMP analogue, 8-bromo-cyclic GMP to probe the NO: cGMP pathway within the vascular smooth muscle cell. In additions, whether release/response of endothelium-derived vasoactive mediators other than NO (e.g. EDHF, prostacyclin, C-type natriuretic peptide, endothelin) are defective in type 1 diabetes should also be investigated.

In this present study, there was no significant sex difference seen in the general population or type 1 diabetic patients to underpin the loss of sex difference in CHD in diabetes. However, vasodilatation is only one aspect of the vascular action of endothelial-derived NO. Potential gender difference in other aspects of NO actions will also need to be studied. For instance, whether NO-mediated anti-platelet, anti-leucocyte actions and anti-smooth muscle cell growth and proliferation effects are impaired to a greater extent in women than men in diabetes compared to the general population. These are potential factors which may contribute to the loss of sex difference in CHD in diabetes.

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Appendices

Appendix 1. **Questionaires for the forearm blood flow study**

Appendix 2. **Questionaires and manual of operations for the EBCT study**

Appendix 1

ID.....

Forearm Study
Questionaire for Type 1 diabetic Subjects

Date: **Time:**

NAME **D.O.B**

TELEPHONE (home) (work)

Handedness: **R** ☐ **L** ☐

Blood pressure: **(Systolic/Diast)** ☐ ☐ ☐ / ☐ ☐ ☐

Infusion arm length:.....

Volume of hand:..... **whole arm:.....** **forearm only:.....**

Any change in smoking habit since the last study? **Yes** ☐ **No** ☐

If yes, specify: **current smoker** ☐ **Ex** ☐

Alcohol intake (units/week) : ☐

Recent infection (within a week): **Yes** ☐ **No** ☐

Past medical history:			
	Hypertension	☐	☐
	Hyperlipidaemia	☐	☐
	Angina / myocardial infarction	☐	☐

***Females**	Yes	No
Are you pregnant?	☐	☐
Menstrual cycle - regular	☐	☐
Are you on the pill	☐	☐

What was the first day of your last period? / /

When do you expect your next period to start? / /

Estimated phase of cycle: Follicular midcycle Luteal Menses

Females who are pregnant are NOT eligible
If unsure about pregnancy status the respondent could contact us again when this has been clarified

Drugs

Are you currently on medication? yes ☐ no ☐

Any change in your medication since last study? yes ☐ no ☐

If yes, fill in the following:
Please list below the names of all medications prescribed by a doctor which the respondent is currently taking (i.e. in the last 7 days), other than insulin. If drug name is not known try to find out what its for and ask for a list to be posted - give SAE - **make sure to include all medications including drops, inhalers, ointment and implanted devices e.g. contraceptive implants**.
*** Record indication as follows:

87 angina = 1 MI = 2 lipid-lowering = 3 BP-lowering = 4
other cardiac = 5 kidney disease = 6 other condition = 7 don't know = 8

generic name - or brand name if generic not known	indication ***	Last dose (nitrate)	BNF code	start date mm/yy
			__ __.__ __.__ __	
			__ __.__ __.__ __	
			__ __.__ __.__ __	
			__ __.__ __.__ __	

Hypoglycaemia over the last 24-48 hours Yes No

Drug combination used: I II III IV V VI

Comments:

Follow-up phone call

Date:

Full Recovery: Yes No, details.....

ID.....

Forearm Study
Questionaire for non-diabetic Subjects

Date: **Time:**

NAME	**D.O.B**
TELEPHONE (home)	(work)

Handedness: **R** ☐ **L** ☐

Blood pressure: **(Systolic/Diast)** ☐ ☐ ☐ / ☐ ☐ ☐

Infusion arm length.....

Volume of hand..... **whole arm**..... **forearm only**.....

Any change in smoking habit since the last study? **Yes** ☐ **No** ☐

If yes, specify: **current smoker** ☐ **Ex** ☐

Alcohol intake (units/week) : ☐

Recent infection (within a week): **Yes** ☐ **No** ☐

Past medical history:		
Hypertension	☐	☐
Hyperlipidaemia	☐	☐
Angina / myocardial infarction	☐	☐

***Females**	Yes	No
Are you pregnant?	☐	☐
Menstrual cycle - regular	☐	☐
Are you on the pill	☐	☐

What was the first day of your last period? / /

When do you expect your next period to start? / /

Estimated phase of cycle:	Follicular	Mid-cycle	Luteal	Menses

Females who are pregnant are NOT eligible
If unsure about pregnancy status the respondent could contact us again when this has been clarified

Drugs

Are you currently on medication? yes ☐ no ☐

Any change in your medication since last study? yes ☐ no ☐

If yes, fill in the following:
Please list below the names of all medications prescribed by a doctor which the respondent is currently taking (i.e. in the last 7 days), other than insulin. If drug name is not known try to find out what its for and ask for a list to be posted - give SAE - **make sure to include all medications including drops, inhalers, ointment and implanted devices e.g. contraceptive implants.**
*** Record indication as follows:

87 angina = 1 MI = 2 lipid-lowering = 3 BP-lowering = 4
other cardiac = 5 kidney disease = 6 other condition = 7 don't know = 8

generic name - or brand name if generic not known	indication ***	Last dose (nitrate)	BNF code	start date mm/yy
			__ __.__ __.__ __	
			__ __.__ __.__ __	
			__ __.__ __.__ __	
			__ __.__ __.__ __	

Drug combination used: I II III IV V VI

Comments:

Follow-up phone call

Date:

Full Recovery: Yes No, details.....

Appendix 2

Bar code label----------

CORONARY ARTERY CALCIFICATION In IDDM Study- patient

Department of Epidemiology and Public Health

University College London
1-19 Torrington Place London WC1E 6BT

This questionnaire asks you about your medical history, your lifestyle and your background.
All your answers will be kept strictly confidential. It will not be possible to identify any individual person from the results of this study.

Please fill out the identification details below. When you return this questionnaire to us, this page which includes your name and address will be removed.

Surname _____

Forenames _____

Home address _____

_____

_____

Date of Birth | / / | day mth yr |

What is your National Health Service Number? _____

You can find your National Health Service number on the medical card that you are sent when you register with a doctor. If you do not have a card ask the receptionist at your doctor's surgery to tell you your National Health Service Number.

Note that your National Health Service Number is NOT the same as your National Insurance Number [1]

[1] 8.12.98

General instructions

The answer to most questions can be indicated by simply ticking the appropriate box
For example

Do you ride a bicycle regularly? Yes 1 [3]

No 2 [ ]

Sometimes the questions ask you to fill in the actual number

e.g. How old were you when you started to smoke cigarettes regularly . [2 0 ___ ___yrs]

When asked to write a few words you are given a box to write in- please use block letters

e.g. What is the name of your GP? [DR A BLOGGS]

CORONARY ARTERY CALCIFICATION- Patient

dentification number (paste bar code label)

EMOGRAPHIC CHARACTERISTICS

1 Date of birth — dd / mm / yy

2 What did you weigh at birth? (lbs and oz) — lb / oz

3 Sex
- Male 1 ☐
- Female 2 ☐

4 What is your marital status?- Are you...
- Single 1 ☐
- married or living as married 2 ☐
- divorced or separated 3 ☐
- widowed 4 ☐
- other 5 ☐
- don't know 9 ☐

IABETES & CLINICAL HISTORY

5 When was your diabetes first diagnosed? — mm / yy
- don't know 9 ☐

When was insulin therapy (of any kind) started? — mm / yy
- don't know 9 ☐

What is the name of the Consultant who oks after you at the diabetes clinic?

When did you first attend your current diabetes clinic? — dd / mm / yy

What is the name of your GP?

0 What is the address of your GP?

How many times in the last 12 months have you been admitted to hospital for your diabetes? ☐☐

How many times per week do you measure your own blood glucose? ☐☐

Do you use this information to adjust your insulin dosage yourself?
- Yes 1 ☐
- no 2 ☐

Over the past 12 months:

14 How many episodes of ketoacidosis have occurred requiring hospital admission?
(i.e blood sugar going high resulting in unconsciousness) ☐☐

15 How many hypoglycaemic attacks have occurred, serious enough to require help from another person? ☐☐

Over the past month:

16 How many non-serious hypoglycaemic attacks have you had?
(i.e. Not requiring help from another person) ☐☐

17 Can you feel when you are going to have a hypoglycaemic attack?

Always 1 ☐
usually 2 ☐
sometimes 3 ☐
seldom 4 ☐
never 5 ☐

In the last six months

18 Have you felt faint on standing up?

yes 1 ☐
no 2 ☐

19 Have you had trouble controlling your bladder?

yes 1 ☐
no 2 ☐

20 Have you had trouble with diarrhoea at night?

yes 1 ☐
no 2 ☐

PAST MEDICAL HISTORY

21 Have you been told by a doctor that you have had a heart attack?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when did the first attack occur? ☐☐/☐☐
mm yy

don't know 9 ☐

---

22 Have you been told that you have had angina pectoris (chest pain due to heart disease)?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when did the first attack occur? ☐☐/☐☐
mm yy

don't know 9 ☐

---

23 Have you had a coronary angiogram (a dye test of the arteries of the heart) done?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when was this?

mm / yy

don't know 9 ☐

24 Have you had an operation on the arteries of the heart? (Bypass graft or angioplasty)

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when was this?

mm / yy

don't know 9 ☐

25 Have you been told by a doctor that you have had a stroke?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when was the first stroke?

mm / yy

don't know 9 ☐

26 Have you been told by a doctor that you have high blood pressure?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when were you first told this?

mm / yy

don't know 9 ☐

27 Have you had a lower limb arteriogram done?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

a) If yes when?

mm / yy

don't know 9 ☐

28 Have you been told that you have peripheral vascular disease or intermittent claudication (bad circulation in your legs which makes it difficult or painful to walk)

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when was this diagnosed?

mm / yy

don't know 9 ☐

29 Have you had ischaemic gangrene of a toe, foot or leg?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when? / mm yy

don't know 9 ☐

30 Have you had an amputation of a toe foot or leg?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when? / mm yy

don't know 9 ☐

31 Have you had foot ulcers?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when did you first have an ulcer? / mm yy

don't know 9 ☐

32 Have you ever had surgery of the blood vessels in the leg ?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when were you first told this? / mm yy

don't know 9 ☐

33 Have you been told by a doctor that you have protein continuously in your urine which is related to diabetes?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when were you first told this? / mm yy

don't know 9 ☐

34 Have you been told by a doctor that you have kidney disease related to diabetes mellitus?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) If yes when were you first told this? / mm yy

don't know 9 ☐

35 Are you currently receiving kidney dialysis or have you had a kidney transplant?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) when did you start dialysis ?
(if on dialysis before transplant give date started dialysis)

mm / yy

don't know 9 ☐

---

36 Have you been told that you have diabetic neuropathy (nerve damage)?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

a) when were you first told this?

mm / yy

don't know 9 ☐

36b) Have you ever been told that you have damage to the back of the eye caused by diabetes (retinopathy?)

Yes 1 ☐
no 2 ☐
don't know 9 ☐

c) when were you first told this?

mm / yy

don't know 9 ☐

36d) Have you ever had laser therapy to the back of the eye?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

e) whendid you first have this ?

mm / yy

---

37 Have you suffered from any other serious illnesses?

Yes 1 ☐
no 2 ☐
don't know 9 ☐

If yes please specify giving dates: a ☐

mm / yy

don't know 9 ☐

b ☐

mm / yy

don't know 9 ☐

c ☐

mm / yy

don't know 9 ☐

---

38 Thinking back over the last **2 weeks** have you been unwell with?

A cold 1 ☐
chest infection 2 ☐
foot infection 3 ☐
diarrhoea 4 ☐
any other infection 5 ☐

if any other please specify.....

not applicable 6 ☐

SMOKING HABITS

39 Have you ever been a regular **cigar** or **pipe** smoker? Yes 1 ☐

No 2 ☐

a) Are you a *(Go to question 40 next question)* Current smoker 1 ☐

(Skip to Q 44 for ex-smokers) Ex-smoker 2 ☐

(Skip to Q 48 on physical activity) Never smoked 3 ☐

CURRENT SMOKERS

40 About how many cigarettes a day do you usually smoke on weekdays - give the number

If less than 1, put 0 ☐☐

41 About how many cigarettes a day do you usually smoke on weekend days - weekends - give the number - *If less than 1, put 0* ☐☐

42 Do you mainly smoke filter tipped cigarettes 1 ☐

plain or untipped cigarettes 2 ☐

hand rolled cigarettes 3 ☐

43 How old were you when you started to smoke cigarettes regularly - give your age ☐☐ys

For "can't remember" put 99

For "don't smoke regularly" put 98

(now go to question 48 physical activity)

EX - SMOKERS

44 Did you smoke **cigarettes** regularly that is at least one a day 1 ☐

only occasionally 2 ☐

never really smoked cigarettes, just tried them once or twice 3 ☐

FOR EX- REGULAR SMOKERS *(if you never smoked regularly go to question 48 physical activity)*

45 About how many cigarettes did you smoke in a day - write the number ☐☐

46 How old were you when you started to smoke cigarettes regularly - write your age ☐☐yrs

For can't remember put 99

47 How old were you when you stopped smoking cigarettes regularly - write your age ☐☐yrs

For can't remember put 99

PHYSICAL ACTIVITIES

48 Which of the following answers best describe your daily activity (at work or at home if you work at home) - please tick one box per question.

	Never	Seldom	Sometimes	Often	Always
do you sit	1	2	3	4	5
do you stand	1	2	3	4	5
do you walk	1	2	3	4	5
do you lift heavy loads	1	2	3	4	5

49 How many miles do you **walk** on an average weekday?

- up to 1 mile ☐
- 1-3 miles ☐
- 4 or more miles ☐

50 How many miles do you **walk** on an average day at the weekend?

- up to 1 mile ☐
- 1-3 miles ☐
- 4 or more miles ☐

---

51 Do you ride a bicycle regularly?

(Regularly = at least once a week)

(if no then go to question on sport)

- Yes 1 ☐
- No 2 ☐

52 How many miles do you **cycle** on an average weekday?

- Up to 2 miles ☐
- 2-6 miles ☐
- Over 6 miles ☐

53 How many miles do you **cycle** on an average day at the weekend?

- Up to 2 miles ☐
- 2-6 miles ☐
- Over 6 miles ☐

---

54 Do you play any sport (or other recreational exercise such as swimming or dancing)?

(if yes go to next question if no then go to question 62 on TV)

- Yes 1 ☐
- No 2 ☐

55 Which sport do you play **most** frequently - please tick **one** box

1. swimming ☐	2. soccer/football/rugby ☐	3. golf ☐
4. squash ☐	5. jogging or skiing ☐	6.basketball/netball ☐
7. dancing ☐	8. cricket ☐	9. badminton ☐
10. tennis ☐	8. bowling ☐	12. weight training ☐
13. other ☐	if other please specify	

56 How many hours a week do you play this sport?

- Less than 1hr/wk ☐
- 1-2 hrs/wk ☐
- 3-4 hrs/wk ☐
- 5hrs/wk or more ☐

57 How many months a year?

- Less than 1 month/yr ☐
- 1-3 months/yr ☐
- 4-6 months/yr ☐
- 6 months/yr or more ☐

58 Do you have a second sport or recreational physical activity?

(yes go to next question if no then go to question 62 on TV)

Yes 1 ☐

No 2 ☐

59 Which is your **second** most frequently played sport - please tick one box

1. swimming ☐	2. soccer/football/rugby ☐	3. golf ☐
4. squash ☐	5. jogging or skiing ☐	6.basketball/netball ☐
7. dancing ☐	8. cricket ☐	9. badminton ☐
10. tennis ☐	8. bowling ☐	12. weight training ☐
13. other ☐	if other please specify	

60 How many hours a week do you play this sport?

Less than 1hr/wk 1 ☐

1-2 hrs/wk 2 ☐

3-4 hrs/wk 3 ☐

5hrs/wk or more 4 ☐

61 How many months a year?

Less than 1 month/yr 1 ☐

1-3 months/yr 2 ☐

4-6 months/yr 3 ☐

6 months/yr or more 4 ☐

TV

62 For how many hours in an average week do you watch television or video?

Less than 1 hr/wk 1 ☐

1-3 hrs/wk 2 ☐

4-8 hrs/wk 3 ☐

9-15 hrs/wk 4 ☐

16hrs/wk or more 5 ☐

DRINKING HABITS

63 Have you ever had a drink of wine, beer or spirits in your life? Yes 1 ☐

(If yes go to next question, if no go to question 66 on Employment) No 2 ☐

64 Thinking back to the last 12 months, please tick the box that best describes how often you usually drank each of the alcoholic drinks listed below - please exclude any non-alcoholic/low alcohol drinks except shandy

	almost every day	3/4 days/wk	once/ twice/wk	once/ twice/month	every 2 months	once/ twice/yr	never in past year
a) wine	☐	☐	☐	☐	☐	☐	☐
b) beer,lager/ stout/cider	☐	☐	☐	☐	☐	☐	☐
c) spirits/ liqueurs	☐	☐	☐	☐	☐	☐	☐
d) sherry/ fortified wine	☐	☐	☐	☐	☐	☐	☐
e) shandy	☐	☐	☐	☐	☐	☐	☐
any other drinks ? write in names of other drinks and tick how often you drink them							
f).....	☐	☐	☐	☐	☐	☐	☐
g).....	☐	☐	☐	☐	☐	☐	☐

For each group of alcoholic drinks that you have drunk in the last 12 months

65 On the days that you have a drink, how much do you usually drink **on any one day?**
Please enter the number of mls in the box:

a) Wine, (number of glasses) (incl. Sparkling wine) — glasses

b) Beer, lager, stout, cider (number of pints) (*If half put 0.5*) — pints

c) Spirits/liqueurs (number of measures) (*One measure=One shot*) — measures

d) Sherry or fortified wine (incl. Port, martini, campari, cinzano, dubonnet) — measures

e) Shandy (number of pints) — measures

f) Any other alcoholic drinks: state type and amount
Name :
1..... — measures

g) 2..... .. — measures

EMPLOYMENT AND SOCIAL HISTORY

66 Are you currently employed?

Employed 1 ☐
Unemployed 2 ☐

67 If not employed which of the following applies?

Waiting to take up a job which you've accepted? 1 ☐
Unemployed and seeking work 2 ☐
Prevented from working from temporary sickness 3 ☐
Permanently sick or disabled 4 ☐
Full time student 5 ☐
Housewife 6 ☐
Not working for some other reason 7 ☐

68 What is the exact title of your **usual** occupation i.e. the title of the job you have held the longest?
please give the exact title of your job or write "student" if you are a student
please give the rank and grade if you have one)

69. What kind of work do/did you do in this job?
(i.e. *list the main things you did)*

70. What qualifications or training are necessary to do this job?

71 How many people are/were employed at your place of work

72. Are/were you in charge of other people?

Yes 1 ☐
No 2 ☐

73. Are/were you an employee or self-employed?

employee *(go to q 74)* 1 ☐
self employed *(go to q 75)* 2 ☐

74. If an employee what does/did your employer make or do?

75. At what age (yrs) did you finish your continuous full-time school or higher education?

yrs

76. At what age did your husband /wife / permanent partner finish your continuous full-time school or higher education - if applicable?

yrs

77. How many cars or vans are normally available for use by you or members of your household? ☐
(put 0 if none)

78 Tick whether you have / or are able to do the following things

	Yes	No
a.freezer	☐	☐
b.tumble dryer	☐	☐
c.dishwasher	☐	☐
d.CD player	☐	☐
e.Spare room for guests	☐	☐
f. garden	☐	☐
g. Home computer	☐	☐
h. going out to a restaurant/cinema/theatre etc. at least once per week	☐	☐
I. Two annual holidays away from home	☐	☐
j. Enough money to be able to make some savings	☐	☐

79 At the time you were twelve years old was your family home

Owned (with or without a mortgage) 1 ☐
Rented from a local council 2 ☐
Rented from a Private landlord 3 ☐

80 At the time you were twelve yrs old Did your family own a car?

Yes 1 ☐
No 2 ☐

FAMILY HISTORY

81 What ethnic origin would you say you are ☐

82 In what country were you born? ☐

83 In what country was your father born? ☐

84 In what country was your mother born? ☐

---

85 a) Did your father have Diabetes Mellitus diagnosed by a doctor?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

b) If yes, does / did he use insulin?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

86 a) Did your father have angina or a heart attack diagnosed by a doctor?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

b) if yes what age was he when angina or heart attack was first diagnosed

yrs

don't know ☐

86 b) Was your father ever on treatment for high blood pressure

Yes 1 ☐
No 2 ☐
don't know 9 ☐

87 Is your father still alive?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

88 If your father is deceased, what did he die of?

Heart attack 1 ☐
stroke 2 ☐
diabetes 3 ☐
cancer 4 ☐
other 5 ☐

Other please specify).....

don't know 9 ☐

89 How old was he when he died (please write his age in years)

__ __

---

90 a) Did your mother have Diabetes Mellitus diagnosed by a doctor?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

b) If yes, does / did she use insulin?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

91 a) Did your mother have angina or a heart attack diagnosed by a doctor?

Yes 1 ☐
No 2 ☐
don't know 9 ☐

)) if yes what age was he when angina or heart attack was first diagnosed

yrs

don't know ☐

) Was your mother ever on treatment for high blood pressure?

Yes 1 ☐

No 2 ☐

don't know ☐

2 Is your mother still alive?

Yes 1 ☐

No 2 ☐

don't know 9 ☐

3 If your mother is deceased, what did she die of?

(Please tick appropriate box)

Heart attack 1 ☐

stroke 2 ☐

diabetes 3 ☐

cancer 4 ☐

other 5 ☐

(other please specify).....

don't know 9 ☐

4 How old was she when she died (please write her age in years)

__ __

---

5 How many brothers and sisters do you have?

__ __

6 a)How many of your brothers and sisters have diabetes?

__ __

b)How many of your brothers and sisters with diabetes use insulin?

__ __

7 How many brothers and sisters have had angina or a heart attack diagnosed by a doctor?

__ __

a) If yes at what age(s) was the angina/heart attack first diagnosed?

yrs

(if more than one brother/sister affected record age of diagnosis for the others in the margin)

dont know 9 ☐

97b) How many borthers or sisters have been on treatment for high blood pressure?

__ __

You are now finished. Thank you for your help. Please remember to bring this questionnaire with you to your appointment.

PLEASE remember to bring all your medications (including any ointments, inhalers etc) with you to your appointment

Identification number ID _ _ _ _ _

CURRENT THERAPY. --PATIENT

Today's date / / dd mm yy

98 What is your present frequency of insulin injections?

- Once daily 1 ☐
- twice daily 2 ☐
- three times daily 3 ☐
- four times daily 4 ☐
- five or more times daily 5 ☐
- continuous subcutaneous insulin infusion (CSII) 6 ☐
- other 7 ☐

if other please specify.....

99 What is your present daily dose of short acting/soluble insulin (units)? __ __

100 What is your present daily dose of medium/long acting insulin (units)? __ __

101 What is your present daily dose of Lispro if applicable (units) __ __

102 Write down your current regime of insulin

Please list below the names of all medications prescribed by a doctor which the respondent is currently taking (i.e. in the last 7 days), other than insulin. If drug name not known try to find out what it's for and ask for a list to be posted - give SAE - **make sure to include all medications including drops, inhalers, ointment and implanted devices e.g. contraceptive implants.**

*** Record indication as follows:

103 angina = 1 MI = 2 lipid lowering = 3 blood pressure = 4
other cardiac = 5 kidney disease = 6 other condition = 7 don't know = 8

generic name - or brand name if generic not known	indication ***	BNF code	start date mm/yy
		__ __.__ __.__ __	
		__ __.__ __.__ __	
		__ __.__ __.__ __	
		__ __.__ __.__ __	
		__ __.__ __.__ __	
		__ __.__ __.__ __	
		__ __.__ __.__ __	

Drugs contd....

generic name - or brand name if generic not known	indication ***	BNF code	start date mm/yy
		__ __ . __ __ . __ __	
		__ __ . __ __ . __ __	
		__ __ . __ __ . __ __	
		__ __ . __ __ . __ __	
		__ __ . __ __ . __ __	
		__ __ . __ __ . __ __	
		__ __ . __ __ . __ __	

104 Apart from any prescribed aspirin noted above do you take aspirin regularly? Yes 1 ☐
(i.e. at least once a week?) No 2 ☐

105 If yes when did you last take aspirin? dd / mm / yy

105a)Have you taken part in any other research studies in the past 4 years? Yes 1 ☐
No 2 ☐

105b) if yes please give the name of the study or what it involved

Complete this section at the clinic for WOMEN only

106 Have you EVER taken the oral contraceptive pill? Yes 1 ☐
No 2 ☐

107 How many years did you take the oral contraceptive pill? __ __
(add up if used on several different occasions, if less than one year put one)

108 When did you last take the oral contraceptive pill? mm / yy
(put in today's date if still on it)

109 Are you currently using an implanted contraceptive device?

yes 1 ☐
no 2 ☐

a) If yes is this a

IUD (coil)1 ☐
Hormonal implant (e.g. depo provera) 2 ☐
other 3 ☐

if other please specify .....

110 Have you had a menstrual period in the last 12 months?

Including 'periods' you may have had whilst on hormone replacement therapy)

Yes 1 ☐

If no go to question 113 No 2 ☐

For those who had a period in past 12 months

111 What was the first day of your last menstrual period?

dd / mm / yy

112 Is there any possibility you could be pregnant at the moment? Yes 1 ☐

(If so do NOT scan do other tests and arrange a scan for another day) No 2 ☐

113 if no period in the last 12 months were your periods stopped by:

surgery 1 ☐

chemotherapy or radiotherapy 2 ☐

pregnancy or breastfeeding 3 ☐

no obvious reason/menopause 4 ☐

Other 5 ☐

(If other please specify).....

114 Have you EVER taken hormone replacement therapy HRT? Yes 1 ☐

(If No **END**) No 2 ☐

don't know 9 ☐

If yes:

115 How many years did you take HRT
(add up if used on several different occasions, if less than one year put one) ___ ___

116 When did you last take HRT

(put in todays date if still on it)

mm / yy

117 For those currently on HRT - Did your first menstrual period stop before you started HRT?

(i.e. no period for 12 months)

Yes 1 ☐

No 2 ☐

don't know 9 ☐

118 If yes were your periods stopped by:

surgery 1 ☐

chemotherapy or radiotherapy 2 ☐

pregnancy or breastfeeding 3 ☐

no obvious reason/menopause 4 ☐

other 5 ☐

if other please specify.....

HYSICAL EXAMINATION - CRF **Identification number** (paste bar code label) ID _ _ _ _ _

19 Height (cm) (no shoes) ___ ___ ___ . __

20 Weight (kg) (indoor clothing, no shoes) ___ ___ ___ . __

21 Waist (cm) 1st ___ ___ ___ . __

22 Hip (cm) 1st ___ ___ ___ . __

23 CAN

) Respiratory rate (breaths /min)

) Time elapsed since eating breakfast ___ ___hrs___ ___mins

		systolic	diastolic	systolic	diastolic
OMRON 24 LEFT Ankle blood pressure (mmHg)	LEFT	1		2	
RIGHT Ankle blood pressure(mmHg)	RIGHT	1		2	

25 BRACHIAL / LYING BLOOD
Semi recumbent Brachial blood pressures (mmHg)
(lying at 30 degrees / right arm only)

systolic	diastolic	systolic	diastolic	systolic	diastolic
1		2		3	

126 BRACHIAL STANDING BLOOD PRESSURE
(taken within 60 seconds of standing)

systolic	diastolic
1	

127 How many hours was it between taking blood and last meal? ___ ___hrs___ ___mins

128 What time was their last insulin injection - other than this morning _____ : _____ am / pm

129 Early morning urine sample:
Which urine sample is this of the day?

130 How much time has elasped since the last urination of the day ? ___ ___hrs___ ___mins

131 Was the respondent fasting from night before when blood sample was taken? **yes/ no**

CHECK LIST ID NUMBER _ _ _ _ _

	Y/N	if no give reason
Completed patient record form		
Weight, height, waist, hip		
Resting ECG (for Minnesota coding)		
C.A.N.		
Ankle blood pressure-OMRON		
Brachial BP lying - OMRON		
Standing blood pressure -OMRON		
12 hour urine collections (x2)		
early morning urine 70mls		

Identification number (paste bar code label) ID _ _ _ _ _

RINE COLLECTIONS

29 Collection 1

a) Start date of collection 1 — dd / mm / yy

b) Start time (24 hr clock) — ___ ___hrs ___ ___mins

c) End date of collection 1 — dd / mm / yy

d) End time collection 1 — ___ ___hrs ___ ___mins

e) nephur stick leucocytes — positive 1 ☐ negative 2 ☐

f) nephur stick nitrate — positive 1 ☐ negative 2 ☐

g) Volume — mls

---

30 Collection 2

a) Start date of collection 2 — dd / mm / yy

b) Start time (24 hr clock) 2 — ___ ___hrs ___ ___mins

c) End date of collection 2 — dd / mm / yy

d) End time collection 2 — ___ ___hrs ___ ___mins

e) nephur stick leucocytes — positive 1 ☐ negative 2 ☐

f) nephur stick nitrate — positive 1 ☐ negative 2 ☐

g) Volume — mls

---

lentification number (paste bar code label) ID _ _ _ _ _

AMPLES TAKEN

0ML PLAIN TUBE ☐

7ML PLAIN TUBE ☐

ML HEPARIN TUBE ☐

6 ML CITRATE TUBE ☐ 4?.....

ARLY MORNING URINE ☐

erc