Skin microvascular function in type 2 diabetes and related aspects of the metabolic syndrome

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Abstract

Microangiopathy is a major cause of morbidity and mortality in type 2 diabetes. This thesis investigates the factors that may influence the microcirculation in type 2 diabetes and related aspects of the metabolic syndrome, using techniques that interrogate the skin microvascular function.

The first study explores the concept of intrinsic microvascular dysfunction. Low birth weight has been linked with the increased risk of developing type 2 diabetes and cardiovascular disease in adult life. Impaired maximum hyperaemic response to local heating was demonstrated in the lowest-quartile birth weight infants compared to the highest-quartile birth weight group. Skin endothelium-dependent vasodilatory function and capillary density were not different between the two groups. In a separate study involving prepubertal and postpubertal subjects, both skin maximum hyperaemic response and postural vasoconstriction response were not related to birth weight. It is possible that extrinsic factors or perhaps the rapid phase of growth and sexual maturation during childhood may have modified the relationship between birth weight and microvascular function.

There has been much interest in the role of postprandial dyslipidaemia in macrovascular disease but its effect on the microcirculation is not known. In a group of healthy volunteers, skin microvascular response was not significantly attenuated after a high-fat meal. However, the change in vasodilatory response correlated strongly with the postprandial rise in triglycerides. In a corresponding study in type 2 diabetic subjects, skin microvascular function was significantly reduced after a high-fat meal.

Thus it would appear that both intrinsic and extrinsic influences are important factors in determining skin microvascular function and the interplay between the elements
that are present at birth and subsequent exposures is one of the essential challenges for future research.
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Abbreviations

ANOVA - analysis of variance
BMI - body mass index
CETP - cholesterol-ester transfer protein
cGMP - cyclic guanosine monophosphate
CHD - coronary heart disease
cv - coefficient of variation
DCCT - Diabetes Control and Complications Trial
Dpv - foot dorsum postural vasoconstriction response
EDRF - endothelium-derived relaxing factor
EDHF - endothelium-derived hyperpolarising factor
EMLA - eutectic mixture of lidocaine and prilocaine
ET-1 - endothelin-1
FDR - first-degree relatives of subjects with type 2 diabetes
FH - fasting hyperglycaemia
GDM - gestational diabetes mellitus
GTN - glyceryl trinitrate
HDL - high density lipoproteins
HMG CoA - hydroxymethylglutaryl coenzyme A
HOMA - homeostasis model assessment
HPLC - high performance liquid chromatography
HQBW - highest-quartile birth weight
IDL - intermediate density lipoproteins
IFG - impaired fasting glucose
IGT - impaired glucose tolerance
IRS - insulin resistance syndrome
LCAT - lecithin cholesterol acyl transferase
LDF - laser Doppler fluximetry
LDL - low density lipoprotein
LDPI - laser Doppler perfusion imaging/imager
Lp(a)- lipoprotein (a)
LQBW - lowest-quartile birth weight
mhrd - maximum hyperaemic response (foot in dependent position)
mhrh - maximum hyperaemic response (foot in horizontal position)
MABP - mean arterial blood pressure
MBW - middle birth weight
MDA - malondialdehyde
MRFIT - Multiple Risk Factor Intervention Trial
MRI - Magnetic Resonance Imaging
mvr - minimum vascular resistance
NEFA - non-esterified fatty acids
NO - nitric oxide
NOS - nitric oxide synthase
OGTT - oral glucose tolerance test
ox-LDL - oxidised low density lipoprotein
PAI-1 - plasminogen activator inhibitor -1
PCO - polycystic ovaries
Ppv - toe pulp postural vasoconstriction response
PV - postural vasoconstriction response

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SD- standard deviation  
SGA- small-for-gestational-age  
sICAM-1- soluble intercellular adhesion molecules  
sVCAM-1- soluble vascular adhesion molecules  
tPA- tissue plasminogen activator  
TRAP- total radical-trapping antioxidant parameter  
TRL- triglyceride-rich lipoproteins  
UKPDS- United Kingdom Prospective Diabetes Study  
VLDL- very low density lipoproteins  
vWF- von Willebrand factor  
WHO – World Health Organisation  
WHR- waist-hip ratio
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The study on the microcirculation in children was part of a larger study published elsewhere by Dr Katherine Price. Her contribution included the performance of the microvascular studies in the children and the data was recorded using the protocols validated in this thesis. I was responsible for the collation of the birth weight data of the subjects and re-analysing the results. The remainder of the work is entirely my own.
1.0 Introduction

1.1.1 Background

Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia resulting in progressive long-term damage and failure of various organ systems. Type 2 diabetes mellitus (non-insulin-dependent diabetes mellitus) has previously been thought to be a mild form of diabetes compared to type 1 disease (insulin-dependent diabetes mellitus). This misconception stems from the fact that type 2 diabetes can be treated with diet and oral hypoglycaemic agents in the early course of the disease, compared to the need for subcutaneous insulin injections at the outset of type 1 disease. However, it has now become apparent that there is a high prevalence of hypertension, macrovascular and microvascular complications in patients with type 2 disease, thus dispelling the notion that it is a mild disease (Group 1993) (Group 1990). Many of these complications may already be present at the time of diagnosis, in contrast to type 1 disease, where such complications would usually emerge only after 10-20 years’ disease duration.

Functional changes in the microcirculation antedate the advent of microvascular complications in diabetes. The mechanisms underlying abnormal microvascular function in type 2 diabetes are unclear, although abnormalities in both endothelium-dependent and endothelium-independent pathways have been suggested (Morris et al. 1995) (Lim et al. 1999). To date, endothelial dysfunction has received the most attention. The high prevalence of microvascular complications at the diagnosis of type 2 disease may be due to the long duration of undisclosed disease and hyperglycaemia preceding the diagnosis. However, functional impairment in microvascular function has also been demonstrated in subjects with fasting hyperglycaemia, who exhibited
only mildly raised fasting glucose (between 5.5-7.8 mmol/l) (Jaap et al. 1994). Moreover, normoglycaemic individuals who are prone to or display features of the insulin resistance syndrome also display abnormal microvascular function (Liddell et al. 1998) (Hu et al. 1998) (Jaap et al. 1994). This suggests that the influence of some other features of the insulin resistance syndrome may have a pivotal role in the development of microangiopathy in type 2 diabetes. Hypertension, obesity and lipid disturbances have been linked with abnormal microvascular function, in particular endothelial dysfunction (Rossi et al. 1997) (Tur et al. 1994) (Khan et al. 1999). Before accepting that the microvascular dysfunction observed is wholly attributed to extrinsic abnormalities, one needs to consider the possibility that it may be a common antecedent or intrinsically linked to the many component features of the insulin resistance syndrome (metabolic syndrome). To test this hypothesis of ‘intrinsic microvascular dysfunction’ requires the identification of subjects who are predisposed to develop type 2 diabetes but do not yet display the adverse metabolic features of the insulin resistance syndrome. Recent literature have indicated that subjects with low birth weight are at an increased risk of developing insulin resistance syndrome and type 2 diabetes in adult life (Barker et al. 1993) and therefore such individuals would represent an ideal population to investigate this further. In support of the concept that impaired microvascular function may be intrinsic to the insulin resistant state, Leeson et al. have recently demonstrated that low birth weight children have reduced vasodilatory function of the brachial artery, in the absence of any overt metabolic abnormalities (Leeson et al. 1997).
1.1.2 Aim of the thesis

To gain understanding of the aetiology and pathophysiology of microangiopathy is crucial in the prevention and treatment of microvascular complications in type 2 disease. The aims of this thesis are therefore two-fold - firstly, to test the hypothesis that the microcirculation may be intrinsically impaired in subjects at risk of developing type 2 diabetes in early life and secondly, to assess whether the exposure to extrinsic factors later in life is also important in the development of microvascular dysfunction. The extrinsic factor examined in this thesis will be the role of postprandial dyslipidaemia on the microcirculation. The aim is to study the role of both intrinsic and extrinsic influences on the skin microcirculation, which is a vascular bed that is easily accessible and readily examined using non-invasive in-vivo techniques, with little disturbance to the microvascular bed under study.

1.1.3 Scope of chapter

In this introductory chapter, the normal skin microcirculation will be described. The risk factors predisposing to cardiovascular disease in diabetes will be deliberated, along with the current views on microvascular dysfunction in type 2 diabetes and prediabetes, including some discussion of the macrocirculation where evidence may be lacking in the microcirculation. This will lead onto the exploration of the hypothesis that microvascular abnormalities predate the onset of diabetes and clinical insulin resistance and the proposal of the fetal origin of adult diseases, specifically type 2 diabetes and cardiovascular disease.

Next the role of one of the important extrinsic factors that can affect vascular function, namely lipid and lipoprotein fractions, will be discussed. Lipid metabolism and its effect on the endothelium in healthy and type 2 diabetic individuals will be
considered, highlighting the emerging importance of the postprandial state in disease processes.
1.2.0 The normal skin microcirculation

1.2.1 Introduction

The skin is the largest organ of the body and is approximately 3mm thick with a surface area of 2m² and weighs about 2 kg. The anatomy and the function of the skin are not static as it is constantly adapting to a changing external environment. The skin microcirculation plays a central role in enabling the skin to carry out its vital functions.

1.2.2 Structure of the skin microcirculation

In the resting stage the skin contains about 4.5% of the body’s total circulating blood. The skin microcirculation consists of arterioles, capillaries, venules and arteriovenous anastomosis (figure 1.1). The large vessels supplying the skin microcirculation arise from three sources - musculo-cutaneous, fascio-cutaneous and direct cutaneous arterioles. The vessels from the three systems spread throughout subcutaneous tissues and also give rise to branches supplying the overlying dermis. The supplying arterioles (40-60μm) form a network of vessels in the deep dermis called the reticular plexus, which supplies the deep dermal structures such as the sweat glands and the deep part of the hair follicles and then eventually feeding the terminal arterioles. The terminal arterioles (30-40μm) form the papillary plexus, with capillaries (5-10μm) projecting perpendicularly from the plexus to form capillary loops. These capillary loops vary in structure and density according to the region of the body. The epidermis overlying the dermis and superficial to the basal membrane zone has no blood vessels and derives its nutrition through diffusion from the network of capillaries in the dermis. The capillaries feed into the postcapillary venules (10-30μm), which are
followed by the collecting venules that drain the skin. Arteriovenous shunts or anastomosis (50μm) are blood vessels that link the arterial to the venous sides of the circulation bypassing the capillaries, thereby diverting blood towards or away from the nutritive capillary bed. These shunts are present predominantly in the middle or deep dermis and the density vary from different skin regions. These vessels are important for thermoregulatory function and are richly innervated and respond to both electrical and chemical stimuli. The AV shunts are particularly sensitive to sympathetic and general vasoconstrictor neural control and the skin blood flow can vary from 1–150ml/100g of skin in response to thermoregulatory demands (Barker et al. 1995).

1.2.3 Structure of the skin blood vessel

The blood vessels in the skin consist of a number of cellular and non-cellular elements. The endothelial cell separates the intravascular compartment from the extravascular environment. It has been recognized that the endothelial cell layer is not just a passive lining of cells acting as a physical barrier between blood and tissue but plays an active role in many physiological functions. The endothelial cell secretes important vasoactive factors and also regulates coagulation, smooth muscle cell tone, the transport of lipid and immunological functions (Shore 1996).

The basement membrane envelopes the endothelial cells and also cellular elements like smooth muscle cells and pericytes, which have smooth muscle cell properties. The thickness and composition of the vessel wall depend on the site and function of the vessel. For example, in the terminal arterioles, the endothelial cells are surrounded by one to two layers of smooth muscle cells with an internal elastic membrane separating the two cell layers (wall thickness = 1-3.5 μm). As the arterioles decrease
in size, the smooth muscle cell layers become thinner and the elastic membrane forms an incomplete sheath. The skin blood vessels are also surrounded by other important cells and structures like mast cells, macrophages and nerve endings (Swerlick 1997).

1.2.4 Function of the skin microcirculation

The skin is an important protective physical barrier and plays an important role in mediating the relationship with the external environment. Its other principal functions include thermoregulation, blood storage, nutrition and local defence. The nutritive requirement of the skin is minimal at rest with the average blood flow estimated to be 20ml/min per 100g of tissue. There are obviously regional variations and the finger skin in particular can withstand very low flows (0.5ml/min/100g tissue) for a long period and still recover from the ischaemic insult. The resting blood flow of the skin thus far exceeds the nutritional demand of the skin, indicating that the nutritive function is only a minor role of the skin microcirculation. The constant over-perfusion serves to meet the requirements for thermoregulation and repair. Various mechanisms that control skin microcirculation and blood flow enable the skin to carry out these functions.

1.2.5 Regulation of skin blood flow

The regulatory mechanisms that control cutaneous blood flow are complex, with competition between thermoregulatory reflexes, baroreflexes, chemoreceptor reflexes and reflex responses to upright posture and exercise. The mechanisms that govern skin blood flow can be broadly divided into intrinsic and extrinsic control.
1.2.5.1 Intrinsic mechanisms

Various local metabolic and physical factors can affect flow through capillaries by influencing pre- and post-capillary resistance. The metabolic factors include hypercapnia, hypoxia, acidosis and the presence of interstitial potassium but such factors however do not play a crucial role in governing the blood flow of the skin compared to other organs with greater metabolic demands.

Physical factors included the perfusion pressures in the arterioles, local temperature and an increase in blood flow itself. An increase in perfusion pressure distends the blood vessels, triggering vasoconstriction, which in turn results in an increase in vascular resistance. Decreased perfusion pressure has the opposite effect. This pattern of events is called the myogenic response, which has a role in maintaining the arterioles in a constant state of partial constriction (basal tone), autoregulation and hyperaemia that accompanies prolonged periods of ischaemia.

Local changes in temperature can also regulate the function of skin blood vessels by modulating the adrenergic neuroeffector interaction. Thus local cooling accentuate the contractile responses of cutaneous vessels to the stimulation of adrenergic nerves and exogenous noradrenaline, thereby reducing skin blood flow (Pergola et al. 1993). Moderate local warming has the opposite effect on skin blood vessels.

Increased blood flow itself can also alter cutaneous blood flow. High shear stress from the increased flow can stimulate the endothelial cells to release vasoactive agents that cause vasodilatation. The new understanding of the role of the vascular endothelium in secreting vasoactive substances stems from the discovery that nitric oxide (NO) or endothelium-dependent relaxing factor (EDRF) is an important mediator of vasodilatation. Besides shear stress, a variety of compounds like acetylcholine can stimulate the endothelium to generate NO from the conversion of L-arginine to L-
citrulline, catalysed by the enzyme nitric oxide synthase (NOS). The NO generated then diffuses across to the smooth muscle cell layer and stimulates guanylate cyclase, which catalyses the formation of cyclic guanosine monophosphate (cGMP), resulting in smooth muscle cell relaxation and vasodilatation of the blood vessel. Many isoforms of NOS have been identified and the multiple locations of the enzyme indicate that NO is also involved in other biological functions, including immune function and neuronal signal transmission (Baumgardner et al. 2000). Other important vasoactive substances released by the endothelium include vasodilators such as endothelial-derived hyperpolarising factor (EDHF), prostanoids and vasoconstrictors such as endothelins, which can act either in concert or in antagonism with one another to modulate microvascular diameters and blood flows.

Local nervous mechanisms may also operate. Stimulation of pain fibres results in an axon reflex that is responsible for the flare component of the Lewis's triple response. Another important nervous reflex is the postural vasoconstriction response. The change in posture from the lying to the standing position is accompanied by an increase in precapillary resistance. The increase in capillary pressure is therefore less than what would have been expected from the posturally induced increases in hydrostatic pressure. This increase in precapillary resistance is a protective reflex and reduces capillary blood flow and oedema formation. It has been demonstrated that in the foot dorsal skin, this reflex is mediated mainly via a local neurogenic mechanism with small contributions from a centrally-induced reflex and also the local myogenic response (Hassan et al. 1988).
1.2.5.2 Extrinsic mechanisms

Blood vessel diameter is also governed by the autonomous nervous system and humoral factors. The cutaneous blood vessels are under neural control from a rich adrenergic nerve supply. These vasoconstrictor nerve fibres are the efferent arms of various reflex drives. The afferent system consists of thermoreceptors, baroreceptors, chemoreceptors, pain receptors and special senses (Rowell 1977). It is thought that the skin blood vessels in human are predominantly innervated by $\alpha$ receptors. Noradrenaline released from the nerve endings in response to nerve stimulation causes vasoconstriction via stimulation of the receptors on the vascular smooth muscle cell. Vasodilatation of the skin vessels is brought about mainly by decreasing vasoconstrictor tone. There are also non-adrenergic, non-cholinergic sympathetic nerves that may influence blood flow and smooth muscle cell tone. A wide range of peptides has been proposed as the neurotransmitters such as vasoactive intestinal peptide and calcitonin gene related peptide (Shore 1996).

Besides the catecholamines, other humoral agents that are either circulating in the blood stream or endogenously produced, such as serotonin, bradykinin, adenosine, acetylcholine, neuropeptide Y, histamine and angiotensin II, also play a role in controlling the skin microcirculation. These substances may modulate the effects of the perivascular nerves by enhancing or inhibiting noradrenaline release. They may also act on the endothelium to release vasoactive substances or directly on the vascular smooth muscle cell to cause vasoconstriction or vasodilatation.

1.2.6 Summary

The skin microcirculation consists of a network of arterioles, capillaries, venules and arterio-venous shunts. The capillary system is made up of the superficial papillary
plexus and deep plexus, with the arterio-venous shunts linking the arterial and venous sides of the circulation. The microcirculation has an important role to play in the various functions of the skin, such as thermoregulation, nutrition, physical and immunological defences. Skin microvascular control involves the integration of the various components of the regulatory system, comprising the central and the peripheral nervous system, humoral pathways, endothelium and smooth muscle cell function.
Fig 1.1. Diagram illustrating the vascular networks supplying the skin microcirculation

- epidermis
- papillary dermis
- reticular dermis
- subcutaneous fat
- muscle
- fascio-cutaneous perforators
- musculo-cutaneous perforators
- direct cutaneous vessels
- hair follicle
- capillary loops
- papillary plexus
- sweat gland
- reticular plexus

K - J

musculo-cutaneous perforators
1.3.0 Cardiovascular risk in diabetes

Diabetic mellitus is a complex group of diseases that has hyperglycaemia as a common metabolic abnormality. Patients with diabetes mellitus are at increased risk of morbidity and mortality from macrovascular diseases manifesting as coronary heart disease, cerebrovascular disease and peripheral vascular disease and microvascular complications involving the eye, kidney, foot and the nervous system. Thus vascular diseases in diabetes are often referred to as either macroangiopathy or microangiopathy.

The development of diabetic angiopathy is closely associated with poor glycaemic control, a point reinforced by both the DCCT (Diabetes Control and Complications Trial) and UKPDS (UK Prospective Study in Diabetes Study). The DCCT has shown that intensive treatment with glucose concentration close to normal range delays the onset and slows the progression of diabetic retinopathy, nephropathy and neuropathy in patients with type 1 diabetes (Group 1993). In the UKPDS, a clinical trial of a policy of intensive control of blood glucose in type 2 diabetes, achieved a substantial reduction in the risk of microvascular complications and also a reduction in the risk of myocardial infarction of borderline significance (Group 1998). Relative hyperglycaemia accounts for part but not all of the increased vascular risk. Raised blood pressure is also more common in people with type 2 diabetes than in the general population and the incidence of clinical complications is strongly associated with raised blood pressure. The improvement in blood pressure achieved a 24% reduction in diabetes-related end points, 32% reduction in deaths related to diabetes, 44% reduction in strokes and 37% reduction in microvascular end-points, predominantly owing to a reduced risk of retinal photocoagulation. There was also a non-significant reduction in all cause mortality (Group 1998). Other risk factors such as obesity,
dyslipidaemia, physical inactivity and smoking that influence the cardiovascular risk in general population also play an important role in diabetic angiopathy (Rios 1998) (Shelgikar et al. 1997) (Lehto et al. 1997) (Wei et al. 2000).

Although type 1 and type 2 patients are both prone to developing microvascular complications, there are some differences in the expression of vascular complications between the two groups. In particular, there is a high prevalence of hypertension and vascular complications at the initial diagnosis of type 2 disease, while complications in type 1 disease usually emerge 10-20 years after diagnosis and the prevalence of hypertension is similar to the normal population in the absence of nephropathy. With type 2 diabetes reported to constitute about 85% of all cases of diabetes in developed countries and reaching epidemic proportions in many developing countries, the emergence of macrovascular and microvascular complications following the increase in the number of cases of type 2 diabetes will become a major threat to future public health throughout the world (Zimmet et al. 1997).
1.4.0 Microvascular function in type 2 diabetes

1.4.1 Introduction

In this section, microvascular abnormalities in diabetes mellitus in general and also in type 1 diabetes will be considered, which is necessary to appreciate the differences in the pathophysiology of vascular complications between type 1 and type 2 disease. Current evidence with regards to vascular function in type 2 diabetes will be reviewed, with emphasis on the microcirculation.

1.4.2 Historical perspective of microvascular complications in diabetes mellitus

The first description of diabetic retinopathy was documented in 1855 in a 22 year-old man with disease duration of 4 years. In 1890, Hirschberg stated that diabetic retinopathy was specific and distinct from albuminuric (hypertensive) retinopathy. De Schweinitz noted in 1921 that the development of retinopathy was duration-related and hence it was a rare entity in the pre-insulin era, during which the outlook for the juvenile form of diabetes was poor and diabetes of long duration was rather uncommon (Tattersall 1994).

With regards to diabetic nephropathy, the link between diabetes and albuminuria was already documented in the early 1800s and by the end of the nineteenth century, the prognostic implications of albuminuria were well recognised. Kimmelstiel and Wilson described typical histological changes in the glomeruli of diabetic patients (glomerular sclerosis) and noted that co-existing hypertension was often a feature. It was however considered at that time that high blood pressure was necessary to perfuse the kidneys of such diabetic patients and it only became apparent in the 1970s
that hypertension was actually detrimental to diabetic renal disease (Mongensen 1995).

With the advent of insulin treatment in 1922, the clinical significance of diabetic microangiopathy became more obvious. This improved the survival of diabetic patients and hence, the associated 'triopathy' of retinopathy, nephropathy and neuropathy became more apparent.

Although microvascular abnormalities have been observed in other conditions such as essential hypertension and Raynaud's disease, the triad of microvascular complications of retinopathy, nephropathy and neuropathy is specific to diabetes. In fact, the likelihood of developing microvascular complications has influenced the diagnostic criteria for diabetes (Group 1979). The 1985 World Health Organisation (WHO) diagnostic criteria (fasting: whole blood glucose ≥ 6.7mmol/l or plasma glucose ≥ 7.8mmol/l and 2 hour-post oral glucose tolerance test (OGTT): whole blood glucose ≥ 10.0 mmol/l or plasma glucose ≥ 11.1 mmol/l) were chosen on the basis of epidemiological data which revealed that individuals with such glucose levels were at increased risk of developing retinopathy.

However, the American Diabetes Association (1997) and WHO recently announced a change in diagnostic criteria, which included a fall in diagnostic fasting plasma glucose level to 7.0 mmol/l and the identification of a new group called impaired fasting glucose (fasting plasma glucose between 6.1 mmol/l and 6.9 mmol/l) (Anonymous 1997) (WHO 1999). The scientific reason behind this alteration was the realisation that subjects with impaired glucose tolerance and those with fasting glucose in the upper part of 'normal distribution' may not be at risk of microvascular complications but are at increased risk of macrovascular disease.
1.4.3 Stages of microvascular damage

The temporal sequence of microvascular damage develops in a similar manner to atherosclerosis in the large blood vessels, in that there is an initial clinically silent phase before overt vascular disease is apparent.

In the large vessels, injury induced by factors such as oxidised low-density lipoprotein (ox-LDL) and hypertension leads to changes in endothelial function. The production of vasodilators and anti-aggregating substances such as prostacyclin and nitric oxide is altered, encouraging adherence and transmigration of macrophages and lymphocytes to the cells. The atheromatous process involves various stages, with the formation of fatty streak, fibro-fatty streak and eventually a fibrous plaque, which represents irreversible structural changes. The terminal event occurs with the formation of platelet mural thrombi on the surface of ruptured plaques at thrombogenic sites, such as the bifurcation of arteries (Ross 1993).

Similarly, there is a functional reversible phase in the development of microangiopathy, where a range of metabolic and haemodynamic insults serve to cause endothelial dysfunction. This leads to structural adaptation with increased capillary permeability, basement membrane thickening and luminal narrowing of the microvessels. These changes eventually culminate in the terminal event with complete microvascular obstruction. Response to permanent injury may elicit reparative mechanisms, depending on the site of injury. For example, neovascularisation occurs in the retina in response to tissue ischaemia. However, these new vessels differ from normal blood vessels in that they are more permeable to macromolecules and prone to leakage and haemorrhage (Zatz et al. 1986) and can therefore cause further tissue damage (figure 1.2).
1.4.4 Microvascular function in type 1 diabetes

Parving proposed the haemodynamic hypothesis in order to link preclinical changes in blood flow to clinical microangiopathy (Parving et al. 1983). The hypothesis states that an increase in blood flow and pressure in the microcirculation, which occurs early in the disease and is related to glycaemic control, induces an injury response involving the endothelium. This stimulates the production of perivascular matrix and basement membrane thickening, with the development of arteriolar hyalinosis and mesangial proliferation. This ultimately limits the maximal vasodilatory capacity and impairs regulatory function (autoregulation) (figure 1.3).

Indirect clinical evidence for the hypothesis is provided by anecdotal observations of apparent protection against the development of ipsilateral retinopathy and nephropathy in those with unilateral carotid stenosis (Duker et al. 1990) and renal artery stenosis (Berkman et al. 1973) respectively. The fact that lower limb capillary basement membrane width increases the further below the heart the tissue sample is taken (Vracko 1970) (Williamson et al. 1971) supports the concept that high hydrostatic capillary pressure is instrumental in the development of capillary basement membrane thickening and microvascular damage.

1.4.5 Skin microcirculation in type 1 diabetes

Further evidence to support the haemodynamic hypothesis comes from the study of human skin microcirculation. With the advent of the technique of cannulating finger skin nailfold capillaries and continuous electronic measurement of capillary pressure, elevated capillary pressure was demonstrated in patients with type 1 diabetes of less than two years’ duration, especially in those with poor glycaemic control (Sandeman et al. 1991). This increase in capillary pressure can respond to improvement in
glycaemic control in those with short disease duration and minimal complications. Capillary hypertension was also proposed to be aggravated through the passage of puberty (Shore et al. 1994) and in the presence of arterial hypertension and to be more pronounced in those at highest risk of developing microangiopathy. These data suggest that capillary hypertension plays a vital role in the development of microangiopathy (Tooke 1995).

Limited skin blood flow responses to various stimuli have also been noted in the later course of type 1 disease. Reduction of skin maximum vasodilatory responses to heat, minor skin trauma (pinprick), arterial occlusion and pharmacological agents have been demonstrated in patients with type 1 disease several years after initial diagnosis (Rayman et al. 1986). Microcirculatory autoregulation has also been shown to be impaired in type 1 diabetes and the degree of impairment was found to correlate with the degree of arteriolar hyalinosis. This would be in keeping with the haemodynamic hypothesis that a build up of extravascular proteins may be contributory to the limited skin vasodilatation (Tooke 1995).

Early changes in microvascular permeability may act in concert with haemodynamic changes in diabetes in the genesis of microangiopathy. Certainly an increase in microvascular fluid permeability has been demonstrated in the forearm tissues of young patients with short duration of type 1 disease and reasonable glycaemic control (Jaap et al. 1993). This suggests that there is a primary change in microvascular permeability in at least a subset of type 1 diabetic patients, perhaps those who are nephropathy-prone. However, other permeability changes such as the increase in transcapillary escape rate of albumin appears to be related to glycaemic control, although once more, the changes are more obvious in those with microalbuminuria.
Fig 1.2 Stages in the development of diabetic microangiopathy.

Hyperglycaemia and other metabolic insults

↓

Functional microangiopathy

↓

Structural adaptation: basement membrane thickening, luminal narrowing

↓

Microvascular obstruction

↓

Repair mechanism: neovascularisation
Fig 1.3 The haemodynamic hypothesis.

Increase in microvascular flow and pressure
(control-related)

↓

Increase in shearing forces with increased flux of water and macromolecules

↓

Basement membrane thickening, arteriolar hyalinosis and mesangial proliferation

↓

Limited vasodilatory capacity; loss of autoregulation
1.4.6 Differences in vascular complications between type 1 and type 2 diabetes

Although patients with type 1 and type 2 diabetes are both prone to developing microangiopathy, closer scrutiny suggests that there are some important differences in clinical expression of large and small vessel disease and hypertension. As mentioned previously, there is a high prevalence of vascular complications at the diagnosis of type 2 diabetes and hypertension commonly co-exists (Group 1990) (Group 1993). In type 1 disease, diabetic complications usually emerge 10-20 years after initial diagnosis and the prevalence of hypertension in the absence of nephropathy approximates that of the normal population. Visual loss in type 1 disease is predominantly due to proliferative retinopathy whereas maculopathy is more common in type 2 disease. In type 1 diabetes, renal complication manifests as a typical albuminuric syndrome. However, in type 2 diabetes, the cause of nephropathy is complex, often caused by, or coexisting with arteriolar disease or other renal pathology (Fioretto et al. 1998) (table 1.1).

1.4.7 Vascular function in type 2 diabetes

There are relatively few studies on the human microvascular function in type 2 diabetes compared to the number of published reports on vascular function in large and medium-sized vessels in type 2 diabetes. A brief synopsis of the evidence of vascular dysfunction in conduit and resistance vessels is vital to the understanding of microvascular function in type 2 diabetes.

1.4.7.1 Conduit artery

Using high-resolution ultrasound and wall tracking software, studies of the conduit artery endothelial function in type 2 diabetes have been assessed by monitoring the
change in vessel diameter in response to an increase in flow, induced by inflation and subsequent release of a cuff around the forearm. This response is termed flow-mediated vasodilatation or flow-related vasodilatation of the conduit artery. In order to examine if the abnormal responses seen may be related to endothelial or smooth muscle cell dysfunction, endothelium-independent function is assessed by the vasodilatory response to sublingual glyceryl trinitrate (GTN) administration. To date, the evidence suggests that in type 2 disease, endothelial function is impaired but vascular smooth muscle cell function appears to be intact (Enderle et al. 1998) (Goodfellow et al. 1996).

1.4.7.2 Forearm blood flow

There have also been many studies that examined the forearm resistance vessel function in subjects with type 2 diabetes. The majority of these studies indicated that endothelium-dependent vasodilatory function is impaired (McVeigh et al. 1992) (Watts et al. 1996) (Ting et al. 1996) (Hogikyan et al. 1998) but the response to sodium nitroprusside (endothelium-independent vasodilator) is more variable, reporting either normal (Hogikyan et al. 1998) (Gazis et al. 1999) (Avogaro et al. 1997) or reduced responses (McVeigh et al. 1992) (Watts et al. 1996) (Williams et al. 1996).

1.4.7.3 In vitro studies

There are also in vitro studies that support the presence of endothelial dysfunction but intact endothelium-independent function in type 2 diabetes. One study employs the subcutaneous vessels obtained from the lower limbs of diabetic and non-diabetic subjects prior to vascular surgery (Cipolla et al. 1996) while another study looked at
the internal mammary artery and saphenous rings from type 2 diabetic and non-diabetic subjects undergoing coronary artery bypass (Karasu et al. 1995). However, many of the subjects in both studies were on multiple medication, which may have influenced the study findings.

1.4.8 Microcirculation in type 2 diabetes

In keeping with the differential clinical expression of microvascular complications seen in type 1 and type 2 diabetes, functional changes observed in the microcirculation prior to the onset of overt microangiopathy are also different between the two groups. In contrast to type 1 disease, nailfold capillary pressure is not elevated in normotensive patients with early type 2 disease. Data on capillary permeability are inconsistent but forearm capillary filtration coefficient is not elevated in normotensive type 2 diabetic patients (Jaap et al. 1994) in comparison to type 1 diabetic patients. However, profound impairment of microvascular vasodilatory capacity is observed in early type 2 disease, similar to that seen in patients with long duration of type 1 disease. These differences in microangiopathic disease expression and functional microcirculatory changes suggest a different pathophysiology for microangiopathy in type 2 disease (Tooke 1995) (table 1.2).

Although the mechanisms underlying reduced vasodilatory capacity in type 2 disease are unclear, there is evidence suggestive of abnormalities involving both endothelium-dependent and endothelium-independent pathways (McVeigh et al. 1992) (Morris et al. 1995). Skin microvascular responses to iontophoresis of acetylcholine (endothelium-dependent vasodilator) and sodium nitroprusside (endothelium-independent vasodilator) were found to be reduced compared to healthy controls (Morris et al. 1995). Study of skin capillary density in type 2 diabetic subjects did not
reveal any evidence of capillary rarefaction that may account for the reduced skin vasodilatory responses observed in type 2 diabetes (Jaap et al. 1996). High prevalence of hypertension and microvascular complications at the diagnosis of type 2 disease could represent a long duration of occult disease preceding diagnosis. The alternative explanation is the influence of pre-diabetic/insulin-resistant state on the vasculature, increasing pre-capillary resistance thereby limiting vasodilation and resulting in a high prevalence of hypertension, preventing an early rise of capillary pressure and filtration as opposed to that observed in type 1 disease.

1.4.9 Summary
The clinical significance of microvascular complications became more widely recognised as the survival of diabetic patients improved with the discovery of insulin treatment. Clinically silent functional damage occurs in the microcirculation before overt microvascular disease is apparent. The haemodynamic hypothesis was proposed to link preclinical changes in blood flow to clinical microangiopathy in type 1 diabetes. These changes include an elevation in capillary pressure, limited vasodilatory capacity and increased microvascular permeability. There are differences in the clinical expression of microvascular complications between type 1 and type 2 diabetes. Functional microvascular changes preceding these complications also differ between the two groups; capillary pressure is not elevated in type 2 diabetic subjects who are normotensive but profound impairment in skin vasodilatory capacity is already present in early disease. It is postulated that the pre-diabetic/insulin resistant state may have a role in modifying the vasculature, leading to limited vasodilatory capacity and a high prevalence of hypertension in type 2 diabetes.
Table 1.1 Differences in expression of angiopathy in type 1 and type 2 diabetes.

<table>
<thead>
<tr>
<th>Vascular complication</th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common cause of visual loss</td>
<td>proliferative retinopathy</td>
<td>maculopathy</td>
</tr>
<tr>
<td>Aetiology of nephropathy</td>
<td>microvascular disease</td>
<td>often complicated by arteriolar disease/ other renal pathology</td>
</tr>
<tr>
<td>Prevalence of hypertension</td>
<td>present in those with incipient and overt nephropathy; prevalence otherwise equates to normal population</td>
<td>40%; often present at the time of diagnosis</td>
</tr>
</tbody>
</table>
Table 1.2 Comparison of microvascular function in type 1 and type 2 diabetes.

<table>
<thead>
<tr>
<th>Microvascular parameter</th>
<th>Type 1</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting blood flow</td>
<td>increased</td>
<td>increased</td>
</tr>
<tr>
<td>Vasodilatory response</td>
<td>later reduction</td>
<td>early reduction</td>
</tr>
<tr>
<td>Capillary pressure</td>
<td>increased</td>
<td>normal (in early disease)</td>
</tr>
<tr>
<td>Fluid permeability</td>
<td>increased</td>
<td>relatively normal (in early disease)</td>
</tr>
</tbody>
</table>
1.5.0 Microvascular and metabolic abnormalities in subjects at risk of diabetes

1.5.1 Introduction

Impaired microvascular function in early type 2 diabetes suggests that abnormalities in the microcirculation antedate the disease. To understand the cause of such perturbation of the microcirculation requires the examination of current evidence of vascular and metabolic abnormalities in subjects at risk of future development of type 2 disease, with particular reference to the skin microcirculation.

1.5.2 Prediabetes

1.5.2.1 Conduit artery

Caballero et al. demonstrated that subjects with impaired glucose tolerance (IGT) according to the WHO criteria (fasting plasma glucose < 7.0 mmol/l and 2 hour post-OGTT plasma glucose between 7.8-11.1 mmol/l) have reduced flow-related brachial artery vasodilatation compared to healthy controls, although the sphygmomanometer cuff was placed proximal rather than distal to the brachial artery to induce reactive hyperaemia in this particular study and a direct NO donor was also not used in order to exclude an impairment in endothelium-independent function. The subjects were also not sex-matched and female subjects were not matched for menstrual status or hormone treatment (Caballero et al. 1999). In a separate study, subjects with fasting hyperglycaemia (fasting plasma glucose between 5.5-7.8 mmol/l, where 11 subjects had normal glucose tolerance and 2 with IGT) were similarly found to have reduced flow-related vasodilatation but intact vasodilatory response to GTN in the brachial artery compared to age- and sex-matched control subjects (Lee et al. 1998).
1.5.2.2 Forearm blood flow

Impaired forearm blood flow response to intra-arterial acetylcholine infusion (endothelium-dependent vasodilatation) has been reported in male subjects with impaired fasting glucose (IFG) (fasting glucose between 6.1 – 7.0 mmol/l) compared to control subjects. The endothelium-independent function was found to be comparable in both groups of subjects (Vehkavaara et al. 1999).

1.5.2.3 Skin microcirculation

In the study by Caballero et al., the subjects with impaired glucose tolerance (IGT) exhibited both reduced endothelium-dependent and endothelium-independent vasodilatory responses in the skin microcirculation (Caballero et al. 1999). Morris and colleagues have found that subjects with fasting hyperglycaemia (FH) (fasting glucose between 5.5-7.8 mmol/l) have impaired endothelium-dependent function of the skin but the endothelium-independent vasodilatation appears to be intact (Morris et al. 1996). Maximum hyperaemic response to local heating was also reduced in FH subjects (Jaap et al. 1994) but nailfold capillary pressure was found to be normal (Shore et al. 1994). Skin capillary density has also been examined in a group of subjects with impaired glucose tolerance, which was reported to be normal (Jaap et al. 1996).

1.5.2.4 Metabolic and biochemical parameters

Although IGT subjects in the Cabarello’s study were found to be normotensive, their systolic and diastolic blood pressure were significantly higher than the healthy controls and they were also found to be more obese and had higher triglyceride and
cholesterol levels. In the macrocirculation, systolic blood pressure, HbA1c, HDL-cholesterol and homeostasis model assessment (HOMA) values (an index of insulin resistance) had significant correlations with brachial artery diameter changes. Significant inverse correlations were also found between microvascular reactivity and systolic blood pressure, fasting plasma glucose, HDL-cholesterol, fasting plasma insulin and HOMA values.

Endothelin-1 (ET-1), a potent vasoconstrictor, which is raised in essential hypertension and in disease states such as atherosclerosis and heart failure (Luscher et al. 2000), was found to be elevated in these IGT subjects. Cellular adhesion molecules are expressed on endothelial cells in response to inflammation and facilitate the adhesion of leukocytes to their surface. This is an early step in transendothelial migration and atherosclerosis. Increased soluble intercellular adhesion molecule (sICAM-1) level has been associated with an increased risk of myocardial infarction. sICAM-1 has been reported to be raised in type 2 diabetic subjects (Lim et al. 1999) (Marfella et al. 2000) and found to be raised in this group of IGT subjects (Caballero et al. 1999). In a study by Ferri et al. who studied hypertensive subjects with multiple metabolic abnormalities, those with impaired glucose tolerance (with or without hyperlipidaemia) were shown to have raised sICAM-1 and soluble vascular cell adhesion molecule (sVCAM-1) levels compared to hypertensive but glucose tolerant subjects and healthy controls (Ferri et al. 1998).

The fasting hyperglycaemic subjects in Jaap's study were similarly shown to have significantly higher body mass index (BMI) compared to the healthy age- and sex-matched controls (Jaap et al. 1994). The maximum hyperaemic response in the FH subjects correlated with calculated insulin sensitivity rather than ambient plasma glucose (Jaap et al. 1997).
1.5.3 Link between impaired vascular function and insulin resistance

Impaired microvascular vasodilatory capacity is already apparent at the pre-diabetic phase. In order to understand the significance of this, it is necessary to examine if this abnormality is present in all causes of insulin resistance or whether it is associated with the component features of the insulin resistance syndrome.

1.5.4 Microvascular function in other causes of insulin resistance

1.5.4.1 Acromegaly

Normoglycaemic and normotensive subjects with acromegaly have also been shown to have impaired maximum hyperaemic response to local heating. This impairment was found to correlate with fasting insulin levels (Jaap et al. 1993).

1.5.4.2 Polycystic ovary syndrome

The maximum hyperaemic response in women with polycystic ovaries (PCO) was similar to that seen in BMI-matched healthy controls (Liddell et al. 1998). However, the vasodilatory function in the PCO women but not the healthy controls was negatively correlated with BMI, waist circumference and waist-hip ratio (WHR). This suggests that the PCO women may be particularly susceptible to the effects of weight gain. This may explain the uncertainty that surrounds the vascular risk posed by this particular condition, with visceral obesity playing a critical role in determining the risk.
1.5.5 **Insulin resistance syndrome (metabolic syndrome)**

Syndrome X or the insulin resistance syndrome (IRS) was first described by Reaven in 1988. It describes a clustering of adverse metabolic features such as hypertension, dyslipidaemia, glucose intolerance and obesity, which predisposes the individual to increased cardiovascular risk and the risk of developing type 2 diabetes in the future (Reaven 1993). It has now become apparent that many of the individual features of the IRS are associated with endothelial dysfunction in the large blood vessels and also impairment in microvascular responses. Acute hyperglycaemia has been shown to attenuate the increase in forearm blood flow induced by brachial artery infusion of metacholine compared to the euglycaemic state (Williams et al. 1998). However a criticism of the study was that the subjects were studied in a post-absorptive state and moreover, there was no time-control included in the study protocol. The changes in the insulin levels concomitant with acute hyperglycaemia may also confound the interpretation of the study data. Akbari et al. also reported that an acute oral glucose load decreased skin red cell flux response following acetylcholine iontophoresis and also flow-related dilatation of the brachial artery (Akbari et al. 1998). Again, no time-control was used in Akbari’s study and in the assessment of brachial artery vasoactivity; the pneumatic cuff was placed proximal to the brachial artery. There has been concern that the proximal placement of the cuff may induce ischaemia of the brachial artery itself and systemic recirculation of ischaemic metabolites into the blood stream may affect the brachial artery diameter. A small study involving 6 healthy volunteers reported no differences in the flow-mediated vasodilatation between proximal and distal sites of occlusion of the artery (Uehata et al. 1997). However, a recent report involving 16 healthy volunteers revealed that the maximum brachial artery flow-mediated dilatation was greater following upper arm occlusion
compared to forearm occlusion and the time course of the change in brachial artery diameter were different for the two cuff positions (Berry et al. 2000). Subjects with essential hypertension have been shown to have reduced cutaneous and muscle blood flow response to acetylcholine (Rossi et al. 1997). There are also several reports suggesting that dyslipidaemia is also associated with endothelial dysfunction (Chowienczyk et al. 1992) (Tur et al. 1994) (Khan et al. 1999). Patients with untreated hypertriglyceridaemia were found to have reduced peak cutaneous blood flow to post-ischaemic hyperaemia compared to the patients on treatment for hypertriglyceridaemia and healthy controls (Tur et al. 1994). Patients with raised cholesterol and peripheral vascular disease have impaired skin blood flow responses to acetylcholine and sodium nitroprusside iontophoresis (Khan et al. 1999). The endothelium-independent (sodium nitroprusside) vasodilatory response improved significantly with lipid-lowering treatment and the endothelium-dependent response also appeared to alter with treatment but the improvement was not statistically significant.

This indicates that the abnormal vasodilatory capacity seen in pre-diabetes and insulin-resistant subjects may be related to extrinsic metabolic abnormalities. Alternatively, it may represent an impairment in endothelial function that precedes the early clinical features of the IRS. This could be further explored by studying two normoglycaemic populations who have been identified to be at an increased lifetime risk of developing type 2 diabetes – 1) women with a previous history of gestational diabetes mellitus (GDM) and 2) first-degree relatives of patients with type 2 diabetes.
1.5.6 Gestational Diabetes Mellitus

1.5.6.1 Conduit artery

Anastasiou and colleagues have shown that flow-related vasodilatation of the brachial artery were decreased in both obese and non-obese ex-GDM women compared to healthy controls (Anastasiou 1998). The obese ex-GDM subjects were notably older and had higher blood pressure compared to the non-obese ex-GDM and control women. GTN-induced vasodilatation was impaired only in the obese ex-GDM group compared to control subjects. The majority of the subjects have been studied 3-6 months post-delivery and it is unclear if the recent history of gestational diabetes may have influenced these findings. The authors argued that repeated studies performed a year later in some of the ex-GDM subjects have yielded similar flow-related and GTN vasodilation results.

1.5.6.2 Skin microcirculation

Such women have been shown to have an abnormal maximum vasodilatory response of the skin to local heating, in the absence of hypertension or dyslipidaemia (Liddell et al. 1998). They were found to have evidence of endothelial dysfunction in the skin microcirculation in one study (Hu et al. 1998) and normal endothelial function in another study (Hannemann et al. 2000). This discrepancy could be due to the different selection criteria applied to the two studies. In Hu’s study, the women with previous GDM had significantly higher BMI, WHR, heart rate, systolic and diastolic blood pressure than the healthy controls. There was also a higher incidence of family history of cardiovascular disease in the ex-GDM group. In Hannemann’s study, the ex-GDM subjects were matched for BMI, blood pressure, age, lipid profile, smoking history,
phase of menstrual cycle and the time elapsed since last pregnancy. Thus it is possible
that the positive findings by Hu et al. may be related to the subtle dysmetabolic
features present in their ex-GDM subjects rather than the risk conferred by a past
history of gestational diabetes mellitus.

1.5.6.3 Biochemical parameters

Ryan et al. studied a group of normal glucose-tolerant women with a past history of
GDM who were found to have higher plasma glucose fasting and at each time point of
the OGTT compared to the controls without a history of GDM (Ryan et al. 1995).
These ex-GDM women have been shown to display defects in both insulin secretion
and action, with reduced first-phase insulin release, insulin sensitivity index and
glucose disappearance rate.

1.5.7 First-degree relatives of subjects with type 2 diabetes

1.5.7.1 Conduit artery

Balletshofer et al. studied 53 normotensive first-degree relatives of subjects with type
2 diabetes (FDR), 10 control subjects with negative family history of diabetes and 25
type 2 diabetic subjects and reported no significant difference in flow-related
dilatation and GTN-induced vasodilatation between the FDR and control groups
(Balletshofer et al. 2000). However, when the FDR group was subdivided according
measures of insulin sensitivity, the flow-related dilatation was significantly impaired
in the insulin-resistant FDR group compared to insulin-sensitive FDR group. GTN-
induced vasodilatation was however not different between insulin-resistant and
insulin-sensitive FDR groups.
In a separate study, brachial artery flow-related dilatation was also reported to be impaired in normoglycaemic subjects with one or two parents with a history of type 2 diabetes (Caballero et al. 1999) but as mentioned previously, one criticism of this study was that the pneumatic cuff was placed proximal rather than distal to the brachial artery.

Lee et al. however did not show any abnormality in flow-related dilatation and GTN-induced vasodilatation of the brachial artery in normoglycaemic subjects with two parents with type 2 diabetes when compared to sex- and age-matched control subjects (Lee et al. 2000).

1.5.7.2 Skin microcirculation

Impaired endothelium-dependent and endothelium-independent vasodilatory function of the skin has been demonstrated in normoglycaemic subjects with one or two parents with a history of type 2 diabetes compared to healthy controls (Caballero et al. 1999). In contrast, Lee et al. who examined the maximum vasodilatory capacity of the skin microcirculation in normoglycaemic subjects with two parents with type 2 diabetes have failed to demonstrate any abnormality (Lee et al. 1998). The contrasting the findings between the two studies may be related to differences in methodology and cohort characterisation; for example, ethnic status, age.

1.5.7.3 Metabolic and biochemical abnormalities

Balletshofer and colleagues found that the FDR have higher 120-minute blood glucose and insulin levels in response to an OGTT as well as raised plasma cholesterol compared to their control subjects (Balletshofer et al. 2000).
In Cabarello’s study, systolic and diastolic blood pressure of the glucose tolerant FDR of diabetic subjects were found to be significantly raised compared to the controls, even though they were well within normotensive range (Caballero et al. 1999). Raised ET-1 and sVCAM were also noted in these subjects.

In Lee’s study looking at skin microvascular function in normal glucose-tolerant FDR of type 2 diabetic subjects, the FDR exhibited significantly higher 2 hr glucose levels and reduced beta-cell function compared to the control subjects. Although the FDR appeared to have lower insulin sensitivity than the control subjects, this did not reach statistical significance. The two groups of subjects were pair-matched for BMI, age and sex and there were no significant differences in fasting cholesterol or triglyceride levels. Systolic blood pressure was significantly raised in the FDR group but diastolic blood pressure was similar between the two groups. Minimum vascular resistance, which normalises the skin maximum hyperaemic response data for blood pressure, was found to correlate with the indices of the insulin resistance syndrome; PAI-1, t-PA, total cholesterol and triglyceride level and was inversely associated with calculated insulin sensitivity in the total study population (Lee et al. 2001).

Studies looking specifically at the biochemical and metabolic features of FDR of type 2 diabetic subjects have shown that such individuals are often more obese and have an increased prevalence of impaired glucose tolerance (Humphriss et al. 1997) (Forsblom et al. 1995). When glucose-tolerant first-degree relatives were pair-matched with control subjects for age, BMI and WHR to eliminate the influence of adiposity, the first-degree relatives were still found to be more insulin-resistant.
Thus in both ex-GDM and first-degree relatives of subjects with type 2 diabetes, there
are some evidence of impairment of vascular function, especially in those who are
prone to obesity and mildly raised blood pressure. Although the two populations do
not exhibit the full array of the component features of the IRS, it is apparent that
subtle metabolic/biochemical abnormalities are already present in these subjects.

1.5.8 Summary

Microvascular dysfunction and dysmetabolic features are evident in the pre-diabetic
state. Many component features of the metabolic syndrome are also associated with
the impairment of microvascular function. Some studies on normoglycaemic subjects
who are at risk of developing type 2 diabetes, such as women with a previous history
of gestational diabetes and offsprings of subjects with type 2 diabetes, also revealed
evidence of impaired vascular function. Although these subjects had normal glucose
tolerance, they were found to exhibit subtle biochemical and metabolic abnormalities.
Thus, to test the hypothesis that microvascular dysfunction is an intrinsically linked or
an antecedent feature of the IRS requires the identification of at-risk subjects earlier in
life before any clinical evidence of dysmetabolism ensues.
1.6.0 Fetal origin of adult diseases

1.6.1 Introduction

Epidemiological studies have shown that low birth weight or thinness at birth is associated with the increased risk of developing many of the component features of the insulin resistance syndrome in adult life and therefore at risk of developing type 2 diabetes (Barker et al. 1993). The cause of this link is still unclear and several mechanisms have been postulated.

1.6.2 Barker's hypothesis

Barker and colleagues first demonstrated the association between anthropometric measures at birth and the increased risk of adult disease in Hertfordshire men. This relationship has subsequently been replicated in other studies in both men and women in different populations (Barker et al. 1989) (Lithell et al. 1996) (Valdez et al. 1994). Barker proposed that fetal malnutrition in-utero leads to reduced fetal growth and permanent programming of the structure and function of certain vital organs and tissues like the pancreatic β cell mass, liver, vasculature, which leads to the increased risk of adult disease (Barker 1998). Evidence to support this concept comes from animal studies where offsprings of rats subjected to protein deprivation during pregnancy were found to develop raised systolic pressure and glucose intolerance in adult life (Langley et al. 1994). However, equivalent human data may not be entirely convincing. A study of individuals born during the time of the Dutch famine (1944-5) and thus subjected to prenatal exposure to the famine had impaired glucose tolerance (Ravelli et al. 1998). However, the Leningrad siege (Stanner et al. 1997) and the Finland famine (1866-68) studies (Kannisto et al. 1997) showed no evidence that
malnutrition in-utero increases the risk of adult disease despite near starvation. It is thus difficult to entertain the possibility that maternal malnutrition is a common cause of adult disease in the Western world.

1.6.3 Fetal glucocorticoid exposure

Seckl and colleagues proposed that excessive exposure to glucocorticoids during early fetal development results in the development of common disorders in adult life (Edwards et al. 1993). This hypothesis was supported by evidence in rat experiments that a decreased activity of 11-β hydroxysteroid dehydrogenase, was associated with low birth weight. The enzyme acts as a placental barrier to maternal glucocorticoids in the fetus by converting maternal cortisol to inactive cortisone. Low placental levels of this hormone can be congenital or acquired but little is known about its regulation (Benediktsson et al. 1993). In another set of experiments where low-dose dexamethasone was administered to pregnant rats, the offsprings were found to have low birth weight and exhibited subsequent hypertension (Tonolo et al. 1988). Similarly in humans, it has been noted that mothers in whom glucocorticoids were administered for medical reasons tended to produce babies with reduced birth weight. In Hertfordshire men born between 1920-30, fasting cortisol level was found to have an inverse and graded relationship with birth weight and also correlated with features of the insulin resistance syndrome (Phillips et al. 1998)

Thus it is speculated that glucocorticoid exposure at critical periods of growth can exert organisational effects or imprint responses that persist throughout life. Steroids have been shown to cause irreversible programming of microsomal and soluble rat liver enzymes. High concentrations of glucocorticoids in the fetus could also affect the development of fetal vasculature and its responses to pressor agents, since
glucocorticoids regulate vascular tone by many mechanisms (Walker et al. 1992). Glucocorticoids could also affect brain development and this may possibly affect central control over the cardiovascular system growth and maturation, leading to altered responses to vasoactive stimuli and hypertension.

1.6.4 Fetal insulin hypothesis

Hattersley and colleagues have proposed an alternative hypothesis that the low birth weight infant and the insulin-resistant adult at risk of type 2 diabetes are two phenotypes of the same insulin-resistant genotype (Hattersley et al. 1999). Fetal insulin sensitivity determines how the fetus grows but the fetus produces insulin in response to maternal rather than fetal glucose. Thus in the presence of maternal normoglycaemia, the insulin resistant fetus will not be able to produce sufficient insulin to overcome its insulin insensitivity, resulting in limited growth. However, following birth, the neonate will feed back on its own blood glucose, resulting in increasing insulin level and catch-up growth and possibly an increased likelihood of developing visceral adiposity. This fetal insulin hypothesis is supported by evidence that the inheritance of the glucokinase gene mutation, which results in a defect in glucose sensing, can lead to reduced fetal growth and hyperglycaemia (Hattersley et al. 1998).

An extension of this hypothesis suggests that the same genes that affect insulin resistance may also influence vascular endothelial function. Vascular growth in-utero may be impaired in the insulin-resistant fetus resulting in reduced capillary density. When the insulin resistant infant develops compensatory hyperinsulinaemia after birth, different signal transduction pathways may be affected differently. In animals, it has been shown that insulin-induced generation of nitric oxide by insulin receptor
substrate 2 and phosphatidylinositol 3 kinase is adversely affected by insulin resistance but the mitogenic effects of insulin is maintained (Jiang et al. 1997). If a similar process occurs in human, this may lead to hypertrophy of the vascular smooth muscle, thereby increasing peripheral resistance. This coupled with capillary rarefaction will predispose to subsequent hypertension. In support of this concept, offsprings of two hypertensive parents have been noted to have reduced skin capillary density and microvascular vasodilatory reserve compared to others with comparable blood pressure but born of parents without hypertension (Noon et al. 1997).

1.6.5 Summary

Various retrospective studies have linked low birth weight with the increased risk of developing the metabolic syndrome, type 2 diabetes and cardiovascular diseases in adult life. Several mechanisms have been proposed to explain this association, namely intra-uterine malnutrition, excess fetal glucocorticoid exposure and the inheritance of the insulin-resistant genotype resulting in a low birth weight infant and an insulin-resistant adult. It is likely that a combination of genetic and environmental factors (Groop et al. 2001) play a role in the pathophysiology of type 2 diabetes and the associated cardiovascular risk.
1.7.0 Lipid and lipid metabolism

1.7.1 Introduction

Having considered the evidence supporting the hypothesis of intrinsic microvascular dysfunction in type 2 diabetes, the focus of this section will be centred on an extrinsic factor that may influence the microcirculation in the insulin-resistant state and type 2 diabetes. The extrinsic factor to be explored will be dyslipidaemia, in particular postprandial dyslipidaemia. The scope of this section will include a brief introduction to the nomenclature, structure and metabolic pathways of the different lipid and lipoprotein particles. The link between dyslipidaemia and atherosclerosis will be explored and the evidence that postprandial dyslipidaemia may be an important factor in cardiovascular risk will also be considered. The influence of insulin resistance and the diabetic state on lipid metabolism will also be discussed and how this may be linked to endothelial dysfunction in type 2 diabetes.

1.7.2 Plasma lipids

Cholesterol is an important constituent of plasma membranes and also a precursor for the synthesis of steroid hormones and bile acids. It is obtained from diet or synthesised de novo in the liver, catalysed by the hydroxymethylglutaryl coenzyme A (HMG Co A) reductase.

Triglycerides consist of a glycerol backbone with three hydroxyl groups esterified with fatty acids and are a major energy store (fig 1.4). The free fatty acids may be released by hydrolysis, catalysed by lipases.
Phospholipids are similar in structure to the triglycerides except that phosphoric acid is esterified to glycerol at the 3-position. They have an important role in the structure of cell membranes and lipoproteins.

1.7.3 Lipoproteins

1.7.3.1 Structure and nomenclature

Cholesterol ester and triglyceride are insoluble in water and are therefore transported in the form of lipoproteins. A lipoprotein consists of a hydrophobic core of insoluble lipid, enclosed by a surface coat of polar compounds, phospholipid, free cholesterol and apoproteins, which solubilise the lipid for transport in plasma. The apoproteins have an important function in lipoprotein formation, stabilisation and metabolism and also act as ligands for lipoprotein receptors. The family of lipoproteins differs in size, content and metabolic function. The lipoproteins are named according to their separation density by ultracentrifugation.

Chylomicrons

Chylomicrons are secreted in the intestine and are rich in triglycerides absorbed from the intestine.

Very Low Density Lipoprotein (VLDL)

VLDL particles are secreted by the liver and are rich in triglycerides.
Low Density Lipoprotein (LDL)

LDL particles are formed mainly from VLDL and approximately 60% of which are returned to the liver. Modified LDL can bind to receptors on the arterial wall. LDL inhibits LDL de novo synthesis, LDL receptor synthesis and stimulates esterification of cholesterol.

High Density Lipoprotein (HDL)

HDL particles are produced by the liver and intestine. They take up free cholesterol from the periphery, esterify cholesterol and return cholesterol to the liver. Esterified cholesterol can also be transferred to other lipoproteins. HDL is linked with the metabolism of triglyceride-rich lipoproteins (TRL) and also acts as a reservoir of apoproteins.

1.7.3.2 Metabolism

1.7.3.2.1 Exogenous lipoprotein pathway

Dietary lipids are absorbed into the small intestinal mucosa as fatty acids and cholesterol, where they are re-esterified to triglyceride and cholesterol ester and incorporated into the lipid core of chylomicrons. ApoB48 is required for chylomicron secretion from the intestinal cell. On entry into the circulation via mesenteric lymphatics and the thoracic duct, chylomicrons acquire apoC and apoE from HDL. ApoC-II is an activator of lipoprotein lipase, located on the endothelium of fat and muscle, which serves to hydrolyse the triglycerides of the chylomicrons. Non-esterified fatty acids and glycerol may then be used as fuel and the fatty acids can be re-esterified to triglyceride for storage in adipose tissue. The surface components of
chylomicrons after hydrolysis are shed and transferred to HDL and the resulting triglyceride-depleted chylomicron remnants are then taken up by the liver (fig 1.5).

1.7.3.2.2 Endogenous lipoprotein pathway

Hepatic triglyceride and cholesterol are secreted in VLDL. ApoB100 is the major VLDL protein and the VLDL particles derive apoC and apoE from HDL particles. VLDL triglyceride is hydrolysed by lipoprotein lipase and the surface components are transferred to HDL. The resulting VLDL remnants may be directly removed by the liver or processed into intermediate density lipoprotein (IDL) and LDL with gradual triglyceride depletion. The LDL is the major circulating cholesterol-rich lipoprotein. The half-time and the concentration of LDL in the plasma depend on its rate of uptake and catabolism via LDL receptors (fig 1.5).

1.7.3.2.3 Reverse cholesterol pathway

HDL particles are synthesised by the liver and accept free cholesterol from peripheral cells or other lipoproteins, which is esterfied and forms a lipid droplet in the core of the particle. This is catalysed by lecithin cholesterol acyl transferase (LCAT). The cholesterol ester can be transferred to particles of lower density via cholesterol-ester transfer protein (CETP), in exchange for triglyceride. Cholesterol esterification and lipid exchange are linked with the metabolism of triglyceride-rich lipoproteins (TRL). HDL particles accept surface components from TRL and form larger, less dense HDL particles, HDL\(_2\). Under some conditions, HDL\(_2\) may be hydrolysed by hepatic lipase to form smaller, dense HDL\(_3\). HDL has an important role in returning free cholesterol from peripheral cells to the liver (fig 1.6).
1.7.4 Lipids, lipoproteins and atherosclerosis

1.7.4.1 The response-to-injury hypothesis

According to the response-to-injury hypothesis, an injurious insult to the endothelium, such as the presence of ox-LDL, can lead to the adherence of monocytes and lymphocytes to the endothelium. These cells subsequently migrate and then reside in the subendothelial space. The macrophages form foam cells due to lipid accumulation and together with lymphocytes and smooth muscle cells, eventually form a fatty streak. The fatty streak progresses to become a fibro-fatty streak and then a fibrous plaque. In areas of the blood vessels where flow is turbulent, such as the bifurcation of arteries or post-stenosis, the formation of platelet mural thrombi may occur. The release of cytokines and growth factors from the cells within the thrombus can lead to further progression of the lesion, forming an advanced lesion. Lesion regression can potentially occur if the injurious agent is removed or protective substances are administered to reduce the inflammatory process (Ross 1993).

1.7.4.2 Cholesterol and LDL

Many epidemiological studies have demonstrated an association between plasma cholesterol and atherosclerosis. Observations from the Multiple Risk Factor Intervention Trial (MRFIT) have shown that the relationship between coronary heart disease (CHD) risk and serum cholesterol is continuous, graded and strong across the age range and independent of smoking and hypertension (Martin et al. 1986).

LDL largely determines the link between plasma cholesterol and atherosclerosis. The formation of a foam cell from the interaction between LDL and monocytes is an early lesion in atherosclerosis. LDL is internalised by scavenger receptors after chemical
modification, leading to intracellular cholesterol accumulation. Oxidatively modified LDL can also stimulate monocyte adhesion and chemostasis, further contributing to atherogenesis (Goldstein et al. 1979).

Primary and secondary prevention trials of cholesterol-lowering have also demonstrated a reduction in overall mortality and cardiovascular events using HMG-CoA reductase inhibitors (Shepherd et al. 1995) (Pedersen et al. 1998).

1.7.4.3 HDL

HDL cholesterol is inversely related to CHD risk, that is a low HDL level is a cardiovascular risk factor. A total cholesterol/HDL cholesterol ratio predicts vascular risk in both sexes (Gordon et al. 1989).

The mechanism in which HDL may protect against atherosclerosis is not clear. It may be related to its role in reverse cholesterol transport. Alternatively, low HDL levels may not be the primary factor in the pathogenesis of atherosclerosis but rather, a reflection of impaired TRL clearance.

1.7.4.4 Triglycerides

The relationship between triglycerides and CHD risk is controversial. Some studies have implicated plasma triglycerides as an independent cardiovascular risk factor while others have not (NIH Consensus Development Panel on Triglyceride 1993; Jeppesen et al. 1998) (Haim et al. 1999).

There is still much to be learnt about each class of TRL and its potential atherogenicity and also their impact on the metabolism of other lipoprotein moieties and haemostatic factors. There is some evidence to suggest that TRL and their remnant particles accumulate in atherosclerotic plaques (VanLenten et al. 1985). High
triglyceride levels may also alter the metabolism of other lipoprotein fractions and hypertriglyceridaemia has been associated with a preponderance of small dense, highly atherogenic LDL particles. The small LDL particles formed are more susceptible to oxidation and also have a high affinity for LDL receptors on the endothelium.

High plasma triglycerides are also associated with increased levels of factor VII and PAI-1 (plasminogen activator inhibitor-1), which have been linked with increased CHD risk (Meade et al. 1986). Thus it is possible that triglycerides are associated with CHD risk through the effects on coagulation factors and fibrinolysis.

1.7.4.5 Lipoprotein (a)

Lipoprotein (a) (Lp(a)) consists of LDL attached via a disulphide bond to an additional apoprotein, apo(a). Lp(a) concentrations vary immensely within and between populations but remain fairly stable throughout life in a given individual. In the European population, levels are generally low with a pronounced right shift to the distribution, while other races have higher median values. 40-90% of the variability in Lp(a) can be explained by the apo(a) gene locus (Boerwinkle et al. 1992).

Lp(a) concentration is positively linked with CHD risk such that the levels in those with coronary atheroma are increased approximately twofold compared to healthy subjects (Armstrong et al. 1986). Indeed Lp(a) has also been demonstrated to be present in atheromatous lesions. Furthermore, the link between Lp(a) and CHD risk is further strengthened in the presence of high LDL cholesterol concentration.
1.7.5 Lipids, lipoproteins and endothelial function

Endothelial cells are continuously exposed to circulating lipids and a link between lipoprotein classes and atherosclerosis is widely established. Studies examining the interaction between lipoprotein particles and the endothelium are therefore particularly relevant as an alteration in endothelial function is seen before the development of atheroma.

Many animal and human studies have demonstrated that raised plasma lipid levels are associated with a reduction in vasodilatory responses in coronary, peripheral conduit and resistance vessels, in response to endothelial NO production (Seiler et al. 1993) (Celermajer et al. 1992) (Chowienczyk et al. 1992) and both LDL and TRL are linked with impaired NO-dependent vasodilatation. However, there have also been some studies which have not demonstrated an association between lipids and endothelial function (Schnell et al. 1999) (Meeking et al. 1999) (McVeigh et al. 1992). The state of oxidisation of the lipid moieties appears to be crucial to their effects on the endothelium (Vakkilainen et al. 2000). Ox-LDL appears to be taken up by endothelial cells in preference to native LDL. It is thought that ox-LDL may reduce endothelium-dependent vasodilatation by interfering with the uptake of L-arginine, receptor-G protein coupling process, NOS protein levels and NO bioavailability. Literature regarding the possible mechanisms of action of other lipoprotein classes on the endothelial vasodilatory function is however limited. Reduction in plasma lipid levels, the use of anti-oxidants and also NOS substrate have been shown to improve the vasodilatory responses (O'Driscoll et al. 1997) (Plotnick et al. 1997) (Creagher et al. 1992) but the underlying mechanisms for these effects are unclear.

The adhesion of leukocytes to the endothelium is an important step in the early stages of atherogenesis, which is dependent on the expression of adhesion molecules on cell
surfaces. Lipoprotein particles such as ox-LDL and TRL can stimulate the expression of such molecules while HDL and apo A1 inhibit the cytokine-dependent expressions of adhesion molecules (Dart et al. 1999) (Khan et al. 1995) (Endemann et al. 1987) (Maier et al. 1994). These lipoprotein effects may be mediated via message transcription and cell signalling pathways (Lin et al. 1996) (Vora et al. 1997).

There is also evidence indicating that lipoproteins may interfere with the production of coagulation factors, creating an environment favouring thrombosis and interfering with fibrinolysis. Cell culture studies have shown that copper-oxidised LDL particles reduce tissue plasminogen activator (tPA) and increase PAI-1 release from macrovascular cells and VLDL has also been shown to stimulate the release of PAI-1 (Eriksson et al. 1998). In the EURODIAB study, von Willebrand factor (vWF) was found to correlate with the plasma cholesterol and triglyceride in men (Greaves et al. 1997).

### 1.7.6 Postprandial lipid metabolism

Another important aspect of lipid metabolism that has attracted much interest is the postprandial handling of lipoprotein fractions. The postprandial period is broadly defined as the period of time following ingestion of a meal, the duration of which depends on the composition of the meal. Postprandial changes in lipid profile are dependent on various factors such as sex, age, nutrition, genetic predisposition and lifestyle factors such as exercise and smoking status (Taskinen 1995) (Bergeron et al. 1997) (Couillard et al. 1999).

Recent evidence indicated that postprandial abnormalities of lipid metabolism are associated with increased cardiovascular risk. Patients with coronary artery disease were found to have higher levels of plasma triglycerides following a fatty meal
compared to healthy controls (Simpson et al. 1990). Vogel et al. demonstrated that a high-fat meal impairs flow-related vasodilatation of the brachial artery compared to a low-fat meal and the impairment correlated with the postprandial rise in triglyceride levels (Vogel et al. 1997). It is however not known if postprandial triglycerides or lipoproteins (e.g. chylomicrons) are directly implicated in endothelial dysfunction (Karpe 1997) or whether they exert their pro-atherogenic effects via modulation of LDL metabolism (Griffin 1999). Alternatively, increased oxidative stress following a meal has also been postulated to be responsible for the postprandial endothelial dysfunction (Plotnick et al. 1997).

However, there were other studies on the effect of a fatty meal on vascular function, which reported different findings. Djousse et al. reported no change in flow-related and GTN-induced vasodilatation with a high-fat meal even though a rise in triglyceride was noted following the fatty meal (Djousse et al. 1999). Williams et al suggested that flow-related vasodilatory response was only impaired when used cooking fat was added to the meal rather than unused fat, suggesting that hydrogenated fat is detrimental to endothelial function rather than the total fat content of the meal per se or the magnitude of the triglyceride rise with the meal (Williams et al. 1999). Gudmundsson et al. also did not report any impairment in endothelium-dependent and endothelium-independent forearm resistance vessel function following a high-fat meal (Gudmusson et al. 2000). Raitakari et al. in contrast found an increase in brachial artery diameter, resting forearm blood flow and post-ischaemic forearm blood flow following consumption of a meal high in saturated fat or high in monosaturated fat (Raitakari et al. 2000). However, neither a time-control nor a control meal was used in this study. The order of the meal was also not randomised.
although assessors of the brachial artery diameters were reportedly blinded to the meal status of the subjects.

1.7.7 Lipid metabolism in type 2 diabetes

Lipid and lipoprotein disturbances are more common in type 2 diabetes compared to type 1 disease. In type 1 diabetes, the presence of dyslipidaemia is associated with poor glycaemic control, advancing age and the presence of nephropathy while in type 2 disease, characteristic dyslipidaemia may be already be present at diagnosis (Betteridge 1997).

The characteristic abnormality in type 2 diabetes is the presence of hypertriglyceridaemia, associated with low HDL level and the preponderance of small, dense LDL particles. These abnormalities are observed even in the presence of good glycaemic control.

1.7.8 The role of insulin resistance in lipid metabolism

Certain risk factors associated with atherosclerosis are much more likely to occur in type 2 diabetes, including insulin resistance. Lipid and lipoprotein abnormalities in type 2 diabetes are closely linked with insulin resistance and hyperinsulinaemia (Garg 1996) (Howard 1999). Kinetic studies have revealed that overproduction of VLDL particles is observed in states of insulin resistance (Grundy et al. 1979). This is primarily due to an increase in liver VLDL triglyceride synthesis. Reduced insulin effect in the insulin-resistant state may lead to increased adipose lipolysis and increased flux of free fatty acids to the liver and may therefore enhance hepatic triglyceride synthesis. Lipoprotein lipase is an insulin-sensitive enzyme and may demonstrate reduced activity in the presence of tissue insulin resistance (Eckel et al.
1987), therefore reducing VLDL and chylomicron breakdown and accentuating hypertriglyceridaemia. Hepatic lipase activity is upregulated in the insulin-resistant state, which may lead to reduced HDL levels and reduced ratio of HDL$_2$ to HDL$_3$ cholesterol (Baynes et al. 1991). The preponderance of small, dense LDL in insulin-resistant state appears to be linked with hypertriglyceridaemia. The abundance of large triglyceride-rich VLDL particles leads to the formation of large LDL particles through the activity of CETP. The large LDL particles are then broken down by hepatic lipase to form small, dense LDL.

1.7.9 Insulin resistance and postprandial dyslipidaemia

Subjects with type 2 diabetes are also particularly susceptible to postprandial dyslipidaemia. The major abnormality seen is the large rise in triglyceride and TRL post-meal. Such exaggerated triglyceride responses have also been described in first-degree relatives of type 2 diabetic patients (Axelsen et al. 1999) and viscerally obese subjects (Guerci et al. 2000). Interestingly, Schrenzenmeir et al. reported that there is a bimodal distribution in peak postprandial triglyceride response in the population and that those with high triglyceride responses also have higher fasting insulin levels (Schrezenmeir et al. 1993). Thus it would suggest that there is a link between the postprandial handling of a fat load and insulin resistance.

Several studies have shown that both endogenous and exogenous triglyceride contribute to postprandial dyslipidaemia, with endogenous lipaemia accounting quantitatively for the majority of the postprandial increase (Coppack 1997). Physiological mechanisms underlying postprandial dyslipidaemia in type 2 diabetes are not fully understood but are thought to be due to either an excessive production of VLDL, a defective clearance in TRL or a combination of both defects. In healthy
subjects, insulin released postprandially inhibits the synthesis of VLDL particles in the liver and also suppresses the release of non-esterified fatty acids (NEFA) from the adipose stores. In the insulin-resistant state, postprandial insulin release does not adequately suppress VLDL synthesis or the increase of NEFA substrate delivered to the liver. Defective clearance of TRL has been suggested to contribute to the postprandial dyslipidaemia. Mechanisms proposed include a decrease in lipoprotein lipase activity, defect in receptor-mediated clearance of TRL, which may give rise to hypertriglyceridaemia postprandially. A change in the composition of TRL particles may also affect the susceptibility of the particles to enzymic breakdown or an alteration in HDL particle composition may affect its ability to accept triglycerides from TRL (Garg 1996) (table 1.3).

1.7.10 Lipids and lipoproteins and cardiovascular risk in type 2 diabetes

Several studies have shown that coronary artery disease mortality is 2-4 times greater in patients with diabetes than those without (Kleinman et al. 1988) (Schemthaner 1996). This increase is seen in both type 1 and type 2 diabetes but the vast majority of coronary artery disease is seen in type 2 disease, which is common and occurs later in life. The increase in atherosclerotic disease in diabetes is also seen in peripheral vascular and cerebrovascular beds, resulting in major morbidity and mortality.

Coronary artery disease mortality is related to the cholesterol level in the diabetic population, similar to the non-diabetic population. However, the absolute risk for any given cholesterol level is increased four-fold compared to the non-diabetic population (Steiner 1997). This would suggest that factors other than cholesterol contribute to this increased cardiovascular risk or for a given amount of LDL-cholesterol, the LDL particles in diabetic subjects have more atherogenic properties.
In type 2 diabetes, angiographic evidence of the severity of coronary artery disease correlated positively with the level of TRL (Mero et al. 2000). However, it is unclear if the atherogenic potential of hypertriglyceridaemia may be mediated via TRL remnant particles, which have been demonstrated in atherosclerotic lesions (VanLenten et al. 1985). Alternatively, the cardiovascular risk of hypertriglyceridaemia may be related to a low HDL, which has a well-recognised inverse relationship with triglycerides. There is also a preponderance of small dense LDL particles in type 2 diabetic subjects, which has been found to be more atherogenic than larger and lighter LDL particles. Small dense LDL particles are more prone to oxidation, which increases its atherogenic potential.

1.7.11 Lipids, lipoproteins and endothelial function in type 2 diabetes

There are various studies that have illustrated that impaired vascular function in type 2 diabetes is associated with dyslipidaemia. Impaired endothelium-dependent vasodilatory function of the brachial artery was associated with the rate of oxidation and the concentration of LDL-III, which is the small, dense LDL subfraction, while impaired endothelium-independent function was linked to the concentration of LDL-III and conjugated dienes, which was used in the respective study as a measure of the LDL susceptibility to oxidation (Tan et al. 1999). Makimattila and colleagues have also shown that impaired acetylcholine-induced forearm blood flow in type 2 diabetes correlated with LDL size. O'Brien et al. similarly found that impairment of forearm blood flow correlated with LDL particle size and HDL-cholesterol level also predicted the reduction of forearm blood flow responses to acetylcholine challenge in type 2 disease (O'Brien et al. 1997). There is also evidence that conduit artery vasoactivity is reduced following consumption of a high-fat meal in patients with type
2 diabetes (Evans et al. 2000). In contrast, McVeigh et al. did not report any significant correlation between LDL-cholesterol with the impairment of forearm blood flow response to acetylcholine or GTN in type 2 diabetic subjects (McVeigh et al. 1992).

Type 2 diabetes is also associated with abnormal endothelial activation and hypercoagulability. Elevated levels of fibrinogen, fibrin monomers, thrombin-antithrombin III complex, factor VIIIc and factor VII have been reported in patients with type 2 disease and serum cholesterol has been found to correlate with the concentration of factor VII and fibrin monomers (Donders et al. 1993). Drug treatment for hyperlipidaemia has also been associated with some improvement in anti-coagulant activity, indicating that dyslipidaemia in type 2 disease may mediate its cardiovascular effects via interference with the production and activity of the clotting and fibrinolytic factors (Tan et al. 1999).

1.7.12 Summary

Metabolism of the different classes of lipoprotein particles is inter-related. Different lipoproteins, in particular LDL particles, have all been implicated in atherogenesis. Various mechanisms have been proposed to explain the changes in endothelial vasodilatory function and the thrombogenic potential in association with dyslipidaemia. The role of postprandial lipid metabolism in endothelial dysfunction and cardiovascular disease has also received growing attention.

Lipid and lipoprotein abnormalities are common in type 2 diabetes and are closely linked with insulin resistance. The characteristic abnormality is hypertriglyceridaemia, reduced HDL level and a preponderance of small, dense LDL particles and also a susceptibility to display postprandial dyslipidaemia. Increased
cardiovascular risk in type 2 disease is related to lipid abnormalities – LDL, TRL and low HDL have all been implicated. Endothelial dysfunction in diabetic subjects without cardiovascular disease has also been linked with characteristic dyslipidaemia. The evidence reviewed in this section with regards to lipids and endothelial function pertains mainly to the conduit and resistance vessels, as there are few studies in the literature concerned with dyslipidaemia and the microcirculation.
Fig 1.4 The chemical structure of triglycerides.

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&\quad | \\
&\quad | \\
&\text{O} \quad \text{CH}_2\text{-O-C-R}_1 \\
&\quad \| \\
&\quad | \\
&\quad | \\
&\quad | \\
&\text{R}_2\text{-C-O-CH} \\
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&\quad | \\
&\quad | \\
&\quad | \\
&\text{CH}_2\text{-O-C-R}_3 \\
&\quad \| \\
&\quad | \\
&\quad | \\
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&\text{O} 
\end{align*}
\]
Fig 1.4 Diagram illustrating the endogenous and exogenous pathways of lipoprotein metabolism.

INTESTINE

- Chylomicron
- Lipoprotein lipase
- Chylomicron remnant

LIVER

- NEFA & glycerol

VLDL

- NEFA & glycerol
- Lipoprotein lipase

IDL

- Lipoprotein lipase
- NEFA & glycerol

LDL

- Other tissues

ARTERIAL WALL
Fig 1.5 Diagram illustrating the role of HDL and reverse cholesterol transport.
Table 1.3 Summary of potential defects in postprandial lipid metabolism in type 2 diabetes.

<table>
<thead>
<tr>
<th>1</th>
<th>Failure to suppress postprandial VLDL production due to a combination of insulin resistance in the liver and increased fatty acid substrates from the periphery.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Defective postprandial clearance of TRL.</td>
</tr>
<tr>
<td></td>
<td>– rise in postprandial chylomicron and VLDL and their remnants and hence competition for lipoprotein lipase action</td>
</tr>
<tr>
<td></td>
<td>- ? defect in lipoprotein lipase in insulin-resistant state</td>
</tr>
<tr>
<td></td>
<td>- ? defect in receptor-mediated clearance in remnant particles</td>
</tr>
<tr>
<td>3</td>
<td>Acceleration of neutral lipid exchange reactions due to increased residence time of TRL – promotes proatherogenic changes in LDL (small, dense LDL particles) and HDL metabolism.</td>
</tr>
</tbody>
</table>
2.0 Methods

2.1.0 Introduction

There are inherent difficulties in studying the microcirculation in man due to the inaccessibility and sensitivity of the systems involved. However, peripheral tissues such as the skin possess the advantage of accessibility and techniques have been developed in the recent years to investigate and to improve understanding of the skin microcirculation. This chapter describes the methods that have been used to study the skin microcirculation in this thesis.

2.1.1 Reproducibility, within-subject and between-subject variability

Reproducibility or reliability of a measurement refers to the degree of stability when a measurement is repeated under identical condition. Repeated measurements made on an individual are likely to vary. This is within-subject or intra-subject variability and a standard deviation can be calculated from these observations. The within-subject coefficient of variation (cv) (the intra-subject standard deviation divided by the mean, expressed as a percentage) can be used to assess the reproducibility of the measurement. Measurements made on different subjects vary according to between-subject or inter-subject variability. If many observations were made on each individual, and the average (mean) is taken, the intra-subject variability is assumed to be 'averaged out' and the variation in the average values (standard deviation, SD) is mainly due to the inter-subject variability. Knowing the inter-subject variability of the study population and the required difference (the smallest difference that is considered to be clinically important and biologically plausible) permits the
estimation of the study sample size that has the power to detect a clinically significant result (usually at 5% level of significance)

The techniques described in this thesis have been assessed for reliability with regards to the equipment and the study protocol in the Department of Vascular Medicine. Measurements collected from the study of subjects on at least two separate occasions have yielded data for the calculation of intra-subject and inter-subject variability, intra-observer and inter-observer variability and also intra-instrument and inter-instrument variability (when more than one set of equipment is available for the same experimental technique). These data are used as the institutional standard and also facilitates power calculation and the estimation of study sample size. The description for each of the experimental technique described in this chapter will include data from my own (intra-subject) reproducibility studies as well as the departmental reproducibility data. In the chapters to follow, the inter-subject variability of the study population and the power of the study will be stated where appropriate. The microvascular techniques for all the studies in this thesis were performed by a single observer and on the same set of equipment throughout, thus avoiding variability arising from inter-observer and inter-instrument factor.

2.2.0 Laser Doppler techniques

2.2.1.0 Laser Doppler fluximetry

Non-invasive optical methods have been used to study the microcirculation for many years and laser Doppler fluximetry is one such technique. The introduction of this technique has enabled the monitoring of relative blood flow rate at a tissue surface
without disturbance of the perfusion steady state in both experimental and clinical settings.

2.2.1.1 Equipment
The Periflux Pf2B laser Doppler fluximetry (LDF) (Perimed, Stockholm, Sweden) has been used to measure the skin microvascular red cell flux in some of the experiments reported in this thesis. This equipment consists of a main unit, fibre-optic cable, a probe and a chart recorder. The main unit houses the Class 2 2mW Helium-Neon laser (wavelength 632.8nm). The fibre-optic cable houses the afferent and efferent fibres carrying the light to the probe. The chart recorder records the output signal from the main unit (fig 2.1).

2.2.1.2 Operating principle
LDF measures microvascular blood flow through tissue utilising Doppler shift, that is, the frequency change that occurs when radiation of a wave nature, such as light, is reflected by moving objects, such as red blood cells. All wave motion is governed by this equation:

\[ v = f \times \lambda, \]

where \( v \) = propagation velocity

\( f \) = wave frequency and

\( \lambda \) = wavelength.

Therefore the wavelength will change when the frequency changes and this results in a change in the quality of the radiation, for example the colour of light or the pitch of sound.
The laser light from the main unit is transmitted to the skin surface via the afferent fibre in the fibre-optic cable to the probe head. The laser beam impinging on the tissue is scattered both by static structures and moving red cells. The light scattered by the moving cells undergoes a frequency change according to the Doppler effect while the backscattered light from the static structures remains unshifted in frequency.

A proportion of the frequency-broadened light is received by the efferent fibres, which are arranged in parallel to the afferent fibre in the fibre-optic cable. The dual efferent light guides are used to produce a cell-motion correlated Doppler signal and to reduce the noise resulting from variations in the light signal and other sources. The signal is then received by the photo-detectors and converted to an electrical signal, which is then processed by the lineariser, so that the resulting signal is directly proportional to the quantity of tissue blood flow (the velocity and the number of red cells). This output signal is then represented on a chart recorder in arbitrary units of volts (Almond 1994).

2.2.1.3 Nature of the measured quantities

The LDF has a measuring volume with a semisphere of 1-1.5mm radius, with its centre at the tissue surface under the end points of the optical fibres. It can perceive signals from a tissue depth of 1-1.5mm. Thus the signal reflects blood flow from a variety of capillaries, arterioles and venules and constitutes a mean value of blood cell motion in many small vessels.

2.2.1.4 Precautions to observe

The skin microcirculation is known to display large periodic oscillations in tissue perfusion within tissue regions of the order of 1 mm³. This is probably related to the
vasomotor activity of the precapillary vessels of the vascular bed. Thus to overcome this problem of temporal fluctuation in blood flow, the data should be recorded over sufficiently long time periods and the average over the time period should be taken as the representative reading. In order to overcome the spatial variation in blood flow, the average reading of measurements over several independent sites should be taken. Measuring relative changes in blood flow in response to a specific provocation would result in more reproducible readings as the LDF is designed to measure relative rather than absolute blood flow. In addition, blood flow also fluctuates in response to metabolic and thermoregulatory demands. Thus to compare skin perfusion levels in a series of subjects would require standardising the environmental conditions, physical and mental stress.

The LDF employs flexible optical fibres to transmit signals and is therefore prone to movement artefact, which would superimpose on the flux signal. This could be eliminated by attachment of the probe to the skin surface using a probe holder and double adhesive tape. The fibre-optic cable should also be fixed to rigid structures and the subject has to remain stationary during the measurement. It is also important that the probe should also be applied in such a manner so as to avoid compression of the vessels, which would give rise to an artificially low reading.

2.2.1.5 Instrument zeroing

Prior to measuring the blood flow, the instrument needs to be zeroed by directing the laser light at a stationary target with a reflectivity similar to the skin. The delrin disk, which is a semi-transparent polyacetal disk, is used for this purpose. The laser probe is placed in a hole in the delrin disc and the chart recorder pen is adjusted to read 0
volt on the chart paper and then the maximum deflection to read 10 volt on the chart paper.

2.2.1.6 Biological zero

As the LDF measures the movement relative to the instrument’s probe head, the predominant contribution comes from the red cells. There is also contribution from leucocytes and thrombocytes but they do not disturb the reading because they form part of the blood flow. However, the signal also encompasses non-blood flow related components from the movements of muscle cells, vessel wall, which are insignificant as a rule. During a complete arterial occlusion, these interstitial movements and also the Brownian motion of the red cells, can produce a small Doppler signal termed the ‘biological zero’. In principle, LDF output should be measured relative to this ‘biological zero’ as this may vary in different skin sites. However, arterial occlusion is impossible to achieve in areas other than the limbs. In addition, post-occlusion hyperaemia may also be an unacceptable disturbance to the measurements.

2.2.1.7 Calibration

The LDF lacks a universal ‘in vivo’ calibration standard, since the calibration will be affected by uncontrollable factors like skin epidermal thickness, pigmentation, which will interfere with light penetration and also the heterogeneous nature of the red cell distribution in tissues. The LDF however is very stable and gives consistent readings over long periods of time in vitro. In the studies described in this thesis, the LDF is calibrated against a physical standard, PF100 motility solution, as recommended by the manufacturer. The calibration is performed by placing the probe in the motility solution with a probe holder and ensuring that the tip of the probe is submerged in the
solution. The chart-recorder reading is then noted and recorded in the LDF file. This allows an investigator to check that the LDF gives the same reading for the same measurement over a period of time. If the electronic components should need replacing due to age, the physical standard would permit the LDF to be restored to its previous calibration.

2.2.2.0 Laser Doppler perfusion imager

In some experimental and clinical settings, it is more important to map the spatial variation of the tissue perfusion rather than measuring the temporal variations at a single point. Thus the laser Doppler perfusion imaging technique was developed for this purpose.

2.2.2.1 Equipment

The laser Doppler perfusion imager (LDPI) (Lisca PIM 1.0, Lisca Development AB, Linkoping, Sweden) comprises a laser, a scanner, an optical detector system, an optical isolation unit, a tripod, a computer with a colour monitor and a colour plotter (fig 2.2). The computer is installed with a self-instructive software and all the operations of the system are performed using the keyboard. The laser used for generating the monochromatic light is a 3mW solid state Helium-Neon laser with an output intensity of less than 1mW (wavelength 635nm).

It works on the same Doppler principle. The monochromatic laser light penetrates the tissue and a fraction of the backscattered and Doppler-broadened light is detected by a photo-detector housed in the scanner head, about 13cm above the tissue surface. A black cloth is placed over the LDPI in order to reduce optical interference with the LDPI's signals. The signal from the photo-detector is then processed to generate an
output signal that is proportional to tissue perfusion. The LDPI sequentially scans the
tissue under study up to 4096 measurement points. A full format scanning procedure
lasts about 4 minutes and covers an area of 12 x 12 cm². This generates a signal that is
proportional to tissue perfusion, which is stored in the computer hard disk. This red
cell flux response is represented by a colour-coded image of tissue perfusion on the
computer monitor and is expressed in arbitrary units of volts (V). The size of the area
scanned can be adjusted by the operators (Nilsson et al. 1994).

2.2.2.2 Calibration
The LDPI’s signal is calibrated against the manufacturer’s motility standard and
recorded in the LDPI file book. The motility standard consists of a suspension of
polystyrene microspheres in water. A correction factor is then worked out over an 8-
week period based on the calibration readings. This is based on the manufacturer’s
recommendation to ensure that the LDPI signals obtained from the studies over a long
period of time may be adjusted to the standard.

2.2.2.3 LDF versus LDPI
The LDF has the advantage of permitting continuous recording of temporal changes
in tissue perfusion at a single point. However, the LDF signal output from a single
spot is not representative of the blood flow over a tissue region due to its high spatial
resolution. The laser Doppler perfusion imager allows the mapping of a specific tissue
area at a discrete point in time. This system also eliminates the need for fibre lines and
therefore permits the recording of tissue blood flow without touching the tissue. This
also has the advantage of eliminating fibre-line movement artefacts but problem with
tissue movement still persists.
Fig 2.1 Laser Doppler fluximetry

a) The main unit of the Periflux Pf2B laser Doppler fluximetry system.

b) This illustrates the attachment of the laser probe to the foot to permit measurement of skin red cell flux.
Fig 2.2 Laser Doppler perfusion imager (LDPI).

a) Computer hard disk and the monitor displaying the PIM software on the screen.

b) The head of the LDPI placed approximately 13 cm above the skin site.
2.3.0 Iontophoresis

2.3.1 Introduction

Transdermal drug delivery has become increasingly exploited as an alternative method of administration of active substances in humans. This method of drug transport depends on the understanding and the manipulation of the biological aspects of the skin.

Iontophoresis is one such technique that transfers substances across the skin by application of an electrical charge. It is based on the general principle of electricity, that like charges repel each other and opposite charges attract one another. Hence to deliver a positively charged substance, the drug has to be placed under a positive electrode (anode) where it would be repelled and also attracted to a negative electrode (cathode) placed elsewhere on the body.

This technique has many applications in field of medicine and therapeutics, such as the delivery of local anaesthetics and analgesics and the treatment of hyperhydrosis (Ledger 1992). This mode of drug delivery is also useful in the investigation of skin microvascular function in response to pharmacological agents. It has the advantages of introducing vasoactive substances into the dermal vascular bed with minimal trauma and also enabling a delivery of relatively high concentration of drug using only a small amount of the drug, thereby minimising any possible systemic side effects.

2.3.2 Equipment

A battery-powered iontophoresis controller (MIC 1, Moor Instruments, Axminster, Devon, UK) provides a direct current for iontophoresis and the LDPI is used to
measure the skin blood flow response. A perspex chamber (Moor Instruments, Axminster, Devon, UK) is used for the application of the study substance. The chamber has a total diameter of 30mm, height of 3 mm and an inner chamber diameter of 10mm. The outer ring of the chamber is covered with non-reflective black tape to minimise artefacts on the LDPI recordings and also to permit identification of the site of drug delivery on the image produced. The chamber is attached to the skin by means of a double-sided adhesive disc and an indifferent electrode is attached to another area of skin some distance away to complete the circuit (see later). The chamber is filled with study solution with a glass cover slide on top of the chamber to prevent reflection artefacts from the solution (fig 2.3).

2.3.3 Drugs
Many different drug substances can be delivered into the skin microcirculation using iontophoresis. The substances used routinely to assess skin endothelial cell and smooth muscle cell functions are listed below.

Acetylcholine
1 % acetylcholine (Miochol, Ciba Vision, Southampton, UK) is used to test the endothelial vasodilatory function of the skin. Acetylcholine acts on the muscarinic receptor on the endothelium of the blood vessel to increase the synthesis of nitric oxide, which diffuses across to the smooth muscle cell layer to cause vasodilatation via cGMP-dependent pathway (figure 2.4).

Mannitol
3% Mannitol (Royal Devon & Exeter Hospital Pharmacy) is used as a drug vehicle for acetylcholine
Sodium nitroprusside

0.25% sodium nitroprusside (Nipride) (Roche, Welwyn Garden City, Herts UK) is used as a direct nitric oxide donor to the vascular smooth muscle cell layer, which causes vascular smooth muscle cell relaxation.

Water

Sterile water for injection (Baxter Healthcare Ltd, Thetford, Norfolk, UK) is used as a drug vehicle for sodium nitroprusside.

2.3.4 Study protocol

All iontophoresis studies in this thesis were conducted with the subject lying supine in a temperature-controlled environment (22±0.5°C) following a 30-minute period of acclimatisation.

Iontophoresis experiments in the adults were all performed on the forearm. The flexor surface of the forearm was cleaned using an alcohol wipe and followed by deionised water. The perspex chamber was attached to the forearm skin and the indifferent electrode was attached to the volar surface of the wrist. The position of the chamber was chosen to avoid hair, skin pigmentation and broken skin.

In order to test the endothelium-dependent vasodilatory function of the skin, iontophoresis was conducted using the drug carrier (3% mannitol) on one skin site (control experiment) followed by iontophoresis of 1% acetylcholine on three skin sites. The drugs were delivered under an anodal current with 5 pulses of 100μA for 20s with intervening period of 60s with no charge. This was because previous studies in the department have shown that 60s interval was required between each charge period to achieve a plateau of the response to each delivery of the acetylcholine. The head of the LDPI is positioned approximately at 13 cm above the centre of the
chamber with a black cloth covering the LDPI and the area to be scanned to minimise optical interference. Forearm red cell flux was measured at baseline and also after each period of drug application using the LDPI, set to scan 32X32 measurement sites, covering an area of about 4cm by 4cm, with each scan taking 20 seconds to perform.

To test the endothelium-independent vasodilatory function of the skin, iontophoresis of sodium nitroprusside was administered using a cathodal current with one 60s pulses of 200μA and the vasodilatation was monitored for a further five minutes by scanning at one-minute intervals. This was performed on three skin sites. Control experiment with the drug vehicle (water) was also performed on one skin site. The skin sites for the sodium nitroprusside and water iontophoresis were pre-treated with EMLA cream (eutectic mixture of lidocaine and prilocaine) 2 hours before the experiment. This was because previous studies have shown that the application of a cathodal current results in a large neurogenic response, which could be blocked by EMLA pretreatment (Morris et al. 1996).

2.3.5 Biological zero

At the end of each study, biological zero, that is flux value without arterial inflow, was measured at both an untreated and a vasodilated site on the forearm during arterial occlusion of the upper arm by an inflated cuff set at 220 mmHg.

2.3.6 Analysis

The colour-coded images shown on the computer monitor were analysed using the Pim 2.3 software package (Lisca Development AB, Linkoping, Sweden). The results were expressed as red cell flux in arbitrary units of volts (V). The response to the drug was calculated by subtracting the response to the drug carrier (mannitol or water)
from the absolute response to the drug. This was calculated for each time-point throughout the study and plotted against time (fig 2.5). The peak skin vasodilatory response in response to the drug was calculated by taking the mean value of the skin red cell flux obtained at the last two time-points.

2.3.7 Reproducibility

The reproducibility of the peak skin vasodilatory response (cv) to acetylcholine iontophoresis determined in one subject on five occasions was 21.1%. This was compatible with the departmental reproducibility determined in 4 subjects on three to five occasions, which was 19.7%. The reproducibility of the peak vascular response to sodium nitroprusside protocol determined in one subject on 5 occasions was 16.8%. The departmental reproducibility determined in 5 subjects on four to five occasions was 21.8%.

The peak vasodilatory vascular response was found to be generally more reproducible than the vasodilatory response for each individual time-point in the iontophoresis protocol. For example, the cv for the vascular response for the acetylcholine protocol for 4 subjects were: 0s- 275.7%, 80s- 44.7%, 160s- 24.8%, 240s-16.7%, 320s- 19.6%, 400s -20.8% (cv for peak vasodilatory response =19.7%). The cv for the sodium nitroprusside protocol in 5 subjects were: 0s- 68.1%, 60s- 52.1%, 120s- 39.1%, 180s-32.2%, 240s- 28.6%, 300s- 24.1%, 360s- 20.2% (cv for peak vasodilatory response =21.8%).
Fig 2.3 Measurement of skin red cell flux in response to the iontophoresis of a drug using laser Doppler perfusion imaging technique.
Fig 2.4 A schematic diagram illustrating how vasoactive substances like acetylcholine act on the endothelium to cause vasodilatation of the blood vessel.

**Acetylcholine**

L-Citrulline  R  L-Arginine

Endothelium

NO  O₂

Smooth muscle cell

NO  GTP  sGC  cGMP

---

**smooth muscle relaxation**

R – receptor  sGC – guanylate cyclase
O₂ – oxygen  cGMP – cyclic guanosine monophosphate
NO – nitric oxide  GTP – guanosine triphosphate
NOS – nitric oxide synthase
Fig 2.5 Graph illustrating skin red cell flux in response to the iontophoresis of a vasoactive substance.

Skin red cell flux is a measure of the number and the velocity of the red cells expressed in arbitrary units of volts (V).
2.3.8 Adaptation of the technique for studies in infants

The studies on the infants were performed on the abdomen rather than the forearm because a relatively flat surface was required for the placement of the chamber and to avoid spillage of the study substance. It was easier to keep the infant’s abdomen relatively stationary compared to the forearm to permit the LDPI to scan the skin site where iontophoresis was performed, thus reducing the movement artefacts on the LDPI signals.

Iontophoresis of acetylcholine was performed on one skin site and mannitol on another skin site on the abdomen. The same charge protocol was adopted for this study with 5 pulses of 100μA for 20s, each followed by an intervening period 60s with no charge applied. The LDPI was once again set at 13 cm above the area to be scanned. However, the LDPI was set to scan the skin sites only at the end of the iontophoresis study, rather than after each charge period. In the pilot studies, it was found that scanning the skin site using the LDPI was more difficult in a neonate compared to an adult. Attempts to keep the neonate’s abdomen stationary and in line with the LDPI scanner in conjunction with placement of the black cloth to minimise optical interference were time-consuming. The pilot study revealed that the study would be unnecessarily prolonged and extremely difficult to sustain the infant’s interest if baseline and serial scans were performed throughout the study. Hence the protocol was modified by just performing a single scan at the end of the entire iontophoresis protocol. The skin vascular response to acetylcholine was represented by the absolute response to acetylcholine minus the response to mannitol. Biological zero was also not feasible on the infants as iontophoresis was performed on the abdomen rather than the limbs.

Endothelium-independent vasodilatory function was not performed. It has been
demonstrated previously that the application of a cathodal current, which is required to administer sodium nitroprusside, results in a large non-specific vascular response (Morris et al. 1996). This will be discussed in greater detail later. This non-specific increase in perfusion can be blocked by the application of EMLA cream but its use is contraindicated in infants less than 6 months old and therefore smooth muscle cell function cannot be interrogated in this manner in the infants.

2.3.9.0 Experiment to investigate the role of methylcellulose carriers in reducing current-induced vasodilatation in iontophoresis studies

A criticism of the use of the iontophoresis technique to study skin vascular function is that the electrical current used to transfer the vasoactive drug can itself cause an increase in skin blood flow (Berliner 1997) (Grossmann et al. 1995). In particular, a cathodal current has been shown to give rise to a greater increase in skin perfusion compared to an anodal current. This can be a major problem when iontophoresis is used to assess skin vascular response to various vasoactive substances. The vascular response to the iontophoresis of drug carrier alone can sometimes be as great as the response to the drug due to this current-induced response. As mentioned previously, Morris et al. have shown that this response can be eliminated by prior application of EMLA to the skin. Noon et al. reported that the use of methylcellulose gel could also reduce this current-induced vascular response effectively (Noon et al. 1998). If indeed the use of methylcellulose carrier could substantially reduce this non-specific vascular response, this would facilitate the investigation of endothelium-independent vasodilatory function in infants using iontophoresis of sodium nitroprusside. This was investigated further in the protocols used by the department and Noon et al.
2.3.9.1 Aims

The aims were several folds:

1) to compare the effect of applying cathodal versus anodal current on the skin perfusion response when methylcellulose gel was used as the carrier

2) to investigate if pre-treatment of the skin with EMLA would alter the skin perfusion response when methylcellulose gel carrier was used

3) to compare the skin perfusion response of methylcellulose carrier versus water carrier when cathodal current was applied

4) to compare the skin perfusion response of methylcellulose gel carrier using cathodal current versus the current departmental protocol of using water carrier and EMLA pre-treatment when cathodal current was applied

2.3.9.2 Study protocol

These studies were performed on 6 healthy volunteers on two separate occasions.

Study 1 examined the perfusion response to 2% methylcellulose carrier (Sigma Chemicals Ltd, Poole, Dorset, UK) using

(i) cathodal current,

(ii) cathodal current on EMLA sites and

(iii) anodal current.

Two skin sites were studied for each of the above experiments. The protocol was as follows: 100μA for 10s, 200 μA for 10s, 200 μA for 20s, 200 μA for 40s and 200 μA for 80s. This was the protocol used by Noon’s group when they reported a substantial
reduction in current-induced perfusion response when methylcellulose gel was used as the drug carrier.

Study 2 examined the perfusion response to the application of a cathodal current using
i) water carrier,
ii) water carrier on EMLA pre-treated skin sites and
iii) methylcellulose carrier.

Two skin sites were used for each of the above experiments. The protocol selected for this study was in accordance with the departmental protocol for the iontophoresis of sodium nitroprusside (endothelium-independent vasodilator), which was administered under a cathodal current: current of 0.2mA was applied for 60s, followed by 5 periods of monitoring skin perfusion responses at one-minute intervals.

2.3.9.3 Statistics

The overall skin perfusion responses for the different drug carriers/ interventions in each study were compared using ANOVA for repeated measures. The mean responses (± standard error of the mean) of the 6 subjects were illustrated in figures 2.6 and 2.7. The increase in vascular response for each drug vehicle was also calculated by subtraction of the red cell flux at baseline from the peak red cell flux response attained at the end of each experimental protocol (results expressed as median value with interquartile range) and comparisons between the different drug carriers were made using Wilcoxon signed ranks tests.
2.3.9.4 Results

In study 1, ANOVA for repeated measures indicated that the skin perfusion responses when methylcellulose gel carriers were used under i) cathodal current, ii) cathodal current and EMLA pre-treatment and iii) anodal current were significantly different (p<0.001). The application of a cathodal current when methylcellulose carrier was used resulted in a large increase in perfusion (1.130 (0.882 to 1.558)) V, which was blocked by EMLA (-0.0496 (-0.070 to -0.021)) V (p=0.028). Methylcellulose carrier with anodal current also increased perfusion (0.415 (0.097 to 0.701) V although this was smaller than when cathodal current was applied (1.130 (0.882 to 1.558)) V (p=0.028) (fig 2.6).

In study 2, ANOVA for repeated measures indicated that skin perfusion responses to i) water carrier ii) water carrier on EMLA-treated sites and iii) methylcellulose carriers were significantly different (p=0.019). The increase in perfusion induced by cathodal current and water (0.977 (0.249 to 1.492)) V was blocked by EMLA (0.017 (-0.022 to 0.050)) V (p=0.028). Methylcellulose also increased perfusion (0.519 (0.016 to 1.065)) V. This was less than the response with water alone (p=0.028) but greater than the response with water vehicle and EMLA pretreatment (p=0.028) (fig 2.7).

2.3.9.5 Discussion

This confirms that current alone induced a substantial increase in skin perfusion response, which appears to involve a neurogenic pathway. This study in conjunction with other studies (Berliner 1997) (Morris et al. 1996) have shown that cathodal current in particular induces a greater response than anodal current and this reinforced the importance of performing both cathodal and anodal current alone controls in any
iontophoresis experiments. Although the current-induced response with methylcellulose gel was less pronounced than with water carrier alone, it was nevertheless a substantial perfusion response and not as effective as the combination of water and pretreatment of the skin with EMLA. Thus methylcellulose gel carrier cannot be used as an effective drug carrier to study endothelium-independent vasodilatory function in infants.
Figure 2.6 Changes in skin perfusion in response to the iontophoresis of methylcellulose drug carrier (no drugs).

Change in perfusion is represented by red cell flux, which is a measure of the number and the velocity of the red cells expressed in arbitrary units of volts (V).
Figure 2.7 Changes in skin perfusion in response to the iontophoresis of various drug carriers using cathodal current (no drugs).

Change in perfusion is represented by red cell flux, which is a measure of the number and the velocity of the red cells expressed in arbitrary units of volts (V).
2.4.0 Postural vasoconstriction

2.4.1 Introduction

There is a fine balance of forces in the capillaries to control the rate of fluid movement between the blood and the tissues. In the supine position with the feet positioned at heart level, the pressure within the capillaries is dependent on systemic blood pressure as well as the resistance pre- and post capillaries and the venous pressure. However, on changing from a supine position to an erect posture, extra hydrostatic pressure load is acting on the capillary bed in the lower limb, especially in view of the increased venous pressure which is easily transmitted to the capillary bed. Much damage in the capillary structure would occur if compensation for this great increase in pressure load created by a change in posture does not occur. The postural vasoconstrictor response is a mechanism whereby the posturally induced increase in capillary pressure is minimised. This involves the vasoconstriction of the pre-capillary arterioles, which is thought to be mediated by a local neurogenic axon response, with a small contribution from a centrally-induced reflex and local myogenic response (Hassan et al. 1988).

2.4.2 Equipment

The laser Doppler fluximeter (Perimed Pf2, Stockholm, Sweden) described in section 2.2.1.0 is used to measure the skin blood flow.

2.4.3 Calibration

At the beginning of each study, the LDF was calibrated with the delrin disc and motility solution as recommended by the manufacturer as described previously.
2.4.4 Study protocol

With the subject lying in the supine position, the laser probes were placed on (i) the pulp of the big toe and (ii) the dorsal surface of the foot. The 2 sites were selected because of the different architecture of the microvessels in the two different sites. In the dorsum of the foot, the blood flows mainly through capillaries, with little passing through shunt vessels while the majority of the blood in the pulp passes through shunt vessels at 22°C. Temperature was recorded at both skin sites using adherent thermocouple (Comark Electronic, Littlehampton, Sussex). The red cell flux was recorded for the two skin areas with the foot at heart level. The readings were taken during the 2 minutes preceding a change in posture of the foot. Next the foot was passively lowered to a standard distance of 40 cm by flexing the leg at the knee and providing support for the leg. The red cell flux was then recorded over the 4th and 5th minute after dependency. Three readings of heart rate and blood pressure (Dinamap, Critikon, Tampa, FL, USA) were recorded at the beginning and at the end of the study. The mean of the final two readings for each recording period was taken as the subject's true blood pressure.

2.4.5 Analysis

The red cell flux was expressed in units of volts. ‘Blood flow at heart level’ (HF) was calculated by taking the mean value of the red cell flux at the final two minutes prior to dependency. ‘Blood flow dependent’ (DF) was the mean of the red cell flux during the 4th and 5th minutes after dependency. Postural vasoconstriction (PV) was expressed as:

\[ PV = (DF/HF) \times 100\% \]
2.4.6 Biological zero

An ankle cuff was inflated to 220mmHg to induce arterial occlusion and the laser Doppler signals from the two skin areas were recorded. This was taken as the biological zero, which was subtracted from the HF and DF readings before PV was calculated.

2.4.7 Reproducibility

The reproducibility of this method was determined in several ways. In 6 subjects on three separate occasions, the change in posture reduced the skin blood flow to (mean±SD) 18.3±4.5, 18.6±3.1 and 19.2±4.3% of the supine values respectively. In three postpubertal subjects measured on two occasions more than a year apart, the blood flow dependent as a percentage of basal flow was 12.9 and 12.0 % in subject 1, 10.7 and 3.4 % in subject 2 and 5.9 and 11.3% in subject 3. In three prepubertal children measured on two occasions more than one year part, blood flow in the dependent position as a percentage of basal flow was 40.0 and 43.3 % in subject 1, 51.7 and 51.3% in subject 2 and 30.5 and 25.9% in subject 3.
2.5.0 Maximum hyperaemic response to local heating

2.5.1 Introduction

In the face of an injurious insult to the skin, a dramatic increase in the local blood flow occurs by an increased vasodilation of the microvasculature. This increase in blood flow may be initiated by the release of vasodilator substances as well as local neurogenic processes. This increases the amount of nutrients and oxygen for tissue repair and also the delivery of white blood cells and antibodies for defence against any potential infection.

The maximum hyperaemic response to local heating can be used as an indicator of the vasodilatory capacity of the skin microcirculation in response to injury. It has been shown that when the skin is heated to between 42 – 43 °C, this is a temperature at which all vasodilatory processes have been initiated and thereby causing a maximum vasodilation of the microvessels (Johnson et al. 1986). This response is then measured using laser Doppler fluximetry, which has been validated against direct measurements of capillary flow velocity (Tooke et al. 1983). The power of this technique has been illustrated by the fact that reduced hyperaemic response was demonstrated in diabetic children before the onset of puberty (Shore et al. 1994).

Most studies examining maximum hyperaemic response to local heating are performed on the dorsum of hands and feet in order to give a reasonable estimate of the nutritive flow as thermoregulatory shunts are relatively absent in these areas. In addition, patients with diabetes are prone to developing vascular disease and neuropathy in the feet and hence, it is of particular relevance to assess the ability of the foot skin microcirculation to mount a vasodilatory response to injury.
2.5.2 Equipment

The equipment consists of a small brass heater of approximately $1\,\text{cm}^2$ in size, attached to a heater box. The heater system is actually a modified transcutaneous oxygen tension monitor used on Special Care Baby Unit. The heater has a narrow hole drilled acentrically through it to permit the laser probe to be positioned for measuring blood flow. A small nylon collar is fitted around the heater and a double-sided adhesive tape is used to secure it onto the skin.

The same LDF system used to measure postural vasoconstrictor reflex is also used to assess the maximum hyperaemic response.

2.5.3 Calibration

At the beginning of each study, the LDF was calibrated with the delrin disc and motility solution as recommended by the manufacturer.

2.5.4 Study protocol

At the start of the study, a thermocouple (Comark Electronic, Littlehampton, Sussex) was placed on the foot, which was then warmed gently with a hair dryer till the temperature was stable at $36^\circ\text{C}$. This was to ensure that the application of the heater to a cold foot would not result in the cooling of the heater due to its small surface area. The heater was placed in a plastic collar and attached to the skin using a double-sided adhesive tape. A thermocouple was also placed beneath the heater to register the temperature between the heater and the skin. If the temperature recorded between the heater and the skin fell below $42^\circ\text{C}$, the foot would be gently warmed again with a hair-dryer until $42^\circ\text{C}$ was restored. After 30 minutes of heating, the maximum blood flow was assessed by using the LDF. The laser probe was placed in the small hole in
the heater such that it was just touching the skin. Rotation of the heated probe holder about the plastic collar permitted blood flow to be measured in an area that has been directly heated immediately before rotation. Eight equidistant sites were marked on the plastic collar and the probe holder was rotated through the eight sites to determine the mean blood flow over the sites. This has been previously demonstrated to be more reproducible than a single measurement.

Next the foot was lowered to a standard distance of 40cm by flexing the knee and providing support for the leg. The mean blood flow over the eight heated skin sites was measured again. Heating of the foot skin to this temperature has been shown to impair the postural vasoconstriction reflex (Hassan et al. 1988).

2.5.5 Analysis

The maximum hyperaemic response was expressed as the mean of the readings obtained on the chart recorder from the eight skin sites in arbitrary units of volts (V). This was determined when the foot was in the horizontal position (mhrh) and then when the foot was in a dependent position (mhrd).

Minimum vascular resistance (mvr) was calculated using the mean arterial blood pressure (MABP), determined by the oscillometric technique (Dinamap, Critikon, Tamp, Florida) and the maximum hyperaemic response with the foot in the horizontal position.

$MABP = \text{Diastolic blood pressure} + \frac{1}{3} \text{pulse pressure}$

$mvr = \frac{MABP}{mhrh}$
Blood vessels are elastic structures and distend with increasing internal pressure.

Distensibility of the blood vessels in this study was expressed as the maximum hyperaemic response of the foot in the dependent position divided by the maximum hyperaemic response of the foot in the horizontal position.

\[
\text{Distensibility} = \frac{\text{mhrd}}{\text{mhrh}}
\]

### 2.5.6 Adaptation of the technique for studies in infants

To adapt this technique to study the skin microcirculation in infants, it was extremely difficult to obtain stable readings of red cell flux using the LDF system due to movement artefact. Hence the LDPI was chosen to quantify this response, which could be achieved much quicker than obtaining point fluximetry readings rotating the laser probe over eight different sites, and therefore less susceptible to movement artefact. As it was difficult to keep the dorsum of the infant's foot perpendicular to the direction of the laser beam, it was more feasible to perform the maximum hyperaemia experiment on the infant's thigh.

At the start of the study, a thermocouple was placed on the thigh, which was warmed up to a stable 36°C before the heater was applied. A plastic collar was placed around the heater and attached to the skin using double-sided adhesive disc. A thermocouple was also placed between the heater and the skin. Heating period was shortened to 20 minutes in this study as it has been shown recently that this length of heating time was sufficient to induce maximum hyperaemia (Gooding et al. 1999). It was also advantageous to shorten the time period required for the study in order to sustain the subject's interest. After the heating period, the heater and the collar were removed and a black chamber, similar to that used in the iontophoresis studies, was placed on the
heated area of the thigh. The black chamber was used to define the heated skin area on the LDPI scan, as the diameter of the inner chamber was equivalent to the diameter of the heated area. The LDPI signal was recorded as colour-coded images on the computer hard disk.

2.5.7 Analysis (infant studies)

The colour-coded images were analysed using Pirn 2.3 software package as described previously and the results were once again expressed in arbitrary units of volts.

2.5.8 Reproducibility

The reproducibility of this technique was assessed in several ways. In 2 adult subjects studied on 4-5 occasions. The cv was 5.7% using the LDF and 8.2% using the LDPI to quantify the hyperaemic response. This was in keeping with the departmental reproducibility where the cv was 7.6% when maximum hyperaemia was determined in duplicate experiments (using LDF) in 9 subjects. In 3 infants subjects studied on 2 occasions 1-2 weeks apart using LDPI, the cv was 1.6%.
Fig 2.8 Measurement of the skin maximum hyperaemic response on the foot dorsum using laser Doppler fluximetry.
Fig 2.9 Measurement of skin maximum hyperaemic response in an infant.
2.6.0 Skin capillary density

2.6.1 Introduction

Any impairment in skin vasodilatory response may be related to either an alteration in the structure or function of the blood vessels or a reduction in the number of capillaries perfusing the skin. In particular, when the laser Doppler technique is used to quantify vascular response, either a reduced number or a reduced velocity of the red cells can give rise to a low flux signal. Therefore, if the capillary density is reduced, this could result in a low flux signal due to a decrease in number of circulating red cells. Hence it is important to incorporate the examination of skin capillary density into the study when comparing skin vasodilatory responses between different subject groups. Although the capillaries only account for approximately 10% of the blood flow detected by the laser Doppler fluximetry, a decrease in capillary numbers may reflect a generalised rarefaction. This can be performed using a videomicroscopy system that allows direct visualisation of the superficial dermal vessels.

2.6.2 Equipment

The equipment consists of an objective lens, a mercury vapour lamp, video monitor, video camera, video recorder. The magnification of the objective lens is x10 (Leitz, Leica, London, UK). The mercury vapour lamp (Leitz 100W type 307-072.042, Leica, London, UK) has an emission spectrum of the same wavelength as the absorption spectrum of haemoglobin (370-450nm), producing a maximum contrast between moving red blood cells against the background of surrounding structures. The lamp is connected to a fibre-optic cable and a flexible arm is used to position the
end of the cable such that the light is focussed over the skin area to be examined. The whole system has a magnification of x200. A video camera (Hitachi CCTV camera, Japan) and a video tape recorder (Panasonic AG-6200, Japan) with a time-generator is incorporated into the circuit to enable recording of the views of skin capillaries. A monochrome monitor is linked to the video system to enable real time and playback viewing of the video images of the capillaries.

2.6.3 Study protocol

The left middle finger was coated with clear nail varnish to reduce light-scattering effects. Next an inflatable cuff with a width of 1.6cm (mini-penile pressure cuff DC 1.6, PMS Instruments, Maidenhead, UK) was positioned loosely around the base of the left middle finger to allow subsequent venous occlusion. The finger was then positioned under the microscope and following acclimatisation, the recordings of the video images of the capillaries begun. One minute video recording of the skin capillaries was made in each of the six adjacent areas on the finger under study (basal capillary density). Following which, the finger cuff was inflated to 40 mmHg to induce venous occlusion using a standard mercury sphygmomanometer. After a period of 10 minutes, video recordings were repeated in the same 6 sites (capillary recruitment by venous occlusion).

2.6.4 Analysis

An acetate film with a circle of known area drawn on the sheet was placed on the television monitor, covering most of the screen. Capillary number was then determined by counting the total number of capillaries which lay within the circle during each one-minute period. This was repeated for the six fields of capillaries
during baseline and after venous occlusion. The mean capillary number at baseline and after venous occlusion was computed from the capillary counts from the six sites. Recording of a graticule was performed at the end of the study so that the magnification of the system could be calculated. Using the magnification and the diameter of the circle drawn on the acetate sheet, the capillary density per mm$^2$ could be estimated.

2.6.5 Reproducibility

Six measurements in 2 normal subjects over the course of three months has shown that this technique has a good intraindividual reproducibility with a mean coefficient of variation of 5% for basal and 7% for post-venous occlusion capillary densities.

2.6.6 Adaptation of the techniques for studies in infants

The medial surface of the infant’s foot was coated with clear nail varnish and allowed to dry. The foot was then positioned under the microscope and recordings of the skin capillaries were made. Attempts were made to record images of the skin capillaries in at least 3 adjacent sites on the foot. Unlike the studies in adults, it was often not possible to record the images for more than a few seconds due to the movement of the infant’s foot. Venous occlusion was not performed as it was not well-tolerated. Capillary density was calculated as the mean capillary number per mm$^2$ for the recorded images from all sites.
Fig 2.10 Measurement of skin capillary density.
2.11 A video image of skin capillaries.
3.0 The relationship between birth weight and skin microvascular function in infancy

3.1.0 Introduction

3.1.1 Skin microcirculation in neonates and infants

After birth, the neonatal macrocirculation and microcirculation undergoes dramatic changes in order to meet the demands of an altered environment from the intra-uterine setting. In particular, the skin microcirculation has a vital role in thermoregulation and needs to undergo rapid adaptation. At birth, the skin microvascular network is relatively inefficient, consisting of a disorderly network of long blood vessels, lacking in papillary loops. Hence, a larger volume of blood flow is required to meet nutritional and thermoregulatory demands. In the first week of life, the capillary networks begin to expand and assume a more orderly pattern. Papillary loops begin to appear as small superficial buds in the second week and clearly defined loops are not visible until the fourth and fifth week. The development of an orderly plexus becomes apparent during the second week of life but this is not characteristic of all areas until the fourteenth to seventeenth week (Ryan 1998).

3.1.2 Relationship between birth weight and type 2 diabetes

There has been much recent interest in the link between low birth weight and the risk of adult disease. Retrospective studies in the United Kingdom have shown that low birth weight is associated with the development of non-insulin dependent (type 2) diabetes, hypertension and increased cardiovascular mortality in adult life (Barker et al. 1993). This association is found to be independent of social class and adult body
mass index and has been confirmed in studies conducted in other populations (Valdez et al. 1994) and countries (Lithell et al. 1996) (Curhan et al. 1996).

Barker and colleagues proposed the fetal programming hypothesis to explain the association, stipulating that undernutrition in utero leads to impaired fetal growth which may permanently programme the structure and function of the pancreas, thus predisposing to the later development of type 2 diabetes (Barker 1998). An alternative or complementary explanation is the fetal insulin hypothesis, which states that the same polygenic factors that increase insulin resistance in utero and in adult life produce two phenotypes – a low birth weight baby and an adult with an increased risk of diabetes and hypertension (Hattersley et al. 1999). It is likely that both genetic predisposition and the intrauterine environment play a role in determining fetal development and these factors in conjunction with other lifestyle and environmental influences in adult life predispose towards the development of obesity, hypertension and glucose intolerance. This clustering of adverse features are collectively referred to as the insulin resistance or metabolic syndrome (Syndrome X) (Reaven 1993).

3.1.3 Hypothesis: the relationship between birth weight and vascular development

The fetal insulin hypothesis also suggests that a genetic predisposition to insulin resistance may also affect fetal vascular development (Hattersley et al. 1999). The fetus produces insulin in response to maternal glucose. In the presence of tissue insulin resistance, fetal insulin secretion may be insufficient to promote normal growth and development in certain tissues such as the vasculature. Insulin causes vasodilatation via an endothelium-dependent nitric oxide mediated pathway (Steinberg et al. 1994). If this pathway shows insulin resistance, generation of nitric
oxide and resulting vasodilatation and blood flow will be impaired, culminating in a reduced stimulus for angiogenesis (Hudlicka et al. 1992). According to the fetal insulin hypothesis the newborn infant will begin to feed back on its own glucose level and increase insulin secretion to overcome peripheral insulin resistance. This in turn may promote vascular smooth muscle mitogenesis, as this particular pathway may be less insulin resistant (Jiang et al. 1997). Vascular smooth muscle mitogenesis in synergism with deficient fetal angiogenesis may predispose the individual to adult hypertension and reduced microvascular vasodilatory reserve. In support of these suggestions are the findings that skin capillary density and microvascular vasodilatory function were found to correlate inversely with blood pressure and positively with insulin sensitivity in young healthy adults (Serne et al. 1999).

In the present study the microvascular function and capillary density in three month-old infants of varying birth weight were examined to test the hypothesis that impaired vascular function may be an intrinsic abnormality present in low birth weight individuals in early infancy.

3.2 Method

3.2.1 Subjects

Two main groups of infants were recruited through the Maternity Unit of the Royal Devon and Exeter Hospital: a lowest-quartile birth weight (LQBW) group with birth weight <25th centile and a highest-quartile birth weight (HQBW) group with birth weight >75th centile on the Castlemead growth chart (Cooney 1995). An additional group of middle birth weight (MBW) infants with birth weight between the 30th – 70th centile, representative of the average weight of the study population, was also
recruited. Only singleton babies born at term, (i.e. gestational age equal to or greater than 37 weeks, confirmed by ultrasound scan), were recruited. The exclusion criteria were: maternal history of heart disease, hypertension, pre-eclampsia, diabetes, epilepsy, alcoholism, smoking, chronic drug treatment (e.g. steroids, anti-convulsants), any serious infections/sepsis during pregnancy, as well as any known genetic/chromosomal or metabolic disorders in the babies.

Mothers were approached by post in the first four weeks after birth with a study information sheet and a covering letter. Mothers who were interested in the study were requested to return a reply slip inviting further contact. Information on the mother’s pregnancy as well as the infant’s anthropometric measures (birth weight, head circumference, length) were obtained from the obstetric records. Written consent was obtained from the mothers. The study was approved by the Exeter Medical Research Ethics Committee.

3.2.2 Study conditions

The subjects were studied after a feed while lying in a cot in a temperature-controlled environment (21.5 – 22.5°C). A thermocouple (Comark Electronic, Littlehampton, Sussex UK) was used to record skin temperature on the abdomen and thigh. Heart rate and blood pressure were also recorded in subjects who tolerated the procedure using a semi-automatic blood pressure recorder (Dinamap, Critikon, Tampa, USA). An electrocardiograph recorder (Sicard 440, Siemens plc, USA) was used to assess heart rate in infants who did not tolerate blood pressure measurement. Skin vascular function tests were modified from the protocols used in previous studies on adults.
3.2.3 Maximum hyperaemic response

Each infant had the thigh warmed up gently by a hairdryer to 36°C before the skin was heated up to between 42 - 44°C by the application of a small brass heater. The heater was removed after 20 minutes and the heated area was then scanned using the LDPI. The skin red cell flux was expressed in arbitrary units of volts. Minimum vascular resistance was calculated by dividing the mean arterial blood pressure by the maximum hyperaemic response.

3.2.4 Response to iontophoresis of acetylcholine

The microvascular endothelium-dependent function of the skin was examined by measuring the skin vasodilatory response to iontophoresis of acetylcholine (endothelium-dependent vasodilator). The abdominal skin was chosen to provide a relatively flat area for the attachment of an iontophoresis chamber containing the study substance. One skin site was used for the iontophoresis of mannitol (acetylcholine vehicle) and another skin site for acetylcholine. The charge protocol has been described in section 2.3.8. At the end of the protocol, the skin sites were scanned using the LDPI.

Endothelium-independent function of the skin (using sodium nitroprusside) was not studied in this group of subjects. Previous studies indicate that iontophoresis of the sodium nitroprusside drug vehicle (water) induces a large increase in vascular response, which may be blocked by the pretreatment of the skin site with eutectic mixture of lidocaine and prilocaine (EMLA) (Morris et al. 1996) (Goh et al. 1999). However, the use of EMLA is contraindicated in infants below the age of six months.
3.2.5 Capillary density

The infant’s foot was placed under the videomicroscopy system after the application of nail varnish on the skin. Recorded images of the foot skin capillaries were examined by an independent assessor for the quality of the images using a scale of 1-5. The video images were then analysed (blinded to the subject’s birth weight status) by placing an acetate sheet on the monitor screen and marking the presence of all capillary images within the defined area. Capillary density for each subject was determined by taking the mean value of the capillary density in all of the images obtained for that subject.

3.2.6 Statistics

Given the mean and variability (SD) in maximum hyperaemic response, endothelium-dependent vasodilatation and capillary numbers observed in healthy subjects were 1.53±0.36V, 1.36±0.52 V and 113.0±13.6/mm² respectively, power calculations suggest that this sample size provided a 90% chance of detecting a 24% difference in maximal hyperaemic response, 39% difference in endothelium-dependent vasodilatation (acetylcholine) and 14% difference in capillary numbers between the LQBW and HQBW groups at 5% level of significance. A greater than 50% reduction in maximal hyperaemic response and a 15% reduction in capillary numbers were observed in a previous study on young (age 23-33) relatives of hypertensive patients, who were at risk of developing hypertension in later life (Noon et al. 1997). A 63% difference in endothelium-dependent vasodilatation was previously reported between small-for-gestational-age and appropriate-weight-for-gestational-age infants (Martin et al. 1998). Comparisons of the microvascular function tests and other parameters between the three groups of infants were made using non-parametric Kruskal-Wallis
test, followed by the Mann-Whitney U test to assess where the differences arise between the groups. The Mann-Whitney U test was also used to compare the differences between the sexes. Spearman rank-correlation coefficients (Rs) were calculated where appropriate. Data are presented as the median value with interquartile range in the text.

### 3.3.0 Results

There were 20 LQBW (8 male and 12 female), 21 HQBW (12 male and 9 female) and 8 MBW subjects (3 male and 5 female) in the study. The median (interquartile range) birth weight, heart rate, systolic and diastolic blood pressure of the LQBW and HQBW groups are listed in table 3.1. There were no significant differences in any of the parameters in the three groups other than the birth weight. The majority of the subjects were breast-fed (LQBW- 13 breast-fed, 2 bottle-fed, 5 both breast and bottle-fed, MQW- 7 breast-fed, 1 both breast and bottle-fed, HQBW- 18 breast-fed, 2 bottle-fed and 1 breast and bottle-fed). None of the mothers smoked during pregnancy; mothers of four subjects were ex-smokers (all in the LQBW group) but have given up smoking prior to the conception period. The parents of the subjects were predominantly from managerial or professional background and hence there were no distinct social class differences between the LQBW and HQBW groups.

### 3.3.1 Basal skin temperature

There were no significant differences in the skin temperature on the abdomen or the thigh between the two group: LQBW thigh 32.3 (31.0-32.8) °C, MBW thigh 30.8 (30.3-32.5) °C, HQBW thigh 32.3 (31.6-32.7) °C, LQBW abdomen 34.4 (33.9-34.7)
°C (p=0.218), MBW abdomen 34.4 (33.4-35.5) °C and HQBW abdomen 34.1 (33.9-34.7) °C (p=0.876).

### 3.3.2 Maximum hyperaemic response

The maximum hyperaemic response were 2.01 (1.53 - 2.26) V in the LQBW group, 2.19 (1.88-2.34) V in the MBW group and 2.44 (1.96 - 2.90) V in the HQBW group (figure 3.1). The responses were significantly different among the three groups (p=0.020), such that the LQBW group had the lowest response and the HQBW infants had the highest response (LQBW vs HQBW, p=0.006, LQBW vs MBW, p=0.286, MBW vs HQBW, p=0.262).

The maximum hyperaemic response correlated with the uncorrected birth weight (Rs=0.388, p=0.006), birth length (Rs=0.374, p=0.009), head circumference (Rs=0.415, p=0.003) (figure 3.2) and placenta weight (Rs=0.439, p=0.002). The vasodilatory response however did not vary with the heart rate, systolic blood pressure, diastolic blood pressure, thigh skin temperature or gestation period. Weight of the infants near the time of the study (recorded by the health visitors) did not correlate with the maximum hyperaemic response although this information was only available for 36 infants. Method of feeding (breast/ bottle or both) also did not influence the results of the hyperaemic response.

The maximum hyperaemic response was also dependent on the sex of the infants, such that the boys had a higher response (2.34 (1.97-2.79)V) than the girls (2.08 (1.77-2.35)V) (p=0.035). There were no significant differences in the birth anthropometric measures, skin temperatures, heart rate and blood pressure between the male and female infants. Weight at the time of the study was higher in the boys.
compared to the girls (p=0.003) but weight at the age of 3 months did not correlate with the maximum hyperaemic response.

Minimum vascular resistance was calculated for 10 LQBW, 3 MBW and 12 HQBW groups because blood pressure readings were only available for the above number of subjects. There were no significant differences in minimum vascular resistance among the three different groups (p=0.407). There was also no significant difference in minimum vascular resistance between sexes but the data was only available for 14 male infants and 11 female infants (p=0.063).

3.3.3 Response to iontophoresis of acetylcholine

The mean red cell flux in response to iontophoresed acetylcholine were 1.02 (0.59-1.32) V, 0.56 (0.40-1.15) V, 0.78 (0.45-1.32) V in the LQBW, MBW and HQBW groups respectively (fig 3.3). There were no significant differences between the three groups (p=0.262). The acetylcholine responses were not related to the birth weight, birth length, head circumference, placenta weight, current weight. However, there was once again a sex difference in that the boys had a higher response to acetylcholine (1.03 (0.70-1.33)V) compared to the girls (0.57 (0.32 -1.23)V) (p=0.011).

3.3.4 Capillary Density

Due to the difficulty in keeping the subjects relatively immobile for video microscopy of the foot, good images of foot skin capillaries were recorded for only 19 LQBW, 7 MBW and 17 HQBW infants. The mean capillary density for the LQBW, MBW and HQBW infants were 49.0 (44.1-67.9) mm$^2$, 58.8 (57.1-64.4) mm$^2$ and 44.1 (41.7-56.0) mm$^2$ respectively (fig 3.4). The capillary density were not different among the three groups (p=0.147) and there were also no sex differences.
3.3.5 Parental characteristics

Information regarding the parents was also obtained from some of the parents, notably, there was difficulty in securing information regarding parental birth weight. Maternal birth weight was known in 42 subjects and data on paternal birth weight was available for 24 subjects. Infant’s birth weight was found to correlate with maternal birth weight (Rs=0.338, p=0.033) and the correlation with paternal birth weight almost reached statistical significance (Rs=0.395, p=0.056). Maternal pre-pregnant weight did not however correlate with the infants’ birth weight (Rs=0.269, p=0.061). Placenta weight also correlated with maternal birth weight (Rs=0.505, p=0.001). Interestingly, paternal birth weight was found to correlate with the maximum hyperaemic response (Rs=0.450, p=0.028).

3.4.0 Discussion

This study has demonstrated that cutaneous microvascular vasodilatory function is influenced by birth weight at the age of three months. There was a graded maximum hyperaemic response to local heating with birth weight, such that a reduced response was seen in LQBW babies compared to their HQBW counterparts. In contrast skin capillary density and the vasodilatory response to iontophoresed acetylcholine were similar in the three groups.

A similar defect in cutaneous maximal hyperaemia induced by local heating has been described in subjects with a recent diagnosis of type 2 diabetes (Jaap et al. 1992) as well as in adults with fasting hyperglycaemia (glucose concentration between 5.5 – 7.8mmol/l), who are at increased lifetime risk of developing type 2 diabetes (Jaap et al. 1994). A further study revealed a correlation between maximum hyperaemic
response and calculated insulin sensitivity in subjects with fasting hyperglycaemia (Jaap et al. 1997), supporting the hypothesis that microvascular functional derangement may be linked with, or be a common antecedent of the insulin resistance syndrome. In normoglycaemic adults the maximum hyperaemic response to local heating was inversely correlated with PAI-1 level (Tooke et al. 1999), itself a feature of the insulin resistance syndrome (Juhan-Vague et al. 1997). Our study has now demonstrated that this response was also impaired in subjects with low birth weight, which has been linked with the increased risk of developing type 2 diabetes and cardiovascular disease in adult life.

The determinants of heat induced vasodilatation remain to be elucidated but at a theoretical level could involve structural factors (microvascular density and microvascular compliance), neural, endothelial and vascular smooth muscle function. Previous studies indicated that cutaneous capillary density was not reduced in normotensive type 2 diabetic patients (Jaap et al. 1996) and in the present study, LQBW babies at risk of this condition had similar baseline capillary density as the HQBW babies. It should be emphasised that the measurements of capillary density obtained in the present study were measures of the number of perfused capillaries at that time. It has been previously demonstrated that the application of a cuff to cause venous occlusion results in an increased number of visible capillaries i.e. capillary recruitment occurs (Katz et al. 1989). The inflation of a cuff around the infant’s ankle to achieve capillary recruitment induced too much movement artefact in this study. It was therefore not possible to confirm or refute the observations of Serne et al., suggesting that skin capillary recruitment correlated with both insulin sensitivity and birth weight in young healthy adults (Serne et al. 1999).
Our study did not reveal a graded relationship between birth weight and endothelium-dependent vasodilatory function. There is however considerable evidence in the literature that impaired endothelium-dependent vasodilatation may precede the development of diabetes and may even contribute to the development of diabetes per se by interfering with insulin induced muscle hyperaemia in the postprandial state thereby limiting glucose disposal (Bergman 1997). In adult subjects with fasting hyperglycaemia microvascular endothelium-dependent vasodilatation assessed in a similar manner to the current study was found to be impaired whereas endothelium-independent vasodilatation was intact (Morris et al. 1996). Similarly endothelium-dependent but not endothelium-independent function was found to be impaired in subjects with impaired fasting glucose (glucose concentration 6.1 – 7.0 mmol/l) by measuring forearm blood flow responses to intra-arterial infusion of vasoactive agents (Vehkavaara et al. 1999). Further evidence that endothelium-dependent vasodilatation might be impaired in subjects at risk of developing type 2 diabetes comes from studies on normoglycaemic women with a past history of gestational diabetes who were shown to have impaired flow-mediated dilatation (a measure of conduit artery endothelium-dependent vasodilation) (Anastasiou 1998) and cutaneous microvascular response to iontophoresis of acetylcholine (Hu et al. 1998). Of particular relevance to the present study, Leeson et al revealed a correlation between flow mediated dilatation and birth weight in healthy school children (Leeson et al. 1997). Similarly Goodfellow et al found an impairment in endothelium-dependent vasodilatation in the conduit arteries of young adults of low birth weight compared to normal birth weight counterparts (Goodfellow et al. 1998). McAllister et al examined the forearm blood flow response to intra-arterial infusion of acetylcholine and sodium nitroprusside in twelve young adults with low birth weight compared to normal weight control.
subjects (McAllister et al. 1999). The low birth weight subjects were found to have elevated von Willebrand factor (regarded as a circulating marker of endothelial activation/damage) but the blood flow responses to acetylcholine and nitroprusside were not impaired.

Martin and colleagues reported an impairment in endothelium-dependent vasodilatation in small-for-gestational age infants compared to the appropriate-weight-for-gestational-age infants but the maximum hyperaemic responses to local heating were similar between the two groups (Martin et al. 2000). The different findings from this present study may be related to the timing of the study; Martin and colleagues studied the infants at the age of 3-4 days while the subjects in this study were examined at the age of 3 months. In the first few days of life, dramatic functional and structural changes are still occurring in the macro- and microcirculation as adaptation to the extrauterine environment takes place (Ryan 1998). Indeed previous studies using laser Doppler flowmetry have documented marked changes in skin blood flow during the first five days of life (Suiches et al. 1990). At the age of 3 months, the transient changes in blood volume (Oski et al. 1982) and metabolism that follow parturition have largely stabilised and the maturation of biological rhythms of the cardiovascular and thermoregulatory system would have occurred (Glotzbach et al. 1994; Jahnukainen et al. 1996). Of particular relevance is the observation that haematocrit, a key determinant of blood viscosity and hence red cell flux, is reported to be high in low birth weight neonates in the first few days of life (Guajardo et al. 1994), which may result in profound reduction in skin blood flow in 3 day old small-for-gestational-age infants. Martin and colleagues argued that the venous haematocrit was found to be normal in all their subjects although the comparison between the haematocrit measures between the two groups
of infants was not performed. Another important difference between this present study and Martin’s study was the recruitment criteria for low birth weight subjects. The small-for-gestational-age (SGA) subjects in Martin’s study had birth weight below the third centile (mean weight 2510g ± 270g) and 12 out of the 20 SGA subjects had evidence of impaired uterine growth based on ultrasound fetometry and umbilical Doppler flow velocity. Among the LQBW subjects in this study, only 2 out of 20 had birth weight below the third centile and the median birth weight of our subjects was 3070 (2778-3244)g. Perhaps impaired endothelium-dependent vasodilatory response may be found in those with extreme low birth weight or who exhibited intrauterine growth retardation and therefore not demonstrated in the LQBW subjects. There were also methodological differences between the two studies. Control experiments with iontophoresis of drug vehicle was not performed on all the infants in Martin’s study and the rationale was that no increase in vascular response was observed when iontophoresis with sodium chloride was performed in some of the infants. The authors have however used water rather than sodium chloride as the drug vehicle in the acetylcholine experiments. Endothelium-dependent function and maximum hyperaemic response were both tested on the same skin site (hand) in Martin’s study while different skin sites were used for iontophoresis (abdomen) and the assessment of maximum hyperaemic response (thigh) in this present study. Different skin sites were used to avoid the possibility that using the same skin site that has been challenged with a vasodilating agent in a previous test may affect the response elicited with respect to the second microvascular function test. It was impossible to perform all the investigations of microvascular function in the same skin region in an infant due to the small skin areas available and the desire to avoid close contact between the infants’ eyes and the mercury vapour light source used in the estimation of capillary
density. However, the use of different skin sites is unlikely to alter the interpretation of the present data because previous studies have demonstrated impaired maximum hyperaemic responses on abdomen (Rayman et al. 1986) (Boolell et al. 1990) as well as on the foot with a good correlation between the two measurements in type 1 diabetes (Rayman et al. 1986) and impaired microvascular responses of both the foot and the forearm of type 2 diabetic (Morris et al. 1995) (Sandeman et al. 1991) and fasting hyperglycaemic subjects (Jaap et al. 1993).

Although this study has failed to demonstrate an impairment of endothelium-dependent vasodilatation in LQBW babies at 3 months of age, the results do not negate the possibility that low birth weight subjects could have an inherited propensity or have been programmed to develop endothelial dysfunction. Besides the production of vasoactive substances, the endothelium has several other functions such as the regulation of haemostasis and cell growth. As this present study has only examined the endothelium-dependent vasodilatory capacity in the skin microcirculation, the possibility that other aspects of endothelial function could be impaired at the age of 3 months cannot be excluded. It is also plausible that endothelial dysfunction may only manifest in childhood or early adulthood in at risk subjects as accompanying subtle metabolic changes emerge or perhaps through the accumulation of visceral fat to which such individuals may be predisposed (Law et al. 1992). Seven year-old black South African children who were small at birth but heavy at the time of the study were found to have higher indices of obesity and exhibited abnormalities in glucose handling and insulin secretion after a glucose load (Crowther et al. 1998). Greater levels of insulin resistance syndrome variables (systolic blood pressure, fasting insulin level, 30-minute post-glucose insulin and glucose concentration and skinfold thickness) and cholesterol level were also reported.
in Indian children who were small at birth but obese at 8 years of age (Bavdekar et al. 1999). In keeping with these findings, a study in 7 year old children in Salisbury also revealed higher plasma glucose levels in response to a glucose challenge in those who were thin at birth and fasting and 30-minute insulin responses were greatest in those who were heaviest at age 7 (Law et al. 1995). Thus the combination of low birth weight and rapid catch-up growth appears to have a negative impact on blood pressure and glucose profiles and may also adversely affect endothelial function.

Interestingly, a gender difference in maximum hyperaemic response and endothelium-dependent vasodilatation was also seen in this group of infants, with the female infants demonstrating lower responses. The number of subjects in each birth weight group was too small and hence there was insufficient power to permit the comparison of the microvascular responses between the sexes within each weight category. The birth weights for the girls were not significantly different from the boys although their weight at the time of the study were lower than the boys. However, the maximum hyperaemic response and the response to iontophoresis of acetylcholine were not correlated with the weight at 3 months of age. Cooke et al. have previously shown that in adults, men have a higher resting hand and finger blood flow and also skin perfusion compared to women (Cooke et al. 1990). However, after total body warming (induction of thermal sympatholysis), hand blood flow in women exceeded that of men, suggesting that lower basal blood flow in women may be due to elevated sympathetic tone compared to men. Thus it is possible that the regulatory mechanisms of the skin circulation in the infants may also show sex differences at the age of three months.

Maternal birth weight rather than the maternal pre-pregnant weight has been found to correlate with the infant’s birth weight and placenta weight in our subjects. This
would suggest that the regulation of growth of the fetus and the placenta may begin before pregnancy (Robinson et al. 2000). It is plausible that genetic factors may be more important than intra-uterine factors in determining fetal size, provided the pregnancy proceeds normally and is not compromised by disorders like pre-eclampsia, maternal smoking and infections. Animal models have shown that the growth of fetuses of hypertensive rats is reduced regardless of the strain (hypertensive or normotensive) of their maternal surrogates (Nicolantonio et al. 2000). The correlation between infants’ birth weight and the paternal birth weight failed to reach statistical significance and this may be related to the small number of subjects where paternal birth weight were available. The association between paternal birth weight and the maximum hyperaemic response however raises the possibility that paternal genetic factors may be important for the development of fetal vasculature since the father does not contribute to the intra-uterine environment of the infant. Obviously this data is somewhat limited in that the parental birth weight information was subject to recall bias.

3.5.0 Conclusion

In conclusion, this study has demonstrated that cutaneous maximum hyperaemic response is reduced in 3 month-old infants in the lowest-quartile birth weight group who are at increased risk of developing type 2 diabetes and cardiovascular disease in adult life. This difference does not appear to be associated with reduced baseline capillary density or the impairment of endothelium-dependent vasodilatation in this vascular bed at this stage in life. Nevertheless the finding of a lower response in this LQBW group soon after birth is compelling evidence that reduced microvascular vasodilatory function is an early antecedent to diabetes in later life.
Table 3.1 Characteristics of the study subjects: lowest-quartile birthweight (LQBW), middle birthweight (MBW) and highest-quartile birthweight (HQBW) infants.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LQBW (n)</th>
<th>MQBW (n)</th>
<th>UQBW (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight (g)</td>
<td>3070 (2778-3244)</td>
<td>3532 (3311-3667)</td>
<td>3920 (3750-4020)</td>
</tr>
<tr>
<td>(weeks)</td>
<td>(20)</td>
<td>(8)</td>
<td>(21)</td>
</tr>
<tr>
<td>Gestation period</td>
<td>40.0 (39.0-40.0)</td>
<td>40.0 (38.3-41.0)</td>
<td>40 (38.5-40.0)</td>
</tr>
<tr>
<td>(weeks)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>8/12</td>
<td>3/5</td>
<td>12/9</td>
</tr>
<tr>
<td>Heart rate (min⁻¹)</td>
<td>147 (130-160)</td>
<td>153 (139-158)</td>
<td>152 (142-157)</td>
</tr>
<tr>
<td></td>
<td>(19)</td>
<td>(8)</td>
<td>(17)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>100 (95-109) (11)</td>
<td>95 (84-106) (4)</td>
<td>101 (90-112) (12)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>62 (50-70) (11)</td>
<td>63 (50-68) (4)</td>
<td>67 (53-83) (12)</td>
</tr>
</tbody>
</table>

Median (interquartile range)

(n) - number of subjects where information was obtained
Figure 3.1 Skin maximum hyperaemic response to local heating in the lowest-quartile birthweight (LQBW), middle birthweight (MBW) and highest-quartile birthweight (HQBW) infants.

Maximum hyperaemic response is represented by red cell flux, which is a measure of the number and the velocity of red cells expressed in arbitrary units of volts (V).
Figure 3.2 Relationship between skin maximum hyperaemic response to local heating and head circumference at birth.

Maximum hyperaemic response is represented by red cell flux, which is a measure of the number and the velocity of red cells expressed in arbitrary units of volts (V).
Skin microvascular response to acetylcholine iontophoresis in the lowest-quartile birthweight (LQBW), middle birthweight (MBW) and highest-quartile birthweight (HQBW) infants.

Skin microvascular response is represented by red cell flux, which is a measure of the number and the velocity of red cells in arbitrary units of volts (V).
Fig. 3.4 Skin capillary density in the lowest-quartile birthweight (LQBW), middle birthweight (MBW) and highest-quartile birthweight (HQBW) infants.

\[ p = 0.247 \]

\[ p = 0.259 \]

\[ p = 0.065 \]
4.0 The relationship between birth weight and skin microvascular function in childhood

4.1.0 Introduction

In the previous chapter, we have demonstrated that low birth weight infants have an impaired maximum hyperaemic response to local heating compared to their higher birth weight counterparts at the age of three months. Low birth weight has been linked with a greater risk of developing the metabolic syndrome and cardiovascular disease in adulthood. This would suggest that microvascular vasodilatory reserve is reduced in early life in those at risk of developing type 2 diabetes and may been an intrinsic feature of the insulin resistant state.

However, it is not known if this relationship between birth weight and microvascular responses in early infancy would still be present in childhood as the exposure to various environmental stimuli occur. At present it is also unclear if the passage through puberty, which is a growth phase characterised by relative insulin resistance, may also influence microvascular function and modify its relationship with birth weight. Hence this study aims to investigate the relationship between skin microvascular function and birthweight in prepubertal and postpubertal subjects.

4.2.0 Method

4.2.1 Subjects

84 healthy children were recruited from the local Exeter community to act as healthy control subjects for a study looking at skin microvascular function in early type 1 diabetes. The study was approved by the local Exeter Medical Research Ethics
Committee and consent was obtained from each child and their guardian for participation in the study. Each child was assessed for pubertal stage using the criteria of Tanner (Dattani et al. 1997). Measurements of height and weight were recorded to facilitate the calculation of body mass index (BMI), BMI centile and percentage BMI for age using Cole BMI slide rule (Cole et al. 1981).

The studies were conducted after a 30-minute acclimatisation period in a temperature-controlled environment (21.5-22.5°C). Blood pressure was measured by the oscillometric technique (Dinamap, Critikon, Tampa, Florida, USA) six times during the study (two periods of three measurements recorded at one-minute intervals). The mean of the final two readings for each period was taken as the subject’s true blood pressure. Skin temperature was recorded continuously using a thermocouple (Comark Electronic, Littlehampton, Sussex, UK) attached to the dorsum of the foot and the pulp of the big toe.

4.2.2 Measurement of skin blood flow

The skin blood flow is measured using the laser fluximeter (Perimed Pf2B, Perimed, Stockholm, Sweden), which has been previously described in section 2.2.1.0.

4.2.3 Postural changes in skin blood flow

This has been described in section 2.4.0.

4.2.4 Skin maximum hyperaemic response to local heating

This has been described in section 2.5.0. Maximum hyperaemic response (mhrh) was determined by taking the mean value of the 8 heated sites with the foot in the horizontal position. Maximum hyperaemic response in a dependent position (mhrd)
was measured by taking the mean value of the 8 heated sites during the 4th and 5th minute after the foot has been passively lowered to 40cm below heart level with the knee bent. Distensibility (dist) of the blood vessels was expressed as the maximum hyperaemic response with the foot in the dependent position divided by the maximum hyperaemic response with the foot in the horizontal position. Minimum vascular resistance (minvr) was calculated by dividing the mean arterial blood pressure of the subjects by the maximum hyperaemic response (mhrh).

4.2.5 Birth weight data

Letters were sent to the subjects or their parents (if the subjects have moved) in 1999 to obtain their permission in order to review their birth records. Only subjects who were studied at the prepubertal (p1g1) or the postpubertal (p5g5) stages and whose birth weight could be validated by hospital birth records were included in this analysis.

4.2.6 Statistical analysis

The data are represented as the median value with interquartile range in the text. Associations between variables were assessed by Spearman’s rank correlation coefficient.

4.3.0 Results

There were 19 prepubertal and 25 postpubertal subjects in whom birth weight data were available; 6 of these subjects have been studied in both the prepubertal and postpubertal stages. Table 4.1 illustrates the characteristics of the subjects. Data on maximum hyperaemic response to local heating and pulp blood flow were obtained
from all 19 prepubertal and 25 postpubertal subjects. Dorsum blood flow data were recorded in 15 prepubertal and 19 postpubertal subjects. Table 4.2 shows the results of the microvascular function tests in the two groups.

4.3.1 Prepubertal group

In the prepubertal group, birth weight was found to correlate with diastolic blood pressure ($Rs=0.581$, $p=0.009$) but not with systolic blood pressure or heart rate. However, weight and BMI at the time of the study did not correlate with the blood pressure or the heart rate.

There were no significant correlations between birth weight and the microvascular function tests (Table 4.3). Figure 4.1 illustrates the relationship between birth weight and skin maximum hyperaemic response. Similarly, weight, BMI and percentage BMI for age at the time of the study did not appear to influence the microvascular function tests (Table 4.3).

4.3.2 Postpubertal group

In the postpubertal group, birth weight did not correlate with the heart rate or the blood pressure. Weight of the postpubertal subjects at the time of the study correlated positively with systolic blood pressure ($Rs=0.500$, $p=0.011$) and there was also a weak negative correlation with heart rate ($Rs=-0.412$, $p=0.041$).

There were however no significant correlations between birth weight and microvascular function tests (Table 4.4). Figure 4.2 illustrates the relationship between birth weight and skin maximum hyperaemic response. Again the weight, BMI and percentage BMI for age at the time of the study did not appear to influence the microvascular parameters (Table 4.4).
4.4.0 Discussion

These results indicate that birth weight in pre- and postpubertal subjects do not appear to influence the postural vasoconstriction reflex and the maximum hyperaemic response to local heating. This is in contrast to the previous study in 3 month-old infants, where the maximum hyperaemic response was found to be impaired in the lowest-quartile birth weight infants compared to their highest-quartile birth weight counterparts.

In healthy subjects, a change in posture from lying to standing invokes an increase in the precapillary resistance in the skin of the foot, which serves to minimise the increase in capillary pressure arising from a posturally-induced increase in hydrostatic pressure. The postural vasoconstriction response is complex, involving a local neurogenic mechanism with a small contribution from a local myogenic response, in addition to a centrally elicited sympathetic component (Hassan et al. 1988). In both the prepubertal and postpubertal groups, the postural vasoconstriction response was not related to weight at birth. Previously this response has been found to be poor in prepubertal subjects, which improved in the postpubertal state (Shore et al. 1994). This suggests that this reflex is acquired through the passage of puberty, perhaps mediated by the effect of pubertal hormonal changes on the regulatory mechanisms controlling the skin microcirculation. Birth weight does not appear to influence the acquisition of this reflex.

As discussed previously, the maximum hyperaemic response to local heating is a robust test to investigate the skin microvascular capacity to respond to an injurious insult. This response was found to be abnormal in type 2 diabetes, pre-diabetes and those at increased lifetime risk of developing type 2 diabetes (Sandeman et al. 1991) (Jaap et al. 1994) (Liddell et al. 1998). Maximum hyperaemic response was also
found to correlate with features of the insulin resistance syndrome, such as raised triglycerides, total cholesterol and PAI-1 levels, in a group of healthy subjects with and without family history of type 2 diabetes (Tooke et al. 1999). As discussed in the previous chapter, an impaired hyperaemic response is likely to involve either structural factors (microvascular density and microvascular compliance) or neural, endothelial and vascular smooth muscle function. Capillary rarefaction does not appear to be a likely candidate mechanism for a reduced hyperaemic response in diabetic and pre-diabetic groups. Adult patients with type 2 diabetes and those with impaired glucose tolerance who have been shown to have a reduced hyperaemic response to local heating also do not demonstrate skin capillary rarefaction (Jaap et al. 1996). In our previous studies in 3 month-old infants, there was no evidence of capillary rarefaction in the lowest-quartile birth weight infants despite a reduction in hyperaemic response. However the possibility of a reduction in the numbers of other blood vessels (e.g. venules and arterioles) in the microvascular bed cannot be excluded and the subpapillary vessels do account for a large proportion of the laser Doppler flux signals. The other factors that are likely to mediate the hyperaemic response have yet to be fully investigated.

The present study did not demonstrate a relationship between birth weight and maximum hyperaemic response in children and adolescents, which contrasts the study findings in infants. To investigate the possibility that the maximum hyperaemic response may be dependent on the systemic blood pressure, minimum vascular resistance was calculated for the prepubertal and postpubertal subjects. However, there was also no significant correlation between birth weight and minimum vascular resistance. One could postulate that skin blood vessels in the low birth weight infants may be structurally or functionally limited compared to their higher birth weight.
counterparts at the age of three months but through the passage of childhood, other extrinsic factors may become more important in modifying the skin microvasculature. Environmental factors such as childhood nutrition, exercise and obesity may play a role. Various studies have linked low level of physical activity and obesity with an unfavorable lipid profile in childhood (Schmidt et al. 1997) (Stewart et al. 1995). Recent studies have raised the possibility that the clustering of common risk factors of coronary heart disease (features of the insulin resistance syndrome) may already be present in childhood (Arslanian et al. 1996) (Raitakari et al. 1994). There is also evidence that insulin resistance and hyperinsulinaemia co-exist in children with moderate and severe obesity (Caprio et al. 1996). In this study, the body weight and BMI at the time of the study did not correlate with the maximum hyperaemic response or postural vasoconstriction response although none of the prepubertal subjects and only one of the postpubertal subjects had a BMI of greater than 25. It has been proposed that the definition of adult obesity (BMI> 25) is not applicable to children and childhood obesity should be assessed using age-related BMI centile charts (Cole et al. 1981) (Cole 2000). Again, only 2 of the prepubertal and 3 of the postpubertal subjects in the present study have a BMI greater than the 90th centile and percentage BMI for age for both groups did not appear to affect the microvascular responses. However, some studies have argued that these parameters may not be sensitive measures of adiposity in children and adolescents. Arslanian et al. have demonstrated that in non-obese normal children at ages as early as 8 years old, high percentage body fat determined by $^{18}$O enrichment of expired carbon dioxide rather than BMI, was associated with the reduced insulin sensitivity and raised total cholesterol and low density lipoprotein levels (Arslanian et al. 1996).
To date, there is one other study looking at the effect of birth weight and vascular function in childhood by Leeson et al., who has shown a graded relationship between birth weight and vascular function in children between the ages of 9-11 (Leeson et al. 1997). The difference with the findings of the present study could be attributed to the differences in the size of the blood vessels under study; they examined the brachial artery vasoactivity while the current study was concerned with the skin microcirculation. Moreover, both studies examined different aspects of vascular function; Leeson’s group studied the flow-mediated dilatation of the brachial artery, which is a test of endothelium-dependent function while this study looked at the skin microvascular response to postural changes, which is dependent on a number of regulatory reflexes, and also to heat stress, where the underlying mechanism is still unclear. Notably, the subjects in Leeson’s groups were not staged for sexual maturity. How is it possible to reconcile the fact that birth weight is linked to abnormal vascular function in infants and also to the increased risk of adult cardiovascular disease and metabolic syndrome but not associated with abnormal vascular reactivity in childhood in this present study? The period of childhood and puberty is a time of dramatic changes, characterised not only by rapid somatic growth but also sexual maturation, which can both influence vascular function (Elhadd et al. 1998) (Shore et al. 1994) (Duckles et al. 1996). In this study, we have looked at children within p1g1 and p5g5 stages in order to control for the level of sexual maturity but this does not take into account the other parameters of growth like height and weight. By studying postpubertal subjects, one would hope that these changes would have begun to stabilise but it has also been reported that insulin levels peak in postpubertal boys before it begins to decrease and level off in young adult life (Taittonen et al. 1997). This may account for the fact that the link between birth weight and adult disease is
stronger in adulthood as it would not be masked by the confounding factors encountered in puberty.

In contrast to the previous study on 3 month-old infants, no gender differences in the microvascular responses have been demonstrated in the pre- and postpubertal subjects. This may be related to the small sample size of the current study compared to the infant study. However, in the post-pubertal subjects, systolic blood pressure was higher in the boys while heart rate and diastolic blood pressure were higher in the girls.

The relationship between growth and development on blood pressure in children is complex. Results from various studies as to which factors are the most important determinants of blood pressure in childhood are conflicting. However, body weight, height, gender, sexual maturation, ethnicity, parental blood pressure all appear to have a role in varying degrees (Gerber et al. 1999). Accordingly, we have demonstrated that childhood weight correlates with systolic blood pressure in postpubertal children in our study. Both systolic and diastolic blood pressure increase through puberty and there is also evidence that there is tracking of blood pressure from childhood to adulthood. The relationship between birth weight and blood pressure in childhood is also controversial, with some studies demonstrating an inverse correlation between the two parameters (Leeson et al. 1997) (Thame et al. 2000) while others did not report any significant findings (Matthes et al. 1994) (Rabbia et al. 1999). One such study revealed that birth weight only had a significant bearing on childhood blood pressure in obese children (Bergel et al. 2000). It is difficult to compare these studies due to the difference in their design; some studies have staged the subjects according to sexual developmental status while others have simply studied children within a specific age group. Moore et al. have shown that the relationship between birth
characteristics and blood pressure amplifies from childhood to adulthood (Moore et al. 1999). This is thought to be due to the increase in standard deviation of blood pressure that occurs with age or perhaps due to the greater rises in blood pressure among those individuals with a low birth weight. It has also been suggested that the inconsistent results from the studies looking at birth weight and blood pressure in childhood may be caused by perturbations in blood pressure caused by adolescent growth spurt.

Limitations

Blood samples were not obtained from the participants of the above study to facilitate measures of insulin sensitivity and blood glucose levels. Other measures of adiposity were also not available such as waist and hip measurements, skinfold thickness or more sophisticated measures like under-water weighing and MRI scanning techniques.

Many studies have stated that it was often subjects who were small at birth who then became obese in childhood (rapid catch-up growth) were at a greater risk of developing adult disease compared to those who remained slim (Law et al. 1995) (Bavdekar et al. 1999). However, the number of subjects in the current study was too small to permit stratifying the subjects into subgroups based on their birth weight and current weight.

In the infant study where there was a demonstrable difference in maximum hyperaemic response between lowest-quartile and highest-quartile birth weight infants, the subjects were selected from both extremes of birth weight (birth weight less than the 25th centile or greater than the 75th centile). In this present study, there were only 7 prepubertal subjects with birth weight below the 25th centile and only 2
prepubertal subjects with birth weight above the 75th centile. In the postpubertal
group, there were 5 subjects with birth weight below the 25th centile and 8 subjects
with birth weight greater than the 75th centile. There were too few subjects in the
lowest and highest birth weight quartiles to permit the comparison of microvascular
responses between the contrasting groups. Thus the birth weight of approximately half
of the subjects in both the prepubertal and postpubertal groups lie between the 25th to
75th centile and this narrow distribution in birth weight centiles may have possibly
influenced the negative findings in the study.

4.5.0 Conclusion

In conclusion, birth weight does not appear to have an effect on the skin
microvascular function in prepubertal and postpubertal subjects. It is possible that
extrinsic factors such as diet, physical activity and obesity may be more important in
determining the microvascular responses in this age group. Alternatively, the
relationship between birth weight and microvascular function may be temporarily
obscured in childhood and adolescence by the compounding effects of rapid somatic
growth and sexual maturation characteristic of this period.
Table 4.1 Characteristics of the subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prepubertal group</th>
<th>Postpubertal group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>9.3 (8.3-10.7)</td>
<td>15.9 (15.1-17.3)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>12 / 7</td>
<td>11 / 14</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.35 (1.30-1.40)</td>
<td>1.70 (1.64-1.76)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>28.0 (26.3-35.4)</td>
<td>60.2 (52.0-67.2)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>16.4 (14.9-17.7)</td>
<td>20.5 (18.8-22.2)</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3310 (3125-3807)</td>
<td>3684 (3284-3853)</td>
</tr>
<tr>
<td>Gestation age (weeks)</td>
<td>39.5 (38.0-41.0)</td>
<td>39.0 (37.0-39.5)</td>
</tr>
<tr>
<td>Heart rate (min⁻¹)</td>
<td>76 (68-80)</td>
<td>63 (59-70)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>101 (98-113)</td>
<td>115 (112-118)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>63 (55-65)</td>
<td>59 (58-62)</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>75 (72-80)</td>
<td>77 (74-81)</td>
</tr>
</tbody>
</table>

Median (interquartile range)
Table 4.2 Results of the microvascular function tests in the prepubertal and postpubertal subjects.

<table>
<thead>
<tr>
<th>Microvascular function test</th>
<th>Prepubertal group</th>
<th>Postpubertal group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsum flow horizontal (dfh) (V)</td>
<td>0.03 (0.02-0.06)</td>
<td>0.03 (0.02-0.04)</td>
</tr>
<tr>
<td>Dorsum flow dependent (dfd) (V)</td>
<td>0.02 (0.01-0.04)</td>
<td>0.01 (0.01-0.01)</td>
</tr>
<tr>
<td>Dorsum postural vasoconstrictor response (dpv=dfd/dfh*100) (%)</td>
<td>53.2 (40.2-70.4)</td>
<td>36.3 (21.8-51.4)</td>
</tr>
<tr>
<td>Pulp flow horizontal (pfh) (V)</td>
<td>0.75 (0.28-1.58)</td>
<td>0.11 (0.06-0.18)</td>
</tr>
<tr>
<td>Pulp flow dependent (pfd) (V)</td>
<td>0.51 (0.08-0.86)</td>
<td>0.03 (0.01-0.06)</td>
</tr>
<tr>
<td>Pulp postural vasoconstrictor response (ppv=pfd/pfh*100) (%)</td>
<td>60.4 (35.0-97.8)</td>
<td>26.7 (14.5-34.8)</td>
</tr>
<tr>
<td>Maximum hyperaemic response horizontal (mhrh) (V)</td>
<td>1.72 (1.52-2.03)</td>
<td>1.57 (1.13-1.84)</td>
</tr>
<tr>
<td>Maximum hyperaemic response dependent (mhrd) (V)</td>
<td>2.21 (1.82-2.87)</td>
<td>1.81 (1.67-2.52)</td>
</tr>
<tr>
<td>Distensibility (mhrd/mhrh)</td>
<td>1.28 (1.19-1.41)</td>
<td>1.28 (1.18-1.52)</td>
</tr>
<tr>
<td>Minimum vascular resistance (MABP/mhr) (mmHg/V)</td>
<td>44.4 (38.9-52.0)</td>
<td>50.3 (41.7-65.5)</td>
</tr>
</tbody>
</table>

Median (interquartile range)
Table 4.3 The relationship between birth weight, childhood weight, body mass index (BMI), percentage BMI for age and microvascular function tests in prepubertal subjects.

<table>
<thead>
<tr>
<th>Microvascular function test</th>
<th>Spearman rank coefficient (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight * dpv</td>
<td>Rs = -0.046 (p = 0.869)</td>
</tr>
<tr>
<td>Birth weight * ppv</td>
<td>Rs = -0.060 (p = 0.808)</td>
</tr>
<tr>
<td>Birth weight * mhrh</td>
<td>Rs = 0.088 (p = 0.721)</td>
</tr>
<tr>
<td>Birth weight * dist</td>
<td>Rs = -0.321 (p = 0.180)</td>
</tr>
<tr>
<td>Birth weight * mvr</td>
<td>Rs = 0.151 (p = 0.538)</td>
</tr>
<tr>
<td>Weight * dpv</td>
<td>Rs = -0.227 (p = 0.416)</td>
</tr>
<tr>
<td>Weight * ppv</td>
<td>Rs = -0.439 (p = 0.060)</td>
</tr>
<tr>
<td>Weight * mhrh</td>
<td>Rs = 0.157 (p = 0.521)</td>
</tr>
<tr>
<td>Weight * dist</td>
<td>Rs = -0.198 (p = 0.416)</td>
</tr>
<tr>
<td>Weight * mvr</td>
<td>Rs = -0.075 (p = 0.762)</td>
</tr>
<tr>
<td>BMI * dpv</td>
<td>Rs = 0.004 (p = 0.990)</td>
</tr>
<tr>
<td>BMI * ppv</td>
<td>Rs = -0.189 (p = 0.437)</td>
</tr>
<tr>
<td>BMI * mhrh</td>
<td>Rs = 0.023 (p = 0.926)</td>
</tr>
<tr>
<td>BMI * dist</td>
<td>Rs = -0.212 (p = 0.383)</td>
</tr>
<tr>
<td>BMI * mvr</td>
<td>Rs = -0.035 (p = 0.887)</td>
</tr>
<tr>
<td>% BMI for age * dpv</td>
<td>Rs = 0.005 (p = 0.985)</td>
</tr>
<tr>
<td>% BMI for age * ppv</td>
<td>Rs = 0.007 (p = 0.977)</td>
</tr>
<tr>
<td>% BMI for age * mhrh</td>
<td>Rs = -0.129 (p = 0.600)</td>
</tr>
<tr>
<td>% BMI for age * dist</td>
<td>Rs = -0.187 (p = 0.444)</td>
</tr>
<tr>
<td>% BMI for age * mvr</td>
<td>Rs = 0.118 (p = 0.630)</td>
</tr>
</tbody>
</table>
Abbreviations:

dpv – dorsum postural vasoconstriction response

ppv – pulp postural vasoconstriction response

mhrh – maximum hyperaemic response horizontal

dist – distensibility

mvr – minimum vascular resistance

weight – weight at the time of the study

bmi – body mass index at the time of the study

% bmi for age – percentage BMI for age
Table 4.4 The relationship between birth weight, childhood weight, body mass index (BMI) and percentage BMI for age and microvascular function tests in postpubertal subjects.

<table>
<thead>
<tr>
<th>Microvascular function test</th>
<th>Spearman rank correlation (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight * dpv</td>
<td>Rs = -0.249 (p = 0.304)</td>
</tr>
<tr>
<td>Birth weight * ppv</td>
<td>Rs = -0.069 (p = 0.743)</td>
</tr>
<tr>
<td>Birth weight * mhrh</td>
<td>Rs = 0.118 (p = 0.575)</td>
</tr>
<tr>
<td>Birth weight * dist</td>
<td>Rs = -0.113 (p = 0.590)</td>
</tr>
<tr>
<td>Birth weight * mvr</td>
<td>Rs = -0.112 (p = 0.595)</td>
</tr>
<tr>
<td>Weight * dpv</td>
<td>Rs = -0.092 (p = 0.707)</td>
</tr>
<tr>
<td>Weight * ppv</td>
<td>Rs = -0.009 (p = 0.965)</td>
</tr>
<tr>
<td>Weight * mhrh</td>
<td>Rs = -0.250 (p = 0.228)</td>
</tr>
<tr>
<td>Weight * dist</td>
<td>Rs = 0.179 (p = 0.391)</td>
</tr>
<tr>
<td>Weight * mvr</td>
<td>Rs = 0.292 (p = 0.157)</td>
</tr>
<tr>
<td>BMI * dpv</td>
<td>Rs = -0.284 (p = 0.238)</td>
</tr>
<tr>
<td>BMI * ppv</td>
<td>Rs = -0.127 (p = 0.544)</td>
</tr>
<tr>
<td>BMI * mhrh</td>
<td>Rs = -0.087 (p = 0.679)</td>
</tr>
<tr>
<td>BMI * dist</td>
<td>Rs = 0.135 (p = 0.520)</td>
</tr>
<tr>
<td>BMI * mvr</td>
<td>Rs = 0.131 (p = 0.532)</td>
</tr>
<tr>
<td>% BMI for age * dpv</td>
<td>Rs = -0.395 (p = 0.094)</td>
</tr>
<tr>
<td>% BMI for age * ppv</td>
<td>Rs = -0.040 (p = 0.848)</td>
</tr>
<tr>
<td>% BMI for age * mhrh</td>
<td>Rs = -0.095 (p = 0.650)</td>
</tr>
<tr>
<td>% BMI for age * dist</td>
<td>Rs = 0.236 (p = 0.256)</td>
</tr>
<tr>
<td>% BMI for age * mvr</td>
<td>Rs = 0.0108 (p = 0.609)</td>
</tr>
</tbody>
</table>
Abbreviations:

dpv – dorsum postural vasoconstriction response

ppv – pulp postural vasoconstriction response

mhrh – maximum hyperaemic response horizontal

dist - distensibility

mvr – minimum vascular resistance

weight – weight at the time of the study

bmi – body mass index at the time of the study

% bmi for age – percentage BMI for age
Fig. 4.1 The relationship between birth weight and maximum hyperaemic response (horizontal) in prepubertal subjects.

Maximum hyperaemic response is represented by red cell flux, which is a measure of the number and the velocity of red cells expressed in arbitrary units of volts (V).
Fig. 4.2 The relationship between birth weight and maximum hyperaemic response (horizontal) in postpubertal subjects.

Maximum hyperaemic response is represented by red cell flux, which is a measure of the number and the velocity of red cells expressed in arbitrary units of volts (V).
5.0 The effect of a high-fat meal on the skin microvascular function in healthy subjects

5.1.0 Introduction

The association between cholesterol and coronary heart disease risk is well-established but this relationship appears to vary in populations with different diets (Verschuren et al. 1995). Traditionally, the low-density lipoprotein (LDL) is considered the atherogenic lipid fraction and atherogenic risk is quantified by its measurement. However, there is now growing evidence that individuals with coronary artery disease have postprandial abnormalities of lipid metabolism. High-fat diet associated triglyceride lipoprotein remnants have been suggested to be atherogenic (Slyper 1992). Patsch et al. found that high postprandial triglyceride concentration was highly predictive of coronary artery disease in a multivariate analysis including HDL-cholesterol (Patsch et al. 1992). Angiographic studies of coronary artery disease have demonstrated that dietary fat and serum lipoproteins in the form of intermediate-density lipoprotein (IDL) and very low-density lipoprotein (VLDL) are independent predictors of coronary artery disease risk (Phillips et al. 1993) (Watts et al. 1994). Postprandial rise in triglycerides has been associated with endothelial dysfunction. Vogel et al. studied the effect of a single high-fat meal on the brachial circulation in healthy individuals and demonstrated a transient impairment of flow-mediated vasodilatory function (endothelium-dependent function) associated with a rise in postprandial triglycerides (Vogel et al. 1997). Lundman has also demonstrated that an intralipid infusion, mimicking the consumption of a fatty meal, also reduced vascular reactivity in young healthy men (Lundman et al. 1997).
These studies on the effect of a lipid load on the vascular function have been performed mainly on large and medium-sized vessels and therefore, it is not known if transient hypertriglyceridaemia would exert a similar effect on the microcirculation. It may not be justified to extrapolate these results to the microcirculation, where the prevailing blood flow and pressure are of a different magnitude and the architecture of the vessel wall is different from the large blood vessels.

The present study investigates the effect of a single high-fat meal versus a fasting state on the skin microcirculation in healthy individuals. The objective is to ascertain if postprandial rise in lipids may affect microvascular function, similar to that observed in the large blood vessels.

5.2.0 Method

5.2.1 Subjects

6 healthy male volunteers were recruited from the community in Exeter. The subjects were all non-smokers with no history of ischaemic heart disease, peripheral vascular disease, hypertension, Raynaud's disease and none of the subjects were on any regular vasoactive medication or antioxidant treatment. The study was approved by the local Exeter Medical Research Ethics Committee.

Power calculations were based on change score data to permit the subsequent comparison of the results of the healthy volunteers in this study with the type 2 diabetic subjects described in chapter 6.0. This sample size provided a 90% chance of detecting a 40% reduction in vasodilatory function at 5% level of significance. The mean and variability (SD) in skin vasodilatory response to acetylcholine observed in the healthy subjects was 1.35±0.52 V. A 60% reduction in vasodilatory function was
the average reduction observed in previous studies investigating the effects of lipids on brachial artery vasoactivity (Vogel et al. 1997) (Plotnick et al. 1997) (Lundman et al. 1997).

5.2.2 Experimental Protocol

All the subjects underwent physical examination together with the measurement of height, weight, hip and waist circumference to facilitate the calculation of body mass index (BMI) and waist:hip ratio (WHR). Electrocardiogram was performed to exclude any evidence of ischaemic heart disease. Heart rate and blood pressure were recorded using a semi-automatic blood pressure recorder (Dinamap, Critikon, Tampa, USA) before each study. Non-steroidal medication was not permitted for at least 10 days prior to the study day. The subjects fasted for 12 hours overnight and also refrained from any undue physical exertion and caffeine intake on the day of the study.

This was a randomised, single-blind crossover study. The experiments were conducted under temperature-controlled environment (21.5 C -22.5 C) with the subjects lying supine in a quiet room. Skin temperature was monitored using a thermocouple (Comark Electronic, Littlehampton, Sussex UK). A venous cannula was inserted in the left antecubital fossa to facilitate blood sampling. After an acclimatisation period of 30 minutes, the subjects had baseline microvascular function measurement performed on the right forearm (pre study (a)). Following that, the subjects were randomised to be given either a standard high-fat meal or to continue to fast. The high-fat meal consisted of a standard milkshake (200ml full cream milk with 50ml double cream) and 2 slices of toasted white bread with 3 butter slices (17.1 g), 4 slices of cheese (24.4g) with a total fat content of 74.3g. After 2.5 hours, the subjects underwent measurement of microvascular function on the same arm (post study (b)),

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avoiding skin sites that have been used previously. The subjects returned on another
day, at least one week apart and the entire procedure was repeated such that those who
were given a high-fat meal previously would fast in the second study and vice versa.
The day where the subjects had to remain fasted throughout was designated ‘day 1’
and the day where the subjects received a high-fat meal was designated ‘day 2’. Table
5.1 illustrates the study design.

5.2.3 Skin microvascular function
The skin microvascular function was assessed by examining the skin vasodilatory
response to acetylcholine, an endothelium-dependent vasodilator, using the technique
of iontophoresis. The equipment and the technique used have been previously
described in detail (Morris et al. 1995) and in section 2.3. Three different sites on the
forearm skin were studied using 1% acetylcholine and control experiment was
performed with 3% mannitol (acetylcholine carrier) on one skin site. Endothelium-
independent vasodilatation of the skin was not examined. Preliminary studies
indicated that inclusion of endothelium-independent vasodilatation in the study
protocol would substantially increase the duration of the study and prolonged fasting
was not tolerated by the subjects. Previous studies have not demonstrated any
impairment of endothelium-independent vasodilatory function with oral fat loading

5.2.4 Blood sampling
Venous blood sampling was obtained before and after each iontophoresis study for
measurement of total cholesterol, HDL-cholesterol, triglyceride (Vitros 950
Analysers, Johnson & Johnson) and plasma glucose concentration (YSI, Yellow
Springs, Ohio, USA). Additional blood samples were obtained before and after the pre study (1a and 2a) for the measurement of insulin using an immunoenzymometric assay (Insulin-EASIA, Biosource, Nivelles, Belgium) calibrated against IRP 66/304 with no cross-reactivity with intact proinsulin or 32-33 split proinsulin. Interassay coefficients of variation were less than 10 % over the range of 95-1038pmol/L. This was to facilitate the calculation of the homeostasis model assessment (HOMA) estimates of insulin sensitivity and beta-cell function of the subjects (Matthews et al. 1985). Further blood samples were also obtained at the end of the post studies (1b and 2b) on the two days for the measurement of malondialdehyde, an estimate of oxidative stress using high performance liquid chromatography (HPLC) method (Hichrom HIRPB 150A 150x4.6 mm column) (Templar et al. 1999).

5.2.5 Statistical analysis
The anthropometric measures and blood results of the subjects were expressed as the median value with interquartile range. Results of the skin red cell flux were expressed in arbitrary units of volts (V). The cutaneous microvascular response to acetylcholine for each time-point was expressed as the skin red cell flux to acetylcholine minus the red cell flux to mannitol. The results of the overall skin red cell flux of the subjects were represented by the mean red cell flux ± standard error of the mean (SE) in the graphs. Analysis of variance for repeated measures was used to assess if acetylcholine iontophoresis changed skin red cell flux.

The peak skin vasodilatory response (plateau of the graph) was taken as the mean of the microvascular response to acetylcholine at 320s and 400s. The effect of the meal on peak skin vasodilatory response was assessed by comparing the difference between the peak skin vasodilatory response between the post and pre studies on day 1 (peak
response \( lb \) – peak response \( la \) versus the difference between the post and pre studies on day 2 (peak response \( 2b \) – peak response \( 2a \)) using non-parametric Wilcoxon signed ranks test. The difference between the lipoprotein fractions and glucose levels on the pre and post studies on the two days were also compared using Wilcoxon signed ranks test. Spearman-rank correlation coefficient was calculated where appropriate.

5.3.0 Results

Table 5.2 lists the characteristics of the healthy volunteers.

5.3.1 Skin microvascular function

Fig. 5.1 shows the mean (± SE) responses for the 6 subjects on the four iontophoresis studies. ANOVA for repeated measures indicated that iontophoresis of acetylcholine increased blood flow from baseline in all four studies (p<0.001 for studies \( 1a \), \( 1b \), \( 2a \) and \( 2b \)). Table 5.3 lists the peak skin vasodilatory responses for studies \( 1a \), \( 1b \), \( 2a \) and \( 2b \). The difference between peak skin vasodilatory response on the fasting day (\( 1b \) and \( 1a \)) versus the difference in peak vasodilatory response on the feeding day (\( 2b \) and \( 2a \)) was not statistically significant (p=0.075).

5.3.2 Biochemical parameters

Table 5.4 and 5.5 shows the lipid profile and blood glucose results of the subjects on day 1 (fasted) and day 2 (fed) respectively. The change in triglyceride level after feeding (peak triglyceride level (4) – baseline triglyceride (1)) on day 2 was significantly different from the triglyceride changes on day 1 (p= 0.028) and changes in HDL-cholesterol levels were also significantly different on the two days (p=0.046).
Cholesterol (p=0.683) and glucose profiles (p=0.463) on the two days were not
different. There was also no significant difference in MDA levels between the post
studies on day 1 and 2 (p = 0.686).

The median value of the HOMA estimates of insulin sensitivity of the subjects was
96.3 (85.4-165.8)% and beta-cell function was 110.3 (79.3-117.0)%. There was no
significant correlation between the insulin sensitivity and the change in triglyceride
levels on the day of feeding (Rs=0.714, p=0.111). The beta cell function was also not
predictive of the size of the postprandial triglyceride rise (Rs=-0.543, p=0.266).
Measures of obesity such as BMI (Rs=-0.200, p=0.704) and WHR (Rs=-0.029,
p=0.952) again did not correlate with the change in postprandial triglyceride levels.

5.3.3 Relationship between skin microvascular function and biochemical
parameters
On the day of fasting, none of the changes in lipoprotein and glucose measurements
correlated with the change in peak blood flow responses between the post (1b) and pre
studies (1a). On the day of feeding, the difference in the peak acetylcholine responses
between the post (2b) and pre study (2a) correlated strongly with the change in
triglyceride levels; that is, those with the larger reduction in blood flow responses
following the high-fat meal also had the greater postprandial triglyceride rise (Rs=-
0.943, p=0.005) (fig 5.2). The change in total cholesterol, HDL-cholesterol and
glucose levels did not correlate with the differences in peak acetylcholine responses.
Both the insulin sensitivity (Rs=-0.600, p=0.208) and beta cell function (Rs=0.600,
p=0.208) did not relate to the change in skin microvascular function.
5.4.0 Discussion

In this study, we have examined the skin microvascular reactivity in 6 healthy men in response to fasting and feeding. Only male subjects were studied as previous studies have indicated that there are gender differences in postprandial lipoprotein responses (Couillard et al. 1999). The subjects were also non-obese and were advised not take part in any undue physical exertion prior to the studies as both obesity and exercise may affect postprandial lipid metabolism (Couillard et al. 1998) (Malkova 1999).

The study was designed in such a manner that there was a pre and post study on both the fasting and feeding days to serve as a time-control. To increase the power of the study, I examined the difference in peak skin vasodilatory response between the pre and post study on the fasting day (1a and 1b) versus the difference between the pre and post study on the feeding day (2a and 2b), which was not significantly different.

In these six subjects, there appears to be a wide variation in the postprandial increment in triglyceride level (range = 0.51-2.75 mmol), with most subjects showing a minimal rise in triglyceride levels. This may account for the non-significant differences in overall skin microvascular responses between the fasting and feeding days. A 60% change in the vasodilatory function was the average reduction seen in the conduit arteries of the healthy volunteers following a lipid load (Vogel et al. 1997) (Plotnick et al. 1997) (Lundman et al. 1997). This study was powered to detect a 40% change in vasodilatory function and thus may not be able to detect a lower level of vasodilatory change in the microcirculation. Since the publication of the work by Vogel’s group (where volunteers on the high-fat meal arm of the study consumed a meal with a fat content of 50g), Djousse et al. have reported that flow-mediated and GTN-induced vasodilatation of the brachial artery was not impaired in a group of healthy volunteers who consumed a high-fat meal (0.8g fat/kg body weight) (Djousse
et al. 1999). In addition, another study looking at resistance vessel endothelial function did not report any impairment in forearm blood flow in response to acetylcholine, bradykinin and nitroprusside after a high-fat meal with total fat content of 50g (Gudmusson et al. 2000).

Schrezenmeir et al. reported that in a healthy population, there is a bimodal distribution in postprandial triglyceride response, a group of low triglyceride responders and a group of high triglyceride responders. The group of high triglyceride responders also had higher fasting insulin levels, suggesting a link to the metabolic syndrome. Normoglycaemic first degree-relatives of type 2 diabetic patients who had normal fasting triglycerides were found to exhibit postprandial hypertriglyceridaemia and had lower insulin sensitivity compared to control subjects (Axelsen et al. 1999). Further support that postprandial triglyceride response may be related to the metabolic syndrome stems from a study looking at the effect of a high-fat meal on normoglycaemic, normolipidaemic obese subjects compared to lean controls (Guerci et al. 2000). The obese subjects were found to have abnormal postprandial lipaemia responses, indicated by a significantly greater triglyceride response in the non-chylomicron subfraction compared to the controls. Parameters relating to obesity showed no relationship with the postprandial parameters but insulin resistance index calculated using HOMA partly explained the increase in non-chylomicron triglyceride levels. Thus the abnormal postprandial response may be a consequence of insulin resistance. Similar to Guerci et al.'s findings, the BMI and waist–hip ratio of the subjects in the current study did not correlate with postprandial triglyceride change. In contrast, the insulin sensitivity index of the subjects in this study did not relate to the postprandial triglyceride change. Perhaps this may be attributed to the small number of subjects in the present study whilst the studies described above involve larger
number of subjects and a comparison between a group of at-risk subjects versus healthy controls. There has also been a recent report on 55 men aged between 34-59 with atherogenic lipid profile, where postprandial triglyceride responses did not correlate with measures of central obesity or insulin levels (Minihane et al. 2000).

On the feeding day, the difference between the peak skin vasodilatory responses in the two iontophoresis studies (2b and 2a) correlated with the change in triglyceride levels, such that those with a large postprandial triglyceride rise had the greatest reduction in microvascular vasodilatory response. This is consistent with Vogel’s findings where the change in brachial artery vasoactivity was found to correlate significantly with the postprandial triglyceride change while fasting triglyceride levels did not relate to the flow-mediated vasodilatation of the brachial artery. Certainly it has been reported that postprandial triglyceride levels may be a better predictor of coronary heart disease compared to fasting triglyceride levels (Patsch et al. 1992). In Vogel’s study, there was no significant difference in the preprandial and postprandial total cholesterol, LDL and HDL- cholesterol levels. In contrast, there was a significant difference in HDL-cholesterol level between the two days in our study but the change in HDL-cholesterol level on the day of feeding did not predict the change in vasodilatory function after the fatty meal. A limitation of our study however was that LDL-cholesterol level was not measured. An estimate of the LDL-cholesterol can be calculated using the Friedewald equation (Friedewald et al. 1972), however this calculation is prone to errors when the triglyceride levels are greater than 2.26mmol/l (Branchi et al. 1998). Two of the subjects in our study had postprandial triglyceride levels greater than 3 mmol/l.

Triglyceride level changes most dramatically compared to other lipid parameters after a fatty meal, therefore the detrimental effects of postprandial dyslipidaemia on the
vasculature have been attributed to the triglyceride-rich lipoproteins (TRL), which have been implicated in foam cell formation and atherosclerotic lesions (Phillips et al. 1993). Alternatively, the TRL may mediate its effects on the vasculature via its influence on the metabolism of LDL and HDL particles. Large TRL promotes the formation of small dense LDL and a decrease in HDL particles, both of which can act in concert to induce endothelial dysfunction and encourage atherogenesis (Ooi et al. 1998).

How does the postprandial increase in triglycerides or specific lipoprotein fractions affect vascular function in healthy subjects? Increased oxidative stress is one proposed mechanism. Reduced brachial vasoactivity following a high-fat meal may be reversed by the consumption of antioxidant vitamin C and E (Plotnick et al. 1997). Administration of vitamin C and E to those subjects who were fed a low fat-meal in their study did not affect the brachial artery vasoactivity. This suggests that postprandial dyslipidaemia may impair vasodilatation, at least in the macrocirculation, via oxidative mechanisms. Folic acid supplementation has also been shown to reverse transient impairment in vascular function following a fatty meal. It has been speculated that the underlying mechanism is likely to be related to the antioxidant effect rather than the homocysteine lowering effect of folic acid, as the treated subjects had lower urinary MDA levels compared to those who received placebo (Wilmink et al. 2000). In contrast, our study has not shown a difference in plasma MDA levels between the fasting and the feeding days. Again this may be related to the small number of subjects in the study but we have also not measured other markers of oxidative stress such as F2-isoprostanes, susceptibility of LDL to oxidation or anti-oxidant capacity such as radical-trapping antioxidant parameter (TRAP) levels, which may have yielded a more complete picture of the in vivo oxidative status.
There is some suggestion that isoprostanes may be the most robust biomarker of lipid peroxidation currently available (Halliwell 2000).

5.5.0 Conclusion

Skin microvascular vasodilatory function is not significantly attenuated in this group of healthy subjects following the consumption of a high-fat meal. However, the change in skin microvascular response is strongly correlated with the postprandial rise in triglycerides such that those with a large triglyceride response also demonstrate the greatest reduction in skin vasodilatation.
Table 5.1 The study design.

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre study (a)</strong></td>
<td><strong>Pre study (a)</strong></td>
</tr>
<tr>
<td>Blood test for insulin, glucose, lipids</td>
<td>Blood test for insulin, glucose, lipids</td>
</tr>
<tr>
<td>Skin microvascular function test</td>
<td>Skin microvascular function test</td>
</tr>
<tr>
<td>Blood test for insulin, glucose, lipids</td>
<td>Blood test for insulin, glucose, lipids</td>
</tr>
<tr>
<td>2.5 hours’ break</td>
<td>2.5 hours’ break – high fat meal consumed</td>
</tr>
<tr>
<td><strong>Post study (b)</strong></td>
<td><strong>Post study (b)</strong></td>
</tr>
<tr>
<td>Blood test for glucose, lipids</td>
<td>Blood test for glucose, lipids</td>
</tr>
<tr>
<td>Skin microvascular function test</td>
<td>Skin microvascular function test</td>
</tr>
<tr>
<td>Blood test for glucose, lipids, MDA</td>
<td>Blood test for glucose, lipids, MDA</td>
</tr>
</tbody>
</table>
Table 5.2 Characteristics of the subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median (interquartile range)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>55.5 (48.0-63.5)</td>
</tr>
<tr>
<td><strong>BMI (kg/ m²)</strong></td>
<td>24.5 (23.6-26.1)</td>
</tr>
<tr>
<td><strong>WHR</strong></td>
<td>0.91 (0.85-0.98)</td>
</tr>
<tr>
<td><strong>Heart rate (min⁻¹)</strong></td>
<td>63 (57-69)</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>124 (112-129)</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td>74 (71-76)</td>
</tr>
</tbody>
</table>
Fig 5.1 Skin microvascular responses to acetylcholine (endothelium-dependent vasodilator) iontophoresis in 6 healthy male volunteers.

Skin red cell flux (measure of the number and the velocity of the red cells) is expressed as the mean value (± standard error of the mean) in arbitrary units of volts (V).

1a) pre-study on day of continued fasting

1b) post-study on day of continued fasting

2a) pre-study on day of feeding

2b) post-study on day of feeding
Table 5.3 The peak skin vasodilatory responses.

<table>
<thead>
<tr>
<th>Day</th>
<th>Peak skin vasodilatory response (V)</th>
<th>Pre</th>
<th>Post</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1 (fasting)</td>
<td></td>
<td>2.29 (1.57-2.85)</td>
<td>2.46 (2.15-3.10)</td>
</tr>
<tr>
<td>Day 2 (feeding)</td>
<td></td>
<td>2.61 (1.82-3.07)</td>
<td>2.13 (1.69-2.58)</td>
</tr>
</tbody>
</table>
Table 5.4 Biochemical parameters on the day of fasting (day 1).

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Pre-study</th>
<th>Post-study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.18 (0.94-1.79)</td>
<td>0.96 (0.75-1.77)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.60 (4.78-6.05)</td>
<td>5.25 (4.65-5.80)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.30 (0.98-1.44)</td>
<td>1.19 (0.95-1.26)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.8 (4.6-4.8)</td>
<td>4.7 (4.3-4.9)</td>
</tr>
<tr>
<td>MDA (μmol/l)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 5.5 Biochemical parameters on the day of feeding (day 2).

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Pre-study</th>
<th>Post-study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>1.06</td>
<td>0.96</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(0.93-1.18)</td>
<td>(0.80-1.14)</td>
</tr>
<tr>
<td><strong>Total cholesterol</strong></td>
<td>5.60</td>
<td>5.25</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(4.78-6.05)</td>
<td>(4.65-5.80)</td>
</tr>
<tr>
<td><strong>HDL-cholesterol</strong></td>
<td>1.33</td>
<td>1.18</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(1.03-1.49)</td>
<td>(0.99-1.41)</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>4.9</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>(4.6-4.9)</td>
<td>(3.4-4.6)</td>
</tr>
<tr>
<td><strong>MDA (μmol/l)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 5.2 The relationship between the change in peak acetylcholine-induced vasodilation and the change in triglyceride levels.

Change in peak vasodilatory response is represented by red cell flux, which is a measure of the number and the velocity of the red cells expressed in arbitrary units of volts (V).
6.0 The effect of a high-fat meal on skin microvascular function in type 2 diabetic subjects

6.1.0 Introduction

It is well known that type 2 diabetic subjects are at high risk of cardiovascular morbidity and mortality (Schernthaner 1996). This increased vascular risk is attributed to the increased prevalence of hypertension, obesity and dyslipidaemia in addition to the hyperglycaemic state posed by type 2 disease (Turner et al. 1998). Subjects with type 2 diabetes display characteristic dyslipidaemia with hypertriglyceridaemia, low level of HDL-cholesterol and a preponderance of small, dense LDL particles (Verges 1999). Postprandial dyslipidaemia is also a feature of diabetic dyslipidaemia (Coppack 1997). This is characterised by increased production and delayed breakdown of large triglyceride-rich lipoproteins (TRL) and altered LDL and HDL metabolism. This abnormal postprandial handling of lipoproteins may have a bearing on the increased vascular risk of such patients (Coppack 1997).

There is some evidence that conduit artery vasodilation is impaired in patients with type 2 diabetes after a standard fatty meal (Evans et al. 1999). As microvascular complications are also common in type 2 disease, even at early stages of the disease (Group 1990), it is plausible that dyslipidaemia may also be a risk factor in the pathogenesis of diabetic microvascular complications.

In this study, we investigated the effect of a high-fat meal on microvascular function in subjects with type 2 diabetes, who are prone to postprandial dyslipidaemia and are at risk of vascular diseases.
6.2.0 Method

6.2.1 Subjects

6 volunteers with diet-treated type 2 diabetes were recruited from the community in Exeter. The subjects were all non-smokers with no history of ischaemic heart disease, peripheral vascular disease or Raynaud's disease. The blood pressure readings of all the subjects were < 160/80mmHg. None of the subjects had any evidence of retinopathy or neuropathy but 2 of the subjects had evidence of microalbuminuria on random morning urine samples collected on two separate occasions. One of the subjects had been treated with losartan for hypertension but this was omitted for one week prior to the study and the rest of the subjects were not on any anti-hypertensive therapy or other vasoactive medication. None of the subjects were on antioxidant treatment. The study was approved by the local Exeter Medical Research Ethics Committee.

Power calculations using change score data suggest that this sample size provided a 90% chance of detecting a 40% reduction in vasodilatory function at 5% level of significance. The mean and variability (SD) in skin vasodilatory response to acetylcholine observed in the type 2 diabetic subjects was 0.86±0.34 V. A 53% reduction in vasodilatory function was the reduction observed in a previous study investigating the effect of a high-fat meal on brachial artery vasoactivity in type 2 diabetic subjects (Evans et al. 1999).

6.2.2 Experimental Protocol

All the subjects underwent physical examination together with the measurement of height, weight, waist and hip circumference to facilitate the calculation of body mass
index (BMI) and waist:hip ratio (WHR). Electrocardiogram was performed to exclude any evidence of ischaemic heart disease. Heart rate and blood pressure were recorded using a semi-automatic blood pressure recorder (Dinamap, Critikon, Tampa, USA) before each study. Non-steroidal medication was not permitted for at least 10 days prior to the study day. Subjects fasted overnight for 12 hours and also refrained from any undue physical exertion and caffeine intake on the day itself.

This was a randomized, single-blind crossover study. The experiments were conducted under temperature-controlled environment (21.5 C -22.5 C) with the subjects lying supine in a quiet room. Skin temperature was monitored using a thermocouple (Comark Electronic, Littlehampton, Sussex UK). A venous cannula was inserted in the left antecubital fossa to facilitate blood sampling. After an acclimatisation period of 30 minutes, the subjects had baseline microvascular function measurement performed on the right forearm (pre study (a)). Following that, the subjects were randomised to be given either a standard high-fat meal or to continue to fast. The high-fat meal consisted of a standard milkshake (200ml full cream milk with 50ml double cream) and 2 slices of toasted white bread with 3 butter slices (17.1 g), 4 slices of cheese (24.4g) with a total fat content of 74.3g. After 2.5 hours, the subjects underwent the measurement of microvascular function on the same arm (post study (b)), avoiding skin sites that have been studied on previously. The subjects returned on another day, at least one week apart and the entire procedure was repeated such that those who were given a high-fat meal previously would fast in the second study and vice versa. The day where the subjects had to remain fasted throughout was designated 'day 1' and the day where the subjects received a high-fat meal was designated 'day 2'.
6.2.3 Skin microvascular function

The skin microvascular function was assessed by examining the skin vasodilatory response to acetylcholine, an endothelium-dependent vasodilator, using the technique of iontophoresis. The equipment and the technique used have been described in section 2.3. Three different sites on the forearm skin was studied using 1% acetylcholine and control experiment was also performed with 3% mannitol (acetylcholine carrier) on one skin site. Endothelium-independent vasodilatation of the skin was not examined.

6.2.4 Blood sampling

Venous blood sampling was obtained before and after each iontophoresis study for serum total cholesterol, HDL-cholesterol, triglyceride (Vitros 950 Analysers, Johnson & Johnson) and plasma glucose concentration (YSI, Yellow Springs, Ohio, USA). Additional blood samples were obtained before and after each pre study (1a and 2a) for the measurement of insulin using using an immunoenzymometric assay (Insulin-EASIA, Biosource, Nivelles, Belgium) calibrated against IRP 66/304 with no cross-reactivity with intact proinsulin or 32-33 split proinsulin. Interassay coefficients of variation were less than 10% over the range of 95-1038pmol/L. This was to facilitate the calculation of indices of insulin sensitivity and beta-cell function of the subjects using the homeostasis model assessment (HOMA) (Matthews et al. 1985). Further blood samples were also taken at the end of the post studies (1b and 2b) on the two days for the measurement of malondialdehyde (MDA) using HPLC method (Hichrom HIRPB 150A 150X4.6mm column) (Templar et al. 1999).
6.2.5 Statistical analysis

The anthropometric measures and blood results of the subjects were expressed as the median value with interquartile range. Results of the skin red cell flux were expressed in arbitrary units of volts (V). The cutaneous microvascular response to acetylcholine for each time-point was expressed as the skin red cell flux to acetylcholine minus the red cell flux to mannitol. The results of the overall skin red cell flux of the subjects were represented by the mean red cell flux ± standard error of the mean (SE) in the graphs. Analysis of variance for repeated measures was used to assess if skin red cell flux changed significantly with acetylcholine iontophoresis. The peak vasodilatory response was represented by the mean value of the skin red cell flux at 320s and 400s. The difference in peak vasodilatory response between the post and pre studies on the fasting day (peak response 1b – peak response 1a) versus the difference between the post and pre studies on the feeding day (peak response 2b – peak response 2a) using Wilcoxon signed ranks tests. The difference between the lipoprotein fractions and glucose levels on the pre- and post-studies on the two days were also compared using Wilcoxon signed ranks test. Spearman-rank correlation coefficient was calculated where appropriate.

6.3.0 Results

Table 6.1 lists the characteristics of the subjects.

6.3.1 Skin microvascular function

Figure 6.1 illustrates the blood flow responses on the four iontophoresis studies. ANOVA for repeated measures has shown that skin red cell flux changed significantly with acetylcholine iontophoresis for each study (1a- p=0.018, 1b-
p=0.025, 2a- p=0.045, 2b- p=0.014). Table 6.2 illustrates the peak skin vasodilatory response for the pre and post studies on the two days. The difference in peak vasodilatory response between the post and pre studies on the fasting day (1b and 1a) versus the feeding day (2b and 2a) was significantly different (p=0.028).

6.3.2 Biochemical parameters

Table 6.3 and 6.4 illustrate the biochemical parameters on the fasting and feeding days respectively. Triglycerides increased from 2.48 (1.27-3.72) mmol/l to 3.89 (1.49-5.99) mmol/l on the day of feeding. This change in triglyceride on the consumption of the high-fat meal was significantly different from the change in triglyceride level (2.55 (1.22-3.80) mmol/l pre to 2.18 (0.96-3.41) mmol/l post) on the day of prolonged fasting (p=0.013). There were no significant differences in the changes in total cholesterol and HDL-cholesterol between the day of fasting and the day of feeding. Changes in plasma glucose concentration were however different on the two days (p=0.005). There was a tendency for the glucose levels to decrease on the day of the fast while the glucose concentration on the day of feeding appeared to be fairly similar at the beginning and at the end of the day. MDA levels between the fasting and feeding days were not different (p=0.917).

The median insulin sensitivity index of the type 2 diabetic subjects was 60.6 (54.5-75.0)% and median beta-cell function index was 50.9 (37.6-64.5)%. There was no significant correlation between the insulin sensitivity index and the change in triglyceride levels induced by the challenge of a high-fat meal (Rs=-0.232, p=0.658). The beta-cell function index was also not related to the magnitude of the postprandial triglyceride rise (Rs=-0.086, p=0.872). Measures of obesity such as BMI and WHR
again did not correlate with the postprandial change in triglycerides (Rs=0.600, p=0.208 and Rs =0.086, p=0.872 respectively).

6.3.3 The relationship between skin microvascular responses and the biochemical parameters

On the day of fasting, the change in peak skin blood flow responses between the post (1b) and pre studies (1a) did not correlate with the change in triglyceride, total cholesterol, HDL-cholesterol and glucose levels. The change in the peak blood flow responses between the post (2b) and pre studies (2a) on the day of feeding did not correlate with the change in peak triglyceride, total cholesterol, HDL-cholesterol and glucose levels.

Both the HOMA estimates of insulin sensitivity and beta-cell function did not correlate with the reduction in peak skin microvascular responses on the day of feeding (Rs=-0.754, p=0.084 and Rs=-0.257, p=0.653 respectively).

6.4.0 Discussion

In this study, we have examined the skin microvascular vasodilatory response in six type 2 diabetic subjects in a fasted state versus the consumption of a high-fat meal. The type 2 diabetic subjects in this study had fairly satisfactory glycaemic control and were not on any pharmacological treatment for diabetes. Staprans et al demonstrated that diabetic subjects with poor glycaemic control (HbA1c >10%) exhibited exaggerated increase in levels of oxidised lipids in chylomicrons, when compared to diabetic counterparts with good glyaemic control (Staprans et al. 1999). There has also been documentation that hypoglycaemic agents such as glipizide and metformin may alter postprandial lipaemia (Jeppesen et al. 1994) (Jeppesen et al. 1994) and
hence we did not include patients on such treatment in this study. The diabetic subjects in the current study were also neither obese nor hypertensive. Various features of the metabolic syndrome such as hypertension and obesity have been associated with endothelial dysfunction in the absence of hyperglycaemia (Taddei et al. 1993) (Steinberg et al. 1996). These exclusion criteria were applied in order to examine the effect of postprandial lipid changes on the microcirculation in relatively healthy and complication-free type 2 diabetic subjects.

The difference between the peak vasodilatory response in the post (1b) and pre (1a) studies on the day of fasting versus the difference in peak vasodilatory response in the post (2b) and pre (2a) studies on the day of feeding were significantly different. The main difference appears to be attributed to a substantial increase in the peak skin vasodilatory response in the post study (1b) compared to the pre study (1a) on the fasting day and a small reduction in the vascular response in the post study (2b) compared to the pre study (2a) on the feeding day.

The increase in peak vasodilatory response in the post study compared to the pre study on the fasting day may be related to the effect of prolonged fasting. Although some of the lipid parameters and glucose concentration showed a tendency to decrease with prolonged fasting, these levels did not predict the increase in skin blood flow in the post study (1b) compared to the pre study (1a). The other possible explanation for this increase in blood flow responses in the post study may be related to the circadian rhythm influencing skin blood flow (Smolander et al. 1993) (Arnold et al. 1997). However, other investigators have refuted that the time of the day affects skin blood flow (Houben et al. 1994) (Sundberg 1984). There is also some evidence that undernutrition may lead to a decrease in sympathetic activity and a lowering of blood pressure (Göhler et al. 2000) and thus it is plausible that prolonged fasting may
lead to a decrease in skin sympathetic activity and increased blood flow. Perhaps caution should be exercised when conducting vascular studies to ensure that all subjects should be studied at around the same time during the day as well as standardising their dietary status on the day of the study.

On the feeding day, it was noted that in one of the subjects, the triglyceride levels initially decreased during the course of the pre study (2a) and then the levels rose back to its previous level post-feeding (2b). Hence the difference in triglyceride levels from the beginning to the end of the study on day 2 was negligible in this particular subject and microvascular responses appeared similar before and after the meal. This may have attenuated the overall results of the group in view of the small number of subjects in this study and hence accounted for a small decrease in peak skin vasodilatory response after the meal.

Unlike the healthy subjects described in chapter 5, the change in triglyceride levels on the feeding day did not correlate with the peak skin vasodilatory response. Perhaps it is not the total postprandial triglyceride levels per se that is the abnormality central to the impairment of vascular function in the type 2 diabetic state. It is possible that the impairment may be related to quantitative or qualitative changes in particular TRL fraction (Verges 1999). It may therefore be important to look at specific measures of subsets of atherogenic TRL such as triglyceride-enriched VLDL or chylomicrons rather than the total serum triglyceride level. Chen et al. reported a higher postprandial increase in TRL of intestinal origin in type 2 diabetic subjects compared to healthy controls, despite a lack of difference in plasma postprandial triglyceride levels between the two groups (Chen et al. 1993). Previous studies have demonstrated that vasodilatory function is impaired in type 2 diabetic subjects compared to healthy controls (Morris et al. 1995) (McVeigh et al. 1992). It is thus possible that skin
vascular response to acetylcholine iontophoresis is impaired in type 2 diabetic subjects to such a degree that the additional insult of postprandial triglycerides cannot aggravate skin vasodilatory function to a dramatic extent.

There have been several studies looking at the effect of a fatty meal on vascular function in type 2 diabetes. Evans et al. studied the effect of ciprofibrate therapy on postprandial flow-mediated dilatation of the brachial artery in subjects with type 2 diabetes (Evans et al. 2000). The diabetic subjects similarly were non-smokers, normotensive and had no evidence of macrovascular disease. The mean glycated haemoglobin was 8.5% but no mention was made with regards to the use of hypoglycaemic agents in this group. The mean BMI of the subjects in their study were greater than 30 and obesity itself has been linked with postprandial dyslipidaemia (Couillard et al. 1998). At baseline prior to ciprofibrate treatment, the consumption of a fatty meal (total fat content = 80g) was associated with an increase level of oxidative stress and a reduction in brachial artery vasoactivity, which correlated with the triglyceride enrichment of VLDL, LDL and inversely with the 4 hour HDL-cholesterol level. After 3 months of ciprofibrate treatment, postprandial impairment in flow-mediated vasodilatation, triglyceride-enrichment of lipoproteins and excursion in oxidative stress improved compared to the placebo group. The improvement in fasting and postprandial endothelial function with ciprofibrate treatment did not correlate with any of the metabolic parameters. Shige et al. studied the effect of a mixed high-fat and high-sugar meal (75g of sucrose) on type 2 diabetic subjects (Shige et al. 1999). However, there was no experimental control in the study and the total fat content of the diet was not specified. Impairment of flow-mediated dilatation but not GTN-induced vasodilatation of the brachial artery occurred postprandially. Glucose, triglycerides, HDL-cholesterol and remnant-like particles (cholesterol) rose
significantly after the meal. The change in flow-mediated dilatation post-meal correlated with the rise in glucose postprandially. The authors concluded that the observed vascular impairment was attributed to the effect of postprandial hyperglycaemia rather than postprandial dyslipidaemia. As glycaemia and insulin release are inextricably linked to lipid metabolism in diabetes, we have tried to minimise the effect of postprandial hyperglycaemia by ensuring that the glucose load of our meal was kept low (total sugar content was only 19.7g) and the subjects also had fairly satisfactory glycaemic control. Although the glucose changes over the fasting and feeding days were significantly different, the plasma glucose concentration was only minimally altered from the beginning to the end of the feeding day and indeed the change in glucose levels did not correlate with the impairment of microvascular response in our subjects.

Insulin plays an important role in the regulation of lipid metabolism such as the inhibition of lipolysis in adipose tissues, the production and breakdown of triglyceride-rich lipoproteins and the metabolism of HDL particles. Type 2 diabetes is a disease state characterised by beta-cell dysfunction and insulin resistance and therefore, diabetic subjects are more likely to exhibit exaggerated postprandial lipid responses (Cooper et al. 1996) (Coppack 1997). Following the consumption of a fatty meal, there is an increased level of intestinally-derived chylomicrons and chylomicron remnants as well as triglyceride-rich VLDL, partly as a consequence of an oversupply of fatty acid substrates from the periphery due to insulin resistance (Griffin 1999). VLDL particles compete ineffectively with the chylomicrons and chylomicron remnants for lipoprotein lipase and this result in a net increase in transfer of triglycerides from VLDL to LDL, leading to the production of small, dense LDL via
hepatic lipase. The increased levels of TRL also result in increased transfer of triglyceride to HDL particles, promoting the catabolism of HDL.

As highlighted previously, the precise mechanism linking triglyceride-rich lipoproteins to endothelial dysfunction and atherogenesis is still unclear. Increased oxidative stress has been associated with postprandial dyslipidaemia in type 2 disease and may be pivotal to the development of vascular dysfunction in type 2 disease (Evans et al. 1999) (Evans et al. 2000). Most attention in the area of lipoprotein induced vascular reactivity to date has been focussed on the effect of LDL-cholesterol on the endothelium-dependent vasodilatation of blood vessels while the role of other lipoproteins are less certain (Dart et al. 1999). In type 2 diabetes, the increased vascular risk associated with the postprandial state is attributed to the direct or indirect effects of TRL (Evans et al. 1999). Postprandial rise in triglyceride-rich remnants has been associated with the progression of coronary artery disease (Phillips et al. 1993) and there is evidence to suggest TRL can cross the endothelium and be incorporated in atherosclerotic plaques (Mamo et al. 1997). In addition, TRL has been associated with the increased release of PAI-1 levels, a marker of endothelial dysfunction (Steiko-Rahm et al. 1990). VLDL particles may also indirectly affect the endothelium via small dense LDL particles, which are the preponderant LDL species in the presence of large TRL. Small dense LDL particles exhibit reduced binding to the LDL receptor and therefore increased residence time in the circulation and increasing the likelihood of penetrance into the arterial wall. These particles also show an increased affinity to proteoglycans and are therefore more likely to remain entrapped in the subendothelial space (Griffin 1999). Small dense LDL particles also exhibit an increased susceptibility to oxidation. There is in vitro evidence to suggest that the degree of oxidation of LDL is related to the extent of impairment in vascular
function (Chin et al. 1992). Oxidised LDL may impair endothelial vasodilatory function by affecting L-arginine activity or impairing nitric oxide synthesis (Chen et al. 1996). These processes act in concert to confer the increased atherogenic risk associated with small dense LDL particles. This coupled with the decreased level of HDL, which has been shown to be a crucial predictor of endothelial dysfunction in type 2 diabetes (O'Brien et al. 1997), may increased the proatherogenic potential of the type 2 diabetes in the postprandial state.

6.5.0 Conclusion

Skin vasodilatory response is impaired in type 2 diabetes following the consumption of a high-fat meal. However, the impairment in vasodilatory response did not correlate with the postprandial rise in triglycerides.
Table 6.1 Characteristics of the subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median (interquartile range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.5 (57.3-66.3)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.8 (22.8-28.0)</td>
</tr>
<tr>
<td>WHR</td>
<td>0.95 (0.87-0.98)</td>
</tr>
<tr>
<td>Heart rate (min⁻¹)</td>
<td>64 (62-73)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128 (113-145)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>72 (67-85)</td>
</tr>
<tr>
<td>Duration of disease (years)</td>
<td>3.5 (1.8-10)</td>
</tr>
<tr>
<td>Glycated haemoglobin (%)</td>
<td>7.8 (6.5-8.2)</td>
</tr>
</tbody>
</table>
Fig 6.1 Skin microvascular responses to acetylcholine (endothelium-dependent vasodilator) iontophoresis in 6 type 2 diabetic subjects.

Skin red cell flux (measure of the number and the velocity of the red cells) is expressed as the mean value (± standard error of the mean) in arbitrary units of volts (V).

1a) pre-study on day of continued fasting
1b) post-study on day of continued fasting
2a) pre-study on day of feeding
2b) post-study on day of feeding
Table 6.2 The peak skin vasodilatory responses.

<table>
<thead>
<tr>
<th>Day</th>
<th>Peak skin vasodilatory response (V)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td></td>
</tr>
<tr>
<td>Day 1 (fasting)</td>
<td>0.97 (0.52-1.64)</td>
<td>1.25 (0.70-2.32)</td>
<td></td>
</tr>
<tr>
<td>Day 2 (feeding)</td>
<td>1.07 (0.43-1.85)</td>
<td>1.03 (0.36-1.53)</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.3 Biochemical parameters on the day of fasting (day 1).

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Pre-study</th>
<th>Post-study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>2.55</td>
<td>2.28</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(1.22-3.80)</td>
<td>(1.06-3.37)</td>
</tr>
<tr>
<td><strong>Total cholesterol</strong></td>
<td>5.15</td>
<td>5.00</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(4.08-6.83)</td>
<td>(3.80-6.50)</td>
</tr>
<tr>
<td><strong>HDL-cholesterol</strong></td>
<td>1.02</td>
<td>0.96</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(0.84-1.24)</td>
<td>(0.82-1.20)</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>10.5</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>(7.6-12.2)</td>
<td>(6.6-11.1)</td>
</tr>
<tr>
<td><strong>MDA (µmol/l)</strong></td>
<td>0.0300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.0232-0.0704)</td>
<td></td>
</tr>
</tbody>
</table>
### Table 6.4 Biochemical parameters on the day of feeding (day 2).

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Pre-study</th>
<th>Post-study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2*</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>2.48</td>
<td>2.00</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(1.27-3.72)</td>
<td>(1.06-3.37)</td>
</tr>
<tr>
<td><strong>Total cholesterol</strong></td>
<td>5.00</td>
<td>4.1</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(4.28-5.85)</td>
<td>(3.95-6.00)</td>
</tr>
<tr>
<td><strong>HDL-cholesterol</strong></td>
<td>0.97</td>
<td>1.01</td>
</tr>
<tr>
<td>(mmol/l)</td>
<td>(0.77-1.24)</td>
<td>(0.79-1.30)</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>8.2</td>
<td>6.70</td>
</tr>
<tr>
<td></td>
<td>(6.6-10.0)</td>
<td>(5.9-8.8)</td>
</tr>
<tr>
<td><strong>MDA (μmol/l)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Glucose and lipid results were missing from 1 subject at this time point due to technical difficulty with venous access.
7.0 The effect of a high-fat meal on skin microvascular function in type 2 diabetic subjects and healthy subjects

7.1.0 Introduction

In this chapter, the aim is to compare the skin microvascular function and biochemical parameters of the healthy subjects (chapter 5) and type 2 diabetic subjects (chapter 6) at baseline and after a high-fat meal. The other objective is to assess the effect of a high-fat meal and the time of the study on the skin microvascular function in the two groups of subjects.

7.2.0 Statistics

7.2.1 Anthropometric measures and biochemical parameters

The various anthropometric and biochemical parameters of the subjects are expressed in the text as the median value with interquartile range and comparisons were made using non-parametric Mann-Whitney U test. Baseline glucose and lipid profiles was calculated by taking the mean value of the fasting glucose and lipid levels from the pre studies on day 1 and 2 (study 1a and study 2a).

7.2.2 Microvascular responses

Baseline data: Baseline skin vasodilatory response for each time-point in the acetylcholine iontophoresis protocol was calculated by taking the mean value of the red cell flux for each time-point in both pre studies (1a and 2a). Two-way ANOVA for repeated measures was used to assess if baseline blood flow responses (acetylcholine response curve) in the pre studies was different between the two
groups. The peak skin vasodilatory function (plateau in the graphs) in response to acetylcholine at baseline (i.e. pre studies) was represented by the mean value of the peak vasodilatory response attained in both pre studies (la and 2a). Mann-Whitney U test was used to compare the peak vasodilatory response of the two groups.

**Post-meal data:** Two-way ANOVA for repeated measures was used to assess the blood flow responses (acetylcholine response curve) in the post study on the feeding day (study 2b in fig 5.1 and 6.1) were different between the two groups. Mann-Whitney U test was used to compare the peak vasodilatory response of the two groups.

**The interaction between the effect of a high-fat meal and the time of study:**
Three-way ANOVA for repeated measures was used to assess the interaction between feeding status (fasting day versus feeding day), the time of the study (the difference between the acetylcholine responses over the six time-points in the post and pre studies) and the subject groups (healthy versus type 2 diabetic subjects). It is recognised that the study power would be reduced in performing a three-way analysis. ANOVA was also used to assess the difference in peak vasodilatory response between the post and pre studies on the fasting day (1b and 1a) and the feeding day (2b and 2a) in the two groups of subjects (type 2 diabetic and healthy subjects).
7.3.0 Results

7.3.1 Characteristics of the two groups

The characteristics, anthropometric measures and biochemical measures of the type 2 diabetic subjects and healthy volunteers are listed in table 7.1. There were no significant differences in the age (p=0.173), the BMI (p=0.423) and the WHR (p=0.749) between the type 2 diabetic and healthy subjects who underwent the high-fat study. Heart rate (p=0.631), systolic (p=0.522) and diastolic blood pressure (p=0.873) were again not different between both groups of subjects.

7.3.2 Baseline results

7.3.2.1 Skin microvascular function

Although the overall response to acetylcholine (acetylcholine response curve) at baseline (pre studies) appeared to be lower in the type 2 diabetic subjects compared to the healthy controls (fig 7.1), this was not statistically significant (p=0.065). However, the peak vasodilatory response (plateau response) at baseline (pre studies) was significantly different between the two groups (1.02 (0.48-1.74) V in the type 2 diabetic subjects versus 2.46 (1.70-2.95) V in the healthy volunteers) (p=0.037).

7.3.2.2 Biochemical parameters

Baseline lipid parameters and glucose levels of the subjects are listed in table 7.2. The only significant difference in the parameters between the two groups was the glucose levels (p=0.004). The type 2 diabetic subjects were less insulin sensitive than the healthy volunteers; median insulin sensitivity index of the type 2 diabetic subjects was
60.6 (54.5-75) % while the insulin sensitivity index of the healthy volunteers was 96.3 (85.4-165.8) % (p=0.025). The beta-cell function index of the type 2 diabetic subjects was 50.9 (37.6-64.5) % and of the healthy subjects was 110.3 (79.3-117.0) % (p=0.006).

7.3.3 The effect of the meal

7.3.3.1 Skin microvascular function

The overall microvascular function in response to acetylcholine (acetylcholine response curve) in the type 2 diabetic subjects following the high-fat meal (study 2b) was reduced compared to the healthy controls (p=0.014) (fig 7.2). The peak vasodilatory response (plateau response) after the high-fat meal was 1.03 (0.36-1.53) V in type 2 diabetic subjects versus 2.13 (1.69-2.58) V in the healthy volunteers (p=0.016).

7.3.3.2 Biochemical parameters

Table 7.3 lists the lipid and glucose levels after the high-fat meal on study day 2. The lipid parameters were not significantly different between the type 2 diabetic and the healthy subjects but glucose levels were significantly different (p=0.004).

7.3.4 The interaction between the effect of the meal and the time of the study

Three-way ANOVA indicated that the interaction between the subject groups (diabetic and healthy subjects), the feeding status (fasting day 1 and feeding day 2) and the change in vascular response with the time of the study (the difference in skin red cell flux between the post and pre studies over the six time-points) was not
statistically significant (p=0.893). However, when the type 2 diabetic and healthy subjects were considered together, there was a significant interaction between the effect of the meal and the time of the study on the skin vasodilatory responses (p=0.031). The interaction between the peak vasodilatory responses in the post and pre studies on the fasting and feeding days were also not significantly different between the type 2 diabetic and the healthy subjects (p=0.813).

7.3.5 The relationship between skin microvascular function and biochemical parameters

As mentioned in the chapter 5, the only biochemical parameter measured that correlated with the change in the skin microvascular function in healthy volunteers post-feeding was the rise in triglyceride level after a high-fat meal. In chapter 6, no such relationship between postprandial triglyceride level and skin microvascular function was demonstrated in type 2 diabetic subjects.

7.4.0 Discussion

There has been a recent plethora of published studies involving the effect of a high-fat meal on the vascular function in healthy controls (Djousse et al. 1999) (Plotnick et al. 1997) (Vogel et al. 1997) (Gudmusson et al. 2000) and type 2 diabetic subjects (Evans et al. 1999) (Evans et al. 2000). It is difficult to make direct comparisons between the different studies, not least due to different study designs but also the different forms and constituents of the high-fat meal. The high-fat meals had a varied amount of total fat content ranging from 50-80g. Some investigators have used a liquid meal in preference to a mixed meal as it was deemed that the preparation of a liquid meal was more accurate and ensured that the composition of the meal was the same for each
subject (Ceriello et al. 1998). However, others have argued that a liquid fat meal causes delayed gastrointestinal absorption, with a biphasic triglyceride response (Bergeron et al. 1997) while others have reported no differences between liquid and solid meals (Mustad et al. 1999). Some investigators have chosen to use fried products in the meal and others have suggested that perhaps the transient defect in endothelial function attributed to the high-fat meal may be related to the high content of lipid oxidation products (Tomaino et al. 1998). Lipid oxidation products are linked with increased platelet thromboxane $A_2$ products and decreased prostacyclin production in animals. Thus it is possible that the impaired vasodilatation observed in those postprandial studies may be related to the presence of lipid oxidation products rather than triacylglycerides per se. Indeed one group of investigators have shown that the impairment in flow-mediated vasodilatation of the brachial artery occurred following the consumption of a meal rich in used cooking fat but not after eating a similar meal rich in unused fat nor a corresponding low-fat meal (Williams et al. 1999). Different classes of fatty acids may also have different effects on the postprandial TRL responses; exaggerated TRL responses are seen with diets high in saturated fatty acids compared to monounsaturated and polyunsaturated fatty acids (Thomsen et al. 1999) (Weintraub et al. 1988). The published studies usually compare the effect of a high-fat meal on a group of subjects who act as their own controls, that is the subjects are exposed to a high-fat meal on one occasion and a low-fat meal on the next occasion. Ideally, a low-fat meal (control meal) should contain similar quantities of glucose, carbohydrates and proteins as the high-fat meal in order to examine the impact of an oral lipid load on the vascular responses. In practice, this may be difficult to achieve, especially when one has to ensure that the meal is palatable as well. Another area of contention in such studies is the content of
antioxidants in the meals. Some studies (Plotnick et al. 1997) (Vogel et al. 1997) have been criticised because the control meals have higher levels of antioxidants compared to the high-fat meal (Tomaino et al. 1998) and may have thus influenced the outcome of the vasodilatory responses in the two limbs of the study. These factors were taken into account in the design of the present study, in that the subjects acted as their own controls and comparison was made between a high-fat meal and the fasted state rather than using a control meal. Our mixed meal could be accurately prepared as the meal consisted of standard processed cheese slices, white bread slices and a milkshake and all the products were obtained from the same manufacturers for each study. The meal was well-tolerated by the subjects. The vitamin content of the high-fat meal was kept to a minimal level (vitamin E= 1.6g and vitamin C= 2.5g).

Considering the baseline biochemical parameters of the type 2 diabetic and the healthy subjects, the only significant differences found between the two groups were the glucose levels, HOMA estimates of the indices of insulin sensitivity and beta-cell function. As type 2 diabetic subjects are prone to exhibit high fasting triglyceride levels and low HDL-cholesterol level, it was therefore surprisingly that this was not clearly demonstrated in this group of type 2 subjects compared to the healthy counterparts. Two of the type 2 diabetic subjects were found to have fasting triglyceride levels of less than 1.50 mmol/l. With the small sample size, this may have diluted the higher fasting triglyceride levels of other subjects in the diabetic group and accounted for the lack of a statistical difference in the triglyceride levels of the two groups. As expected, the glucose levels in the postprandial state were also raised in the type 2 diabetic subjects compared to the healthy controls. Although the postprandial triglyceride levels appeared to be raised in type 2 diabetic subjects compared to the control subjects, this was not found to be statistically significant. Of
the two diabetic subjects who had fasting triglyceride levels <1.50 mmol/l, one subject did not show any noticeable alteration in triglyceride levels postprandially while the other subject exhibited a rise in triglyceride level, but the postprandial triglyceride concentration was still <1.50 mmol/l. This may have diluted the change in postprandial triglyceride levels seen in the rest of the type 2 diabetic subjects. It is also possible that the stringent criteria used to recruit the type 2 diabetic subjects in this study may have identified a group of subjects who may not be representative of the type 2 diabetic patients seen at large in the community. For instance, the UKPDS has indicated that many type 2 diabetic subjects are found to have microvascular complications and hypertension at early onset of the disease. In contrast, none of the subjects in the present study had any overt microvascular complications, only two subjects had evidence of microalbuminuria and one subject had a history of hypertension (who had a blood pressure of 128/84 mmHg despite withdrawal of his medication for a week). In addition, none of the type 2 diabetic subjects were on an oral hypoglycaemic agent and had relatively satisfactory glycaemic control (median disease duration of 3.5 years). As these type 2 diabetic subjects are relatively healthy, non-obese and free of complications, perhaps they do not display the marked fasting dyslipidaemia typical of most of the type 2 diabetic patients in the community.

In the fasted state at baseline (pre studies), the overall skin microvascular response (acetylcholine response curve) in the type 2 diabetic subjects appeared to be reduced compared to the healthy volunteers but this did not reach statistical significance. However, the type 2 diabetic subjects have a significantly reduced peak vasodilatory function compared to the healthy controls. Other studies have revealed that vasodilatory responses are impaired in the type 2 diabetic subjects compared to the healthy controls. McVeigh et al. demonstrated an impairment in forearm blood flow
responses to acetylcholine (endothelium-dependent function) and glycercyl trinitrate infusion (endothelium-independent function) in type 2 diabetic subjects compared to healthy controls (McVeigh et al. 1992). Morris et al. reported an impairment in skin microvascular response to acetylcholine and sodium nitroprusside iontophoresis in 14 male type 2 diabetic subjects compared to male age-matched healthy controls (Morris et al. 1995). In the post-meal microvascular study (study 2b), the overall skin microvascular function (acetylcholine response curve) and the peak vasodilatory response were both significantly impaired in the type 2 diabetic subjects compared to the controls.

Thus it would appear that the healthy volunteers exhibited higher absolute red cell flux responses to acetylcholine compared to the type 2 diabetic subjects both at baseline and after a high-fat meal (although the difference in the acetylcholine response curve between the two groups at baseline did not reach statistical significance). However, the interaction between the subject groups (diabetic and healthy subjects), the feeding status (fasting day 1 and feeding day 2) and the change in vascular response (acetylcholine response curve) with the time of the study (pre and post studies) was not statistically significant. That is, the vasodilatory function in response to the interaction of a high-fat meal challenge and the time of the study was not significantly different between the two groups. When the type 2 diabetic and healthy subjects were considered together, the interaction of the meal and the time of the study had a significant impact on the skin vascular response (acetylcholine response curve). In both the type 2 diabetic and healthy subjects, the microvascular response would appear to be increased in the post study compared to the pre study on the fasting day (day 1) and the high-fat meal (day 2) attenuated this difference (see figures 5.1 and 6.1). When the effect of a high-fat meal on the change in peak
vasodilatory response (rather than the acetylcholine response curve) between the post and pre studies was examined, no significant interaction was once again demonstrated between the two subject groups. As the postprandial triglyceride levels were not significantly different between the two groups in this present study, it was perhaps not surprising that the overall effect of the high-fat meal and the time of the study on the microcirculation were not different between the two groups.

What are the therapeutic options available to minimise the rise in postprandial lipids and the role it plays in the pathogenesis of endothelial dysfunction and atherogenesis? The combination of dietary modifications in terms of reduction in fat, in particular saturated fat and an increase in physical exercise level (Malkova 1999) may be beneficial in reducing the postprandial rise in triglycerides and atherogenic lipoproteins. Candidate therapeutic agents may include oral hypoglycaemic agents such as sulphonylurea and metformin which may reduce the postprandial excursion in triglyceride levels (Jeppensen et al. 1994) (Robinson et al. 1998). Acarbose has also been shown to reduce postprandial hypertriglyceridaemia and also total cholesterol, due to a reduction in VLDL carrier (Hanefield et al. 1991). Treatment with ciprofibrate for three months has also been shown to improve postprandial dyslipidaemia and vascular function in type 2 diabetes (Evans et al. 2000). Fenofibrate treatment has also been shown to reduce alimentary lipaemia and is associated with a rise in plasma HDL-cholesterol level (Simpson et al. 1990). Oral ingestion of vitamin C and vitamin E (Plotnick et al. 1997) and pretreatment with folic acid (Wilmink et al. 2000) have been shown to prevent lipid-induced decrease in flow-mediated vasodilatation. Equally, there have been other studies that have shown no beneficial effects of antioxidant administration on endothelial function (Gilligan et al. 1994) and cardiovascular events (Jha et al. 1995). Interestingly, treatment with
either quinapril or losartan for 2 weeks was found to prevent endothelial dysfunction induced by an acute oral fat load (Wilmink et al. 1999). Angiotensin II has a role in various proatherogenic processes such as LDL oxidation, macrophage activation, smooth muscle proliferation and thrombogenesis (Vogel et al. 1998). Wilmink et al. reported that quinapril had a greater protective effect than losartan and proposed that this may be related to the effect of ACE inhibition on the bradykinin-dependent pathway or possibly a difference in pharmacokinetics and dosages of the two drugs used in the study.

Limitations

As mentioned previously, a limitation of the studies on the type 2 diabetic and healthy subjects were the small numbers of subjects involved. Although initial power calculations did suggest that the studies had sufficient power to detect a difference in vasodilatory responses, perhaps it did not provide sufficient power to assess the relationship between biochemical parameters and skin microvascular function. Specific subsets of lipoproteins such as VLDL, chylomicrons and LDL subclasses, which may have yielded more information about the postprandial lipoprotein metabolism and the link to vascular function were also not measured in the present study. MDA, which is a marker of oxidative stress in vivo, was measured in the current study but only in the post studies. It may also be useful to measure the MDA levels in both the pre and post studies and to examine the differences. Other measures of oxidative stress like isoprostanes, the susceptibility of LDL to oxidation and antioxidant capacity like TRAP were not assessed in this study, which may have provided further useful information. Endothelium-independent function was not examined in our subjects and therefore it could not be deduced if the impairment in
skin vasodilatory function was due to endothelium-dependent or independent mechanisms.

7.5 Conclusion

At baseline, overall skin microvascular function in type 2 diabetic subjects was not significantly attenuated compared to the healthy controls, although the peak vasodilatory response was significantly reduced in the type 2 diabetic group. After a high-fat meal, both overall skin microvascular function and peak vasodilatory response was significantly impaired in the type 2 diabetic subjects compared to the healthy volunteers. However, the impact of the meal and the time of the study on the changes in overall skin vascular function and peak vasodilatory function were not different between the two groups of subjects.
Table 7.1 Characteristics of the subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type 2 diabetic subjects</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>61.5 (57.3-66.3)</td>
<td>55.5 (48.0-63.5)</td>
</tr>
<tr>
<td>BMI (kg/ m²)</td>
<td>26.8 (22.8-28.0)</td>
<td>24.5 (23.6-26.1)</td>
</tr>
<tr>
<td>WHR</td>
<td>0.95 (0.87-0.98)</td>
<td>0.91 (0.85-0.98)</td>
</tr>
<tr>
<td>Heart rate (min⁻¹)</td>
<td>64 (62-73)</td>
<td>63 (57-69)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>128 (113-145)</td>
<td>124 (112-129)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>72 (67-85)</td>
<td>74 (71-76)</td>
</tr>
</tbody>
</table>
Skin microvascular response is represented by red cell flux, which is a measure of the number and the velocity of the red cells expressed in arbitrary units of volts (V).
Table 7.2 Biochemical parameters in the fasting state.

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Type 2 diabetic subjects</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>2.51 (1.24-3.76)</td>
<td>1.06 (1.00-1.51)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.08 (4.18-6.34)</td>
<td>5.38 (4.98-5.99)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.98 (0.81-1.24)</td>
<td>1.34 (1.00-1.45)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>9.1 (7.4-11.1)</td>
<td>4.8 (4.6-4.9)*</td>
</tr>
</tbody>
</table>

*p=0.004
Fig 7.2 Skin microvascular response to acetylcholine iontophoresis in type 2 diabetic and healthy subjects (after a high-fat meal).

Skin microvascular response is represented by red cell flux, which is a measure of the number and the velocity of the red cells expressed in arbitrary units of volts (V).
Table 7.3 Biochemical parameters after the high-fat meal.

<table>
<thead>
<tr>
<th>Biochemical parameter</th>
<th>Type 2 diabetic subjects</th>
<th>Healthy subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>3.89 (1.49-5.99)</td>
<td>2.03 (1.66-3.27)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.80 (3.95-5.80)</td>
<td>5.15 (4.80-5.83)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.88 (0.70-1.49)</td>
<td>1.17 (0.99-1.24)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.8 (6.3-12.1)</td>
<td>4.8 (4.4-5.1)*</td>
</tr>
</tbody>
</table>

*p=0.004
8.0 Summary and Conclusion

There are a number of inherent difficulties associated with the in vivo investigation of human microvascular pathophysiology, such as the general inaccessibility of the microvascular beds. However, the skin microcirculation is a relatively favourable site to study the human microvascular function using the laser Doppler technique, without disturbance to the vascular bed under study. Changes in the cutaneous microcirculation often mirror changes in other vascular beds in advancing complications of diabetes, thus skin microvascular studies may provide a useful insight as to how the microcirculation may be affected in the type 2 diabetic state and also in at-risk subjects. However, data from skin vascular studies should be interpreted with caution as diabetes may have a different impact upon the microcirculation of one organ compared to another, which may be partly related to the differences in microvascular architecture (e.g. surface area, permeability) and haemodynamic factors (e.g. flow rate, pressure) in each vascular bed.

The aims of this thesis were to investigate: 1) the hypothesis that microvascular function is an intrinsic abnormality that is present in subjects at risk of developing type 2 diabetes before the emergence of the clinical features of the insulin resistance syndrome and 2) the impact of an extrinsic factor, namely postprandial lipaemia, on the microvascular function.

Low birth weight has been associated with the increased risk of type 2 diabetes, insulin resistance syndrome and cardiovascular disease in adult life. An extension of the fetal insulin hypothesis proposes that low birth weight infants may have an inherent limitation in microvascular reserve and this precedes the onset of the clinical features of the metabolic syndrome. In support of this concept that microvascular function may be an abnormality intrinsic to the metabolic syndrome, infants with
lowest-quartile birth weight were shown to have impaired maximum hyperaemic response at the age of 3 months in this thesis. In addition, strong correlations between microvascular function and various birth anthropometric measures (weight, length, head circumference) were also demonstrated. These infants were studied at such an early age in order to minimise the influence of the external stimuli on the microvascular function. Majority of the infants in this study were breast-fed rather than bottle-fed and there were no significant differences in microvascular responses between the different modes of feeding. Parents who participated in the study were mostly from managerial or professional background and thus social class differences were unlikely to account for the different microvascular responses in the lowest-quartile and highest-quartile groups.

The mechanisms underlying the maximum hyperaemic response are still unclear but a defect in this hyperaemic response have been demonstrated in type 2 diabetic subjects as well as healthy subjects who are at an increased risk of developing type 2 disease. Other aspects of skin microvascular function – skin microvascular response to acetylcholine iontophoresis (endothelium-dependent function) and skin capillary density were not impaired in the lowest-quartile birth weight group. Endothelium-independent function was not interrogated because EMLA cream, which is used to block the non-specific vascular response associated with the application of a cathodal current in the iontophoresis of sodium nitroprusside (endothelium-independent) vasodilator, is contraindicated in infants of that age. It should be noted that the skin capillary density was confined only to the subpapillary layer and the assessment of capillary recruitment, which would involve the inflation of a sphygnomanometer round the infant’s ankle, was not tolerated by these infants.
The next step was to investigate if the relationship between skin microvascular response and birth weight is still present in childhood when exposure to environmental factors may strengthen or attenuate this relationship. It would be ideal to follow up the infants in the prior study during their childhood to interrogate this relationship. However, this is impractical due to the time-scale to complete the thesis. Hence information from healthy children involved in a previous study was collected and collated to facilitate this investigation. A group of 84 healthy children have been recruited as healthy controls for a study looking at skin microvascular function in type 1 diabetic children. These children were Tanner-staged for pubertal maturity and underwent measurements of postural vasoconstriction responses of the foot dorsum and big toe pulp, maximum hyperaemic response of the foot dorsum in the horizontal and then dependent position and the assessment of minimum vascular resistance and distensibility of the blood vessels. There were a total of 19 prepubertal and 25 postpubertal subjects with validated birth weight data. In contrast to the findings in the infants, birth weight in childhood did not correlate with any of the microvascular responses. Weight at the time of the study, body mass index and body mass index adjusted for age also did not correspond to any of the microvascular parameters. Arguably the small sample size, notably relating to the difficulty in obtaining validated birth weight data, and the narrow distribution of the birth weight of the subjects may have influenced the study findings. The study also did not have sufficient power to permit stratification of the subjects into subgroups based on birth weight and subsequent weight gain to examine the impact of catch-up growth on microvascular function. There was also a lack of other measures of childhood obesity and biochemical measures of glucose and insulin sensitivity in this study, which would have permitted further interrogation of the relationship between the metabolic
syndrome and skin microvascular function. Thus, birth weight did not appear to influence skin microvascular function in the prepubertal and postpubertal subjects in this study. How can this finding be explained in the light of the findings in the infant study and other published studies which have linked low birth weight with reduced vasodilatory responses in adult life? It is possible that other environmental factors such as diet and physical activity may have a greater influence at this stage on microvascular function. Alternatively, the rapid phase of physical and sexual maturation may have temporarily obscured the relationship between birth weight and microvascular function.

There has been much recent interest in the effect of the postprandial state on vascular function. Postprandial lipid metabolism in particular is gathering attention as an important risk factor in the pathogenesis of atherosclerosis, especially when human subjects spend the majority of our time in the postprandial state. Hence in this thesis, postprandial dyslipidaemia is the extrinsic factor of interest and its effect on the microcirculation was studied.

In healthy subjects, skin microvascular response was examined in response to the iontophoresis of acetylcholine (endothelium-dependent vasodilator) and the peak skin vasodilatory function was not significantly attenuated after a high-fat meal. However, the change in skin microvascular function correlated negatively with the size of the postprandial rise in triglycerides, such that those with the greatest reduction in skin microvascular response had the greatest increment in triglyceride level. The lack of a significant reduction of skin microvascular function in the entire group may be related to the small number of subjects and that most of the recruited subjects had a fairly small postprandial rise in triglycerides. Perhaps the small triglyceride responses were not surprising, as these were healthy volunteers with no history of hypertension or any
other serious illnesses. Some studies have shown a statistical correlation between measures of insulin resistance with postprandial triglyceride response while others have not. In the present study, an estimate of insulin sensitivity using HOMA did not reveal such a relationship, which again may be related to the small sample size.

In type 2 diabetic subjects, the peak skin vasodilatory function was found to be significantly impaired by the consumption of a high-fat meal. However, the impairment of the microvascular response did not correlate with the magnitude of the postprandial triglyceride rise. Type 2 diabetes is characterised by insulin resistance and this has a bearing on the already complex postprandial metabolism of lipids. Perhaps in type 2 diabetes, it is the quantitative or qualitative changes in specific lipoprotein fraction, for example triglyceride-enriched VLDL fraction, rather than the total serum triglycerides that is linked with the reduction in microvascular responses.

When the responses of the healthy and type 2 subjects were compared, it was noted that at baseline, the type 2 diabetic subjects have a lower overall skin microvascular response to acetylcholine compared to healthy controls but this was not statistically significant. The peak vasodilatory function at baseline was however significantly different between the two groups. After a high-fat meal, the overall skin microvascular function and peak vasodilatory function was significantly lower in the type 2 diabetic subjects compared to the healthy controls. The interaction between the meal factor (high-fat meal versus fasting), the subject factor (diabetic versus healthy subjects) and the time factor (pre and post studies) on the change in overall and peak vascular function was not significant. Surprisingly, the postprandial triglyceride responses did not differ significantly between the two groups. Intuitively, one would expect a higher triglyceride response in type 2 diabetic subjects, which was present in some of the type 2 subjects, but due to the small sample size, this was diluted by
others in the group who exhibited a minimal postprandial rise in triglycerides. It is also possible that the strict exclusion criteria applied to this study may have identified a number of particularly well-controlled type 2 diabetic subjects, not typical of most of the type 2 diabetic subjects in the community, who display many of the features of the metabolic syndrome and vascular complications. As the overall postprandial triglyceride responses did not differ significantly between the healthy and type 2 diabetic groups in the present study, it was perhaps not surprising that the interaction between the meal factor, the time factor on the change in skin microvascular response was not different between the two subject groups. When the two groups are considered together, the interaction between the meal factor and the time factor on the change in overall microvascular response was significant. Skin blood flow appeared to increase in the post study compared to the pre study on the fasting day (day 1) while skin blood flow on the feeding day (day 2) appeared to decrease in the post study compared to the pre study. This increase in the post study vascular response on the fasting day (day 1) was an unexpected finding, raising the possibility that circadian rhythm, a change in sympathetic tone or an improvement in metabolic factors with prolonged fasting may affect skin vascular function.

What do the findings of the effects of intrinsic and extrinsic factors on the microvascular function mean in clinical terms? The concept of intrinsic microvascular dysfunction in low birth weight subjects predisposed to develop the metabolic syndrome in adult life may not necessarily spell doom and gloom. Animal studies have suggested that the treatment with angiotensin-converting enzyme inhibitor in low birth weight newborn rats may be able to reverse the process of hypertension in adult life. Thus further elucidation of the underlying pathological processes responsible for the impairment of vascular function in susceptible individuals may
have public health implications, enabling the identification and the targeting of preventative treatment at these at-risk subjects. The effect of postprandial dyslipidaemia on the microcirculation may be minimised by dietary discretion. However, it should be noted that various studies have shown that excess endogenous rather than exogenous lipid moieties may be predominantly responsible for generating postprandial dyslipidaemia. The role of pharmacological agents in minimising the postprandial rise in triglycerides and triglyceride-rich lipoproteins have been examined in various experimental settings, which may eventually translate into clinical therapeutic areas.

The studies described have fulfilled the initial aims of the thesis. However, it has also given rise to further questions. As highlighted previously, the underlying structural or functional mechanisms for the impaired maximum hyperaemic response in lowest-quartile birth weight infants remain to be elucidated. The study in the infants suggests that the impairment is not mediated via an endothelium-dependent mechanism and is not related to capillary rarefaction. Although there was no significant relationship between birth weight and skin microvascular function in childhood, the interaction between birth weight, catch-up growth and microvascular function in childhood needs further exploration. It would be interesting to follow up the subjects involved in the current infant study when they reach childhood to study whether catch-up growth may have a modifying influence on the relationship between birth weight and microvascular function demonstrated in infancy. The present studies on the effect of a high-fat meal on microvascular function have several limitations and the exact pathophysiological processes underlying the impact of postprandial lipid metabolism on microvascular dysfunction are unclear. In the macrocirculation, current literature supports the role of increased oxidative stress in the reduction in vasodilatory
responses in the presence of an acute lipid load. Perhaps a study of microvascular function involving a larger sample size together with measurements of specific lipoprotein subfractions and markers of oxidative stress levels may help to elucidate this further. It may also be of interest to examine if antioxidant administration can ameliorate the impairment in skin microvascular function in response to a fat meal challenge in type 2 diabetic subjects.

In conclusion, this thesis has highlighted the importance of both intrinsic and extrinsic influences on skin microvascular function and the interplay between elements that are present at birth and subsequent exposures is one of the essential challenges for future research.
References


List of publications and presentations

Publications


Poster presentations


Oral presentation

