MICROVASCULAR FUNCTION IN NON-INSULIN-DEPENDENT DIABETES MELLITUS

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DECLARATION

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SUMMARY

Epidemiological studies suggest differences in the prevalence and natural history of microvascular complications between subjects with insulin dependent (IDDM) and non-insulin dependent (NIDDM) diabetes. The haemodynamic hypothesis proposes that early functional changes in the microcirculation result in the eventual development of diabetic microangiopathy. There is now a large body of experimental evidence in support of this hypothesis in patients with IDDM, with abnormalities in blood flow, capillary pressure and permeability having been demonstrated. In contrast, there have been few studies investigating microvascular function in NIDDM; however, preliminary work has identified a profound limitation in microvascular vasodilation at an early stage, while capillary pressure does not appear to be elevated. The aim of this thesis was to further investigate functional changes in the skin microcirculation in patients with NIDDM and impaired glucose tolerance (IGT).

1. Using a sensitive plethysmographic system, no difference was found in microvascular fluid permeability between patients with NIDDM and control subjects (5.3 (3.2-9.1) x 10^{-3} ml.min^{-1}.100g tissue^{-1}.mmHg^{-1} vs 5.4 (3.5-8.0) x 10^{-3} ml.min^{-1}.100g tissue^{-1}.mmHg^{-1}, median and range; p = 0.98, Mann-Whitney).

2. In confirmation of previous studies, reduced microvascular hyperaemia in response to local heating of the skin was found (using laser Doppler fluximetry) in NIDDM patients with large vessel disease excluded (0.82 (0.42-1.41) V vs 1.40 (0.89-2.13) V control

subjects; $p<0.005$). Limited vasodilation correlated with fasting plasma insulin (R_s =-0.63, $p<0.04$) but not glycaemic control. Microvascular hyperaemia increased after one year of improved glycaemic control in recently diagnosed patients (1.20 (0.51-3.93) V vs 0.97 (0.22-2.17) V at baseline; $p<0.05$). In hypertensive NIDDM patients, there was no further reduction in microvascular vasodilation (1.05 (0.70-1.42) V vs 1.04 (0.79-1.63) V normotensive NIDDM, $p = 0.82$), although there was an increase in calculated resistance to blood flow (127.2 (87.5-181.3) mmHg.V^{-1} vs 84.7 (61.9-123.0) mmHg.V^{-1} normotensive patients, $p < 0.02$).

3. Reduced microvascular hyperaemia was found in subjects with IGT (1.01 (0.71-1.57) V vs 1.41 (1.32-2.13) V control subjects, $p < 0.001$), and also insulin resistant patients with acromegaly (0.96 (0.56-1.70) V vs 1.46 (1.24-2.13) V control subjects, $p < 0.05$). In subjects with IGT, limited vasodilation was found to correlate with fasting plasma insulin (R_s = -0.7; $p < 0.001$) and insulin sensitivity (R_s = 0.52; $p < 0.02$), but not with ß-cell function, plasma glucose or serum lipid concentrations.

4. Using iontophoresis and laser Doppler fluximetry, defective endothelium-dependent vasodilation was found in subjects with IGT (518 (410-905) AU.min^{-1} vs 1236 (875-1588) AU.min^{-1} control subjects, median and range; $p < 0.003$). In contrast there was no significant difference in myogenic (683 (301-1175) AU.min^{-1} vs 898 (303-998) AU.min^{-1} control subjects; $p = 0.5$) or neurogenic vasodilation (61 (31-109) AU vs 46 (37-146) control subjects; $p = 0.8$).

5. No differences in skin capillary density were found between patients with NIDDM, subjects with IGT and control subjects under basal conditions (112 (71-144) caps.mm^{-2} vs 107 (76-140) caps.mm^{-2} vs 112 (76-138) caps.mm^{-2} respectively; p = 0.9, Kruskal Wallis), or after venous occlusion (122 (87-157) caps.mm^{-2} vs 121 (90-143) caps.mm^{-2} IGT vs 123 (81-147) caps.mm^{-2}; p = 0.9).

In light of the above results, a unifying hypothesis has been proposed to explain the differences in epidemiology and pathophysiology of microvascular disease between IDDM and NIDDM.

ABBREVIATIONS

ACh	acetylcholine
ACR	albumin creatinine ratio
AER	albumin excretion ratio
CIGMA	continuous infusion of glucose with model assessment
CFC	capillary filtration coefficient
EDRF	endothelium-derived relaxant factor
GH	growth hormone
IDDM	insulin-dependent (Type 1) diabetes mellitus
IGF-1	insulin-like growth factor-1
IGT	impaired glucose tolerance
NaNP	sodium nitroprusside
NIDDM	non-insulin-dependent (Type 2) diabetes mellitus
OGTT	oral glucose tolerance test
PAI-1	plasminogen activator inhibitor-1
$tcPO_2$	transcutaneous oxygen tension
VPT	vibration perception threshold

CHAPTER 1

INTRODUCTION

Damage to the microcirculation plays a central role in the development of the long term complications of diabetes, leading to a specific microangiopathy characterised by basement membrane thickening in capillaries, arterioles and venules[1]. Although classically affecting the retina and kidney, the histological features of microangiopathy are apparent in a wide variety of other microvascular beds including skin[2], adipose tissue[3], skeletal[4] and cardiac muscle[5]. In addition, it is increasingly recognised that microvascular abnormalities are involved in the development of neuropathy[6] and diabetic foot ulceration[7]. Microvascular disease is responsible for a substantial amount of morbidity, with diabetic retinopathy and nephropathy being the commonest causes of blindness and renal failure respectively, in the non-elderly population in Western countries[8,9].

Non-insulin-dependent diabetes mellitus (NIDDM) is a disease characterised by a high prevalence of large vessel disease and hypertension, leading to a greatly increased mortality from cardiovascular causes[10]. Although patients with NIDDM are subject to the same spectrum of microvascular complications as patients with insulin-dependent diabetes mellitus (IDDM), the prevalence and natural history of some of these differs markedly in the two types of diabetes, as detailed in Chapter 1.3 (page 8). This suggests that there may be differences in the underlying pathophysiology of microvascular disease in IDDM and NIDDM. Diabetic microangiopathy may result from earlier functional changes in the microcirculation, and a large number of studies have characterised abnormal microvascular function in patients

with IDDM, in contrast to the situation in NIDDM where there have been relatively few studies to date.

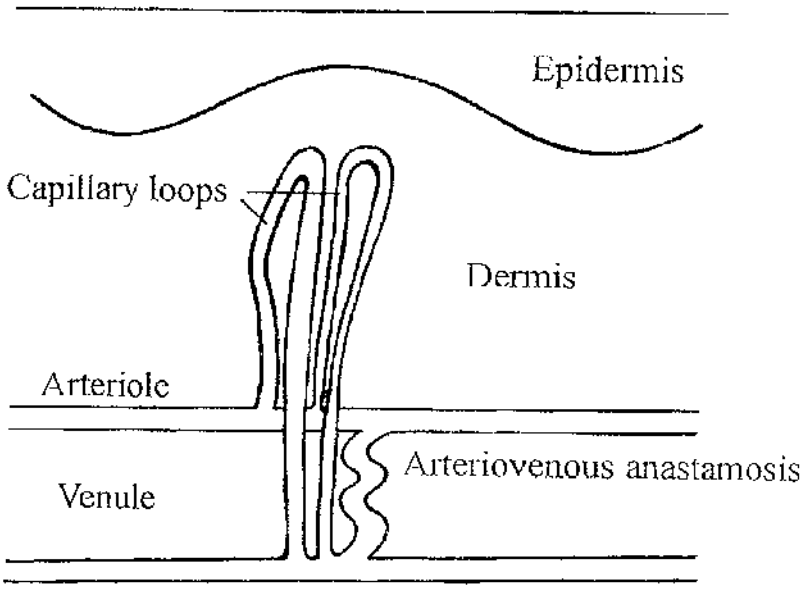
The studies in this thesis attempt to determine the nature and extent of abnormalities in skin microvascular function in subjects with, or at risk of developing, NIDDM; however, before considering the effects of the diabetic state on the microcirculation, it is necessary to define normal microvascular structure and function.

1.1 The normal skin microcirculation.

Microvascular structure:

The microcirculation consists of arterioles, capillaries and venules. In the skin, small branch arteries divide into a subpapillary arteriolar plexus from which capillary loops supplying the skin surface arise[11] (Fig 1.1). These nutritional capillaries are arranged in functional units with one to three capillary loops per dermal papilla[11]. The venous ends of the capillaries merge to form postcapillary venules which unite to form collecting venules in a subpapillary venous plexus[11]. In the skin there are also specialised arterio-venous shunt vessels[12,13] arranged in parallel with the nutritive capillaries, which are involved in thermoregulation. These are coiled channels of average diameter 35μm which are most numerous in the nailbeds and pulps of the digits, and almost absent on the dorsum of the hands or feet[12]. High blood flow through arterio-venous shunts allows the dissipation of heat, although

Fig 1.1 *Diagrammatic representation of the skin microcirculation*



even under resting conditions the bulk of skin blood flow passes through shunt vessels[14].

Capillaries consist of a single layer of endothelial cells surrounding the lumen and an outer layer of basement membrane which encloses occasional pericytes[15]. Arterioles and larger venules have an additional outer layer of smooth muscle cells surrounding the basement membrane[15]. Endothelial cells are joined together by intercellular junctions which are areas of direct contact between specialised regions of the plasma membrane of adjacent cells[16]. The luminal surface of endothelial cells is covered in a layer of glycoprotein material (glycocalyx) which also fills the intercellular junctions[16]. The basement membrane is also composed of a lattice of glycoproteins consisting mainly of Type 4 collagen, laminin and negatively charged heparan sulphate proteoglycan[17]. Capillary endothelium in skin, muscle, fat and retina is of the continuous variety[15], whereas in the glomerulus it is fenestrated (perforated by small holes), although the basement membrane remains intact[15].

Microvascular function:

The main functions of the microcirculation are the delivery and transfer of oxygen and nutrients from blood to the tissues and removal of waste products of metabolism. Exchange of substances across the microcirculation occurs by a variety of mechanisms depending on their exact nature. Fluid and small hydrophilic molecules move through intercellular junctions in capillaries and post-capillary venules[1], whereas small lipophilic molecules (e.g. oxygen) diffuse directly through cell membranes[18]. Larger hydrophilic molecules (e.g. proteins) move largely

with fluid by the process of solvent drag[19], ease of passage being dependent on molecular size and charge, with the glycocalyx and basement membrane acting as a molecular sieve[20], and the strong negative charge of heparan sulphate proteoglycan being an important limiting factor[21]. The passage of fluid is dependent on the transcapillary pressure gradient, which in turn depends on the balance of Starling's forces[18] (the hydraulic pressure of blood inside the capillary, the oncotic pressure of plasma proteins, and interstitial hydraulic and oncotic pressures). As capillary (hydraulic) pressure is arguably the most variable of these, regulation of microvascular pressure and blood flow have a major bearing on the exchange process. Intrinsic rhythmical variations in pre-capillary vascular resistance (vasomotion)[22] allows periods of filtration and reabsorption in individual capillaries. Arteriolar diameter is regulated by both neural and humoral mechanisms, with the predominant mechanism being dependent on the metabolic needs of a particular tissue, and its capacity to withstand circulatory deprivation. In the skin, neural mechanisms supervene to mediate thermoregulation and arteriolar tone is largely maintained by sympathetic vasoconstrictor nerves, which also maintain low arterio-venous shunt flow under normal circumstances[14]. This major neurogenic control mechanism is modulated by local myogenic and humoral mechanisms, such as vasodilation due to the build up of metabolites or release of vasoactive substances by the endothelium[14]. In addition, vascular smooth muscle cells can respond directly to stretch or tension by contracting (myogenic tone)[23], the sensor for which may reside in the endothelial cell[24].

The endothelium has numerous synthetic functions, including involvement in the regulation of vascular tone through the secretion of vasodilator (e.g. endothelium-derived relaxant factor (EDRF)[25],

prostacyclin[26]) and vasoconstrictor substances (e.g. endothelin[27]); and in the control of coagulation (e.g. prostacyclin[28], plasminogen activator inhibitor-1(PAI-1)[29]). Receptors exist on endothelial and vascular smooth muscle cells for a wide variety of peptide and non-peptide neurohumoral mediators, and the particular distribution of these may partly determine the reactivity of a given vascular bed[30]. The function of pericytes, which are more numerous in the retina, is poorly understood, but they may be involved in the regulation of angiogenesis[31], and may have a contractile function with the potential to alter blood flow[32]. With regard to the latter, it is noteworthy that endothelin receptors have been identified on cultured retinal pericytes[33]. Basement membrane, in addition to its role in microvascular permeability, provides structural stability for the microvessels, preventing over distension and acting as a scaffold for endothelial regeneration and repair following injury[17]. Finally, there is a network of lymphatic microvessels which return excess filtered fluid and macromolecules to the circulation to maintain homeostasis. The structure and function of these in health is poorly defined, with even less being known about them in diabetes.

1.2 Non-insulin-dependent diabetes and vascular disease.

As already mentioned, NIDDM is characterised by a high prevalence of large vessel disease and hypertension[10,34]. These problems can already be present at diagnosis, with 35% of newly diagnosed patients having hypertension and 18% electrocardiographic evidence of

ischaemic heart disease[35,36]. It is known that NIDDM may be preceded by a period of several years during which a variable combination of increasing ß-cell dysfunction and decreasing insulin sensitivity[37,38] lead to hyperinsulinaemia and progressive abnormalities of glucose and lipid metabolism, which eventually become manifest as impaired glucose tolerance (IGT). Such subjects are at high risk of developing NIDDM in the future[39]. Hypertension and large vessel disease are even present in people with IGT[40,41] who, despite the fact they exhibit only a modest degree of hyperglycaemia, already have an increased cardiovascular mortality[40,41]. Atherosclerosis in patients with NIDDM differs quantitatively from that in non-diabetic subjects in that it affects an increased proportion of females, is more extensive and diffuse, and tends to affect more distal vessels[42].

As discussed in Chapter 1.6 (page 21), mechanisms other than hyperglycaemia are liable to be important in the pathophysiology of vascular disease in NIDDM, and other metabolic factors such as hyperinsulinaemia have been shown to have a direct effect on large and small blood vessels[43]. There is currently much interest in the role of impaired vascular development in the pathogenesis of adult cardiovascular disease, and low birth weight has been associated with later development of hypertension[44] and premature death from ischaemic heart disease[45]. Low birth weight has also been associated with the development of diabetes and impaired glucose tolerance in adult life[46], possibly due to maldevelopment of the pancreatic ß-cells or islet vasculature. This observation is of potential interest in the context of this thesis as maldevelopment of the microcirculation could underlie abnormal microvascular function.

1.3 Differences in the epidemiology of microvascular disease between non-insulin-dependent and insulin-dependent diabetes.

It has already been stated that differences exist in the prevalence and natural history of microvascular complications between NIDDM and IDDM. Firstly, microangiopathy tends to be related to disease duration in IDDM, with the clinical manifestations first becoming apparent after several years of diabetes; for example background retinopathy is rare in the first few years of IDDM[47] and before puberty, but increases in prevalence with increasing duration of diabetes so that it is present in over 90% of patients after 25 years of diabetes[47]. The prevalence rates for proliferative retinopathy[47] and nephropathy follow a similar pattern[48]. In contrast, and similar to the situation for large vessel disease and hypertension, patients with NIDDM may already have clinical evidence of microangiopathy at the time of diagnosis, with 21% of newly diagnosed NIDDM patients having been shown to have background retinopathy in one study[35]. This phenomenon applies equally to other complications such as maculopathy, neuropathy and nephropathy[35] and may relate to a long period of undiagnosed diabetes or to the metabolic abnormalities of the pre-diabetic phase. Following diagnosis, the subsequent prevalence of microangiopathy tends to be less closely linked to disease duration in NIDDM, so that the overall prevalence of background retinopathy in patients with a long duration of diabetes is lower in NIDDM than in IDDM[47].

Maculopathy is a particular problem in NIDDM, being present in over 15% of patients[34]; however, sight threatening macular involvement is rare in patients with IDDM. On the other hand, proliferative retinopathy affects around 50% of IDDM patients after 25

years of diabetes[47], whereas this is a less common problem in NIDDM patients, being present in only 20%[34].

There is no universally accepted definition of diabetic neuropathy and it is therefore difficult to compare epidemiological data from different studies, with prevalence rates for neuropathy varying from 10% in studies based on clinical findings, to greater than 90% in studies based on electrophysiology. It has previously been suggested that the prevalence of peripheral sensori-motor neuropathy and autonomic neuropathy was similar in the two types of diabetes[49], although recent evidence from neurophysiological and anatomical studies suggest differences between IDDM and NIDDM. A higher degree of both small and large nerve fibre dysfunction being found in NIDDM patients with both subclinical and symptomatic neuropathy in one study[50], whereas a similar pattern of abnormalities has been reported in NIDDM and IDDM patients with subclinical neuropathy in another[51]. Differences have been found in the histopathology of peripheral nerve lesions, with axo-glial dysjunction predominating in IDDM, and focal fibre loss and Wallerian degeneration in NIDDM[52]. It appears that some patients with NIDDM already have disturbed nerve function at diagnosis[53], with only slight deterioration over the next 5 years, except for some patients with poor control[54]. There appears to be a significantly higher prevalence of subclinical autonomic neuropathy (as assessed by cardiovascular reflexes) in NIDDM[55], despite the fact that symptomatic autonomic neuropathy is uncommon.

Diabetic nephropathy affects 30-40% of patients after 15 years of IDDM[48]. In contrast, only 3-8% of elderly Caucasian NIDDM patients reach end-stage renal failure[56,57]; however, the majority of diabetic patients requiring renal replacement therapy in some series have NIDDM as this is the more prevalent form of diabetes[9]. There is evidence that

the rate of decline in renal function may be slower in NIDDM than in IDDM[58,59]. Microalbuminuria is the first indicator of susceptibility to develop diabetic nephropathy in patients with IDDM[60]; however it is a less powerful predictor of nephropathy in NIDDM[58], where an elevated urinary albumin excretion rate (AER) is more associated with macrovascular disease and high cardiovascular mortality[61,62].

Despite the fact that microvascular disease is often detectable at the time of diagnosis in patients with NIDDM, evidence of significant microangiopathy is rarely detectable in subjects with IGT[63]. In one study the frequency of retinopathy in subjects with IGT was no higher than that in a control population, despite the fact that visual impairment occurred more frequently[64]. There is, however, some evidence that microalbuminuria may be present in the pre-diabetic phase, as 15% of Pima Indians with IGT have an elevated urinary albumin excretion[65]. This occurred especially in those with hypertension[65], suggesting that microalbuminuria in this context may relate to other features of the insulin resistance syndrome. There is also some evidence suggesting the presence of subclinical autonomic neuropathy, as characterised by an abnormal pupillary light reflex in subjects with IGT[66].

1.4 Microvascular function in insulin-dependent diabetes.

To attempt to explain the above differences in the pattern and natural history of microangiopathy in NIDDM and IDDM, it is necessary to outline what is known about the pathophysiology of microvascular

disease in the two forms of diabetes. Microvascular function has been extensively studied in IDDM, whereas there have been fewer studies to date concentrating on patients with NIDDM.

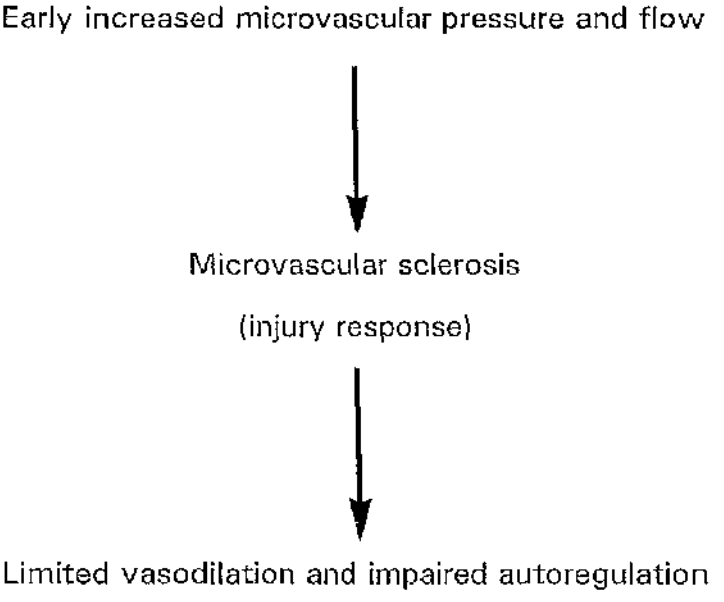
The haemodynamic hypothesis:

It has been proposed that diabetic microangiopathy may be the end result of long-standing functional changes in the microcirculation[67], such as increased pressure, blood flow and permeability, which could eventually lead to microvascular sclerosis, perhaps via an injury response to repetitive endothelial damage (Fig 1.2). In turn, such structural changes could lead to further impairment of microvascular function by limiting flow reserve and the capacity to autoregulate, that is, the ability to maintain a constant flow in the face of varying perfusion pressure. There is now a large amount of experimental evidence in favour of this haemodynamic hypothesis[67-69]. An important feature of such a chain of events is that the early functional changes are potentially reversible, in contrast to established microangiopathy which is largely irreversible.

Microvascular function in skin, subcutaneous tissue and muscle:

Haemodynamic changes have been extensively characterised in these tissues, especially the skin which is easily accessible to investigation using non-invasive techniques. Abnormalities of skin microcirculatory function may have direct clinical relevance in the pathogenesis of diabetic foot ulcers, as regardless of whether

Fig 1.2 ***The haemodynamic hypothesis for the pathogenesis of diabetic microangiopathy***



predominantly due to ischaemia or neuropathy, ulceration ultimately represents microcirculatory failure[7]. In addition, abnormal microvascular function may have a profound effect on the healing of foot ulcers, e.g. limitation of vasodilation could depress the acute inflammatory response to infection and trauma[7].

Increased microvascular blood flow is present in the skin at an early stage after diagnosis in patients with IDDM[70]. Much of this increased flow occurs through arterio-venous shunt vessels as suggested by both early experiments demonstrating histological arterialisation of the venous system in the foot[71] and partitioning of microspheres[72] and more recent studies showing high foot venous oxygen tension[73], abnormal forward flow in diastole on Doppler sonography[74] and high flow in the toe pulp - an area rich in arteriovenous anastomoses - using laser Doppler fluximetry[75]. There is also evidence for increased flow through the nutritive skin capillaries using direct videomicroscopy[75], although it is unknown whether this is adequate to meet the increased metabolic demands of the tissues due to raised temperature and metabolic rate. Microcirculatory overperfusion is associated with poor glycaemic control and the presence of neuropathy[70,74,75], and improvement in diabetic control using continuous subcutaneous insulin infusion has been shown to redistribute blood flow in favour of the nutritive microcirculation[76].

Although microvascular overperfusion has been well characterised, capillary pressure measurements in patients with diabetes have only recently been reported. Studies in IDDM patients have demonstrated an increase in skin nailfold capillary pressure which is present early in the course of the disease and is related to the degree of hyperglycaemia[77]. Capillary pressure is elevated in patients with early evidence of nephropathy (microalbuminuria), in contrast to those with

a long disease duration but minimal evidence of microvascular complications, where capillary pressure values are similar to those found in control subjects[78].

Increased microvascular fluid permeability has been demonstrated in the tissues of the forearm in young IDDM patients with a short duration of diabetes even during reasonable glycaemic control, suggesting a primary change in permeability[79]. In contrast, other early permeability changes, such as an elevated transcapillary escape rate of albumin, are improved with glycaemic control[80]. Permeability changes to a variety of solutes, including an increased capillary diffusion capacity[81,82], and increased transcapillary escape rates for albumin[83] and immunoglobulin G[84] are found in patients with longer disease duration, especially those with microangiopathy.

In addition to such haemodynamic changes under resting conditions, there is evidence for impairment of postural responses, such as postural vasoconstriction[85]. This refers to the increase in precapillary resistance which occurs in the lower limb on standing, thereby limiting the rise in capillary pressure resulting from the vertical column of blood between the heart and foot. Postural vasoconstriction is principally mediated by a local sympathetic axon reflex, the veno-arteriolar reflex[86]. Ineffective postural vasoconstriction leads to increased microvascular pressure and blood flow on dependency, with increased fluid filtration from the microcirculation and ultimately oedema formation. The postural vasoconstriction response is normally acquired during passage through puberty, yet children with diabetes fail to develop this protective reflex[87]. Loss of this and other protective mechanisms results in an increased haemodynamic burden during activity, which could further accelerate the rate of microvascular damage.

Despite microvascular overperfusion under resting conditions, there is limitation of maximum perfusion in response to a number of stimuli. Assessment of skin microvascular hyperaemia using laser Doppler fluximetry has shown a reduction in response to local heating in IDDM patients after several years of diabetes[88]. Similar reductions in maximum vasodilation have been demonstrated following minor skin trauma induced by a needle prick[88,89], and after arterial occlusion[90,91]. Limited microvascular vasodilation has also been found in children who have had diabetes for several years[92], despite the rarity of clinically detectable microangiopathy before puberty.

Diabetic neuropathy leads to abnormalities of neurogenic vasodilation such as the flare response[93] which occurs on stimulation of nociceptive C fibres and may be an important part of the response to foot trauma. A decreased flare response to iontophoretically applied acetylcholine has been demonstrated in the sole of the foot in patients with neuropathic ulcers and Charcot's arthropathy[94]; similar abnormalities are present in the skin of the dorsum of the foot at an earlier stage of neuropathy as defined by a raised vibration sensory threshold[95].

Finally, there is evidence for impaired microcirculatory autoregulation[96]. This has been related to the amount of basement membrane thickening in terminal arteriolar walls[97], supporting the idea that arteriolar sclerosis may contribute to limited vasodilation.

Microvascular function in other tissues:

There are considerable differences in the normal structure of individual microvascular beds in different tissues, allowing

specialisation of function, e.g. filtration in the glomerulus. Despite this, similar haemodynamic abnormalities to those already described in skin, subcutaneous tissue and muscle are present in other tissues and organs in patients with IDDM.

Kidney

In the kidney, an increase in glomerular filtration rate is present at diagnosis in IDDM which relates to glycaemic control. This glomerular hyperfiltration is due, in part, to increased renal plasma flow and an increase in filtration surface area[98]. There is also compelling evidence in favour of a rise in intraglomerular hydrostatic pressure[99], which is supported by the results of direct micropuncture studies in animal models[99]. There is, at present, some controversy regarding the exact relationship of early glomerular hyperfiltration to the subsequent development of diabetic nephropathy. Glomerular permeability to macromolecules, as reflected by a rise in AER, is also increased at this early stage[80]. In advanced nephropathy, there is loss of autoregulation of glomerular filtration rate[100].

Retina

Studies of microvascular function have been more limited in the retina; however, once again a rise in retinal blood flow has been detected at an early stage in IDDM[101], along with increased capillary leakage observed in a proportion of fluorescein angiograms and on vitreous fluorophotometry, although the latter observation has not

been confirmed in more recent studies using sensitive equipment[102]. There is also some evidence suggesting an elevation in retinal perfusion pressure[103]. Loss of pericytes at an early stage in the course of diabetes may play a part in retinal vasodilation[32]. At a later stage, there is maldistribution of retinal blood flow, with areas of non-perfusion adjacent to areas of overperfusion, and loss of autoregulation[104].

Peripheral nerve

There is now increasing evidence for the involvement of microvascular disease in the pathogenesis of diabetic neuropathy. Decreased nerve blood flow and reduced oxygen tension have been observed in animal models of diabetic neuropathy[105] with improvements in nerve conduction velocity with increases in arterial PO_2[106]. Impaired nerve blood flow with arterio-venous shunting[107] and reduced sural nerve oxygen tension[108] have also been found in human diabetic neuropathy. Fibre loss is patchy in sural nerve biopsies from diabetics with neuropathy[109] suggesting local ischaemia. Some studies have demonstrated endothelial abnormalities[110] and increased capillary closure[111] in endoneurial vessels and others have related the degree of endoneurial vessel disease to the severity of the neuropathy[6,112].

1.5 Microvascular function in non-insulin-dependent diabetes.

Microvascular function in skin, subcutaneous tissue and muscle:

In contrast to the large number of studies of skin microvascular function in IDDM, there have been few studies in NIDDM patients to date, although some of these suggest that there may be differences in the pattern of functional abnormalities observed in the two types of diabetes.

Microvascular overperfusion has been observed under resting conditions in patients with NIDDM, particularly during poor glycaemic control[113] or in those with neuropathy[114]. Improvement in diabetic control using intravenous insulin infusion has been shown to redistribute blood flow in favour of the nutritive microcirculation[114]. In contrast to the marked changes described in IDDM[77,78], skin nailfold capillary pressure does not appear to be elevated in normotensive NIDDM patients[115]. On the other hand, NIDDM patients have an abnormal capillary pressure waveform, with a decreased systolic arrival time[115], indicative of an increase in transmission velocity to the capillary bed along stiffened large vessels as a result of reduced arterial wall compliance.

Permeability changes in the skin and subcutaneous tissues are less clear cut than in IDDM. Increased permeability to sodium fluorescein in the skin[116] has been described in a mixed group of IDDM and NIDDM patients, and a rise in the permeability/surface area product of pentetic acid, a small lipophilic molecule, has been found in NIDDM[117]. Increased permeability of the forearm tissues to radio-labelled albumin has also been reported[118]: however, this was most often related to a

slow disappearance of albumin from the tissues, reflecting a decrease in lymphatic washout rather than a true increase in permeability. In addition, this abnormality was most marked in hypertensive NIDDM patients[118], and was only found in a minority of normotensive patients, all of whom had neuropathy and poor glycaemic control[119]. Permeability to albumin assessed by another technique, the transcapillary escape rate of radiolabelled albumin following intravenous injection (which measures mainly extra-renal clearance) is not increased in normotensive NIDDM patients with no clinical evidence of microangiopathy[120]; whereas poor metabolic control, hypertension and microangiopathy have all been shown to independently increase this parameter[120].

The major functional abnormality found in the skin microcirculation in NIDDM is a marked reduction in maximal microvascular vasodilation[121,122]. This has been found to be already present at diagnosis[122], whereas in IDDM a similar degree of vasodilatory impairment may take several years to become apparent[88]. In contrast, cutaneous vasodilation responses to exercise of underlying muscle are similar in NIDDM patients and control subjects[123], although it should be emphasised that this is not a measure of maximum vasodilatory capacity. The mechanism for this response, however, appears to be different in NIDDM patients and control subjects, involving increased capillary flow in the former and capillary recruitment in the latter[123]. Although no studies have looked specifically at microvascular blood flow in skeletal muscle in NIDDM, reductions in total muscle blood flow after exercise[124] and insulin infusion[125] have been described. In contrast, there does not appear to be impairment of muscle reactive hyperaemia following arterial occlusion[126].

Microvascular function in other tissues:

Regarding other microcirculatory beds, increased renal plasma flow can be detected in newly diagnosed NIDDM patients[127,128]. An increase in retinal blood flow has also been observed in patients with NIDDM using the technique of laser Doppler velocimetry[129], and can be reduced by an insulin-induced decrease in blood glucose concentration. There is also reduced retinal vasoconstriction in response to breathing 100% oxygen[129]. Finally, arteriovenous shunting and reduced blood flow have been observed in the endoneurial microvessels of NIDDM patients with neuropathy[130].

There is no data on direct measurement of capillary pressure in other microvascular beds; however, indirect calculations of intraglomerular hydrostatic pressure from changes in renal plasma flow and filtration fraction yield inconsistent results in NIDDM[127,128].

Clinically, marked exudative changes and macular oedema imply that increased permeability is present in diabetic maculopathy; however, a study in NIDDM patients who had no retinopathy, using the technique of vitreous fluorophotometry, showed no evidence of increased retinal permeability at this stage[131], suggesting that permeability changes may be secondary. In contrast, increased permeability to both fluid and macromolecules seem to be present in the kidneys at an early stage in NIDDM: Some studies have found early glomerular hyperfiltration[127,128,132] similar to that found in IDDM, which reduces with improved glycaemic control[127], although other studies have found glomerular filtration rate to be normal[133,134]. Early increased urinary albumin excretion is found in some NIDDM patients[58], which may in part relate to abnormal tubular function[135]; nonetheless, even in the presence of normal albumin excretion, there appears to be

an early loss of glomerular pore size selectivity[132,136] and reversible tubular dysfunction, as reflected by enzymuria[137]. Finally, with increasing albumin excretion there is also loss of glomerular charge selectivity[138].

Microvascular function in impaired glucose tolerance:

To date, microvascular function remains largely unexplored in subjects with IGT. Regarding blood flow, one study suggests a reduction in insulin-mediated increase in skeletal muscle perfusion in obese non-diabetic subjects[125], while there may be increased microvascular permeability, as reflected by the presence of microalbuminuria in some subjects with IGT[65].

1.6 Mechanisms of abnormal microvascular function

The mechanisms underlying abnormal microvascular function at the molecular and cellular level in NIDDM remain to be elucidated, and therefore the following discussion is largely speculative.

The development of diabetic microangiopathy is closely linked with poor glycaemic control. It therefore follows that hyperglycaemia and its biochemical consequences are likely to underlie changes in microvascular function. This is emphasised by the fact that microvascular complications are most marked in cells and tissues in which glucose uptake is largely independent of insulin action, so that

cellular glucose concentrations directly reflect blood levels. Hyperglycaemia has a direct toxic effect on endothelial cells in culture and reduces the rate of cell division[139]. There are several potential mechanisms through which hyperglycaemia could affect microvascular cell function: Firstly, hyperglycaemia may induce alterations in intracellular enzyme systems such as increased protein kinase C activity with subsequent widespread effects on cellular function[140]. In addition, increased activity of the enzymes of the polyol pathway as a consequence of hyperglycaemia may be of relevance due to the osmotic effects of increased sorbitol concentrations, depletion of myoinositol or altered sodium-potassium ATPase activity[141]. Finally, increased non-enzymatic glycation of proteins[142], in addition to interfering with cell enzyme activity, may also lead to permeability changes via altered charge or structural properties of glycoproteins present in intercellular clefts and basement membrane[21].

As microvascular disease can already be detected at the time of diagnosis in NIDDM, when there is only mild hyperglycaemia in some patients, it is unlikely that hyperglycaemia alone is responsible for the development of early microvascular abnormalities. Further evidence for this comes from studies of improved glycaemic control in patients with IDDM to similar levels of mild hyperglycaemia which have resulted in improved microvascular function[76,80].

Other metabolic abnormalities that could affect microvascular function in the early stages of NIDDM include hyperinsulinaemia and lipid abnormalities. Patients with IGT and NIDDM have peripheral hyperinsulinaemia due to increased endogenous insulin secretion as a result of insulin resistance, and, at a later stage in the disease, exogenous insulin therapy. It has been demonstrated that insulin stimulates proliferation of capillary endothelial cells, pericytes and

smooth muscle cells in culture[43,143-145]. In addition, basement membrane producing cells appear to be hypersensitive to the effects of insulin leading to increased type IV collagen and decreased heparan sulphate synthesis[146], the latter of these being important for the maintenance of the charge barrier that is a determinant of permeability[21]. Similarly, lipid abnormalities associated with NIDDM, such as hypertriglyceridaemia and oxidised lipoproteins[147], could alter cellular activity via effects on cell membrane structure and function.

Regardless of the underlying biochemical mechanisms, abnormal endothelial cell function is a plausible candidate to contribute to altered blood flow, perhaps via an imbalance in the secretion of vasodilator and vasoconstrictor vascular mediators. Elevation of endothelium-derived relaxant factor (EDRF) levels[25] early in the course of diabetes due to increased shear stress acting on the endothelium, could lead to initial vasodilation and overperfusion; and as endothelial damage accrues, limitation of EDRF production[28] could contribute to impaired maximal vasodilation. Advanced glycation end products may also 'quench' nitric oxide[148], providing a further mechanism for limitation of vasodilation. Although it is difficult to extrapolate findings from larger vessels to the microcirculation, intra-arterial infusion studies have demonstrated decreases in both endothelium-dependent and independent (i.e. myogenic) forearm vasodilation in NIDDM[126]. The situation is liable to be far more complex as there is evidence for alteration in the secretion or action of numerous other mediators in diabetes, such as catecholamines and components of the renin-angiotensin system[149]. Abnormalities of prostaglandin secretion may be important in the development of glomerular hyperfiltration[150], possibly via differential effects on afferent and efferent glomerular arteriolar tone. It has been proposed that oxidative stress due to

increased free radical production may underlie abnormal microvascular cell function in diabetes[151], and that this may be due to an increase in the intracellular NADH/NAD+ ratio secondary to abnormalities in many of the biochemical pathways mentioned above - 'hyperglycaemic pseudohypoxia'[152]. Increased free radical activity has been demonstrated in NIDDM patients, especially those with microalbuminuria[153].

Altered endothelial cell and platelet function also contribute to the prothrombotic changes reported in patients with NIDDM[154,155]. These, along with changes in microvascular rheology, such as decreased red and white cell deformability[156], could contribute to disordered microcirculatory function; however, there is little evidence correlating such in vitro changes with early changes in microvascular blood flow, making it likely that such abnormalities are of more relevance in the presence of established microangiopathy.

In the presence of impaired autoregulation, further damage to the microcirculation may occur in the absence of hyperglycaemia. Loss of flow regulation in combination with decreased perfusion following improved glycaemic control may compromise tissue nutrition, thus explaining the acute deterioration in microvascular disease observed in some patients after marked improvements in control ('glycaemic re-entry' phenomenon). Likewise, during periods of hypoglycaemia, loss of pressure autoregulation may allow the transmission of associated increases in systolic blood pressure directly to the capillary bed, which along with the prothrombotic changes which accompany hypoglycaemia, may further accelerate microvascular damage[157].

Metabolic abnormalities alone are not sufficient to explain why microvascular complications only affect a proportion of patients with diabetes, making it likely that there is also a genetic component. In

IDDM the tendency to nephropathy may be linked to a genetic predisposition to hypertension as some patients have a family history of this[158]. In addition, increased red blood cell sodium-lithium countertransport activity, which is a marker for other cell membrane cation exchange pumps and is elevated in essential hypertension, has been reported in both patients with nephropathy and their parents[159]. The exact genes involved in the pathogenesis of microangiopathy in NIDDM have still to be identified.

1.7 Effect of peripheral vascular disease and hypertension on microvascular function.

Peripheral vascular disease:

Given the high prevalence of hypertension and peripheral arterial disease in NIDDM, it is important to consider the impact of these disease processes on microvascular function if microvascular behaviour in NIDDM is to be properly understood.

Peripheral vascular disease leads to several abnormalities of microvascular function which have been best described in non-diabetic patients. The prime importance of the microcirculation in the pathogenesis of critical limb ischaemia is emphasised by the fact that some patients with significant large vessel disease, as manifest by low toe systolic blood pressures, do not develop ischaemic lesions, while, conversely, others with high toe systolic pressures do.

Resting laser Doppler flux and transcutaneous oxygen tension ($tcPO_2$) measurements are normal until the ankle : brachial pressure index falls below 0.3[160]. In the presence of significant proximal arterial obstruction there is maldistribution of blood flow between the nutritive vessels and arterio-venous shunts[161]. Further evidence for this comes from fluorescein studies which have revealed a heterogeneous distribution of microvascular perfusion in both time and space in patients with severe lower limb ischaemia[162], although such patients may retain a normal skin capillary density on direct microscopy. Some patients with critical limb ischaemia actually have a higher than normal total foot blood flow at rest[163], suggesting that a maldistribution of flow, or microvascular 'steal', may be important in the development of ischaemic ulceration. Although the mechanism of this maldistribution is currently unclear, there is evidence for a disturbance of vasomotion[164] which could explain the loss of dynamic equilibrium.

Postural vasoconstriction is impaired in those with rest pain or ankle brachial pressure index less than 0.5 when compared to controls[160]; or in those with unilateral severe ischaemia, when compared to the other leg[165]. In addition, visible capillary density increases in the skin of severely ischaemic feet on dependency[166], a fact which may explain the characteristic relief of rest pain by lowering the foot in such patients. The loss of postural vasoconstriction may be due to the build up of vasoactive metabolites, secondary to tissue ischaemia, leading to local vasodilation of pre-capillary resistance elements. Such a response may be a compensatory mechanism to maintain nutritional blood flow in the presence of proximal arterial obstruction.

Despite the fact that there is overperfusion under resting conditions, there is, not surprisingly, a limitation of maximum microvascular hyperaemia. Post-occlusive reactive hyperaemia is impaired in severely

ischaemic limbs and seems to affect both the nutritive and thermoregulatory microcirculation to a similar degree[161]. Maximum blood flow and $tcPO_2$ measurements on locally heated skin are also reduced in the face of severe ischaemia[160].

These microvascular abnormalities appear to be functional as there is evidence that they are improved following angioplasty or reconstructive arterial surgery[167].

Essential hypertension:

Studies of microvascular function in non-diabetic subjects with essential hypertension have shown that skin nailfold capillary pressure is increased in untreated subjects compared with normotensive controls[168]. In contrast to the situation in both major forms of diabetes, studies of maximum microvascular hyperaemia yield inconclusive results, with some showing no reduction[169,170], while others do show limited vasodilation[171]. In any case correction for elevated perfusion pressure in essential hypertension reveals increased structurally-based resistance to blood flow[169,171] which probably represents an adaptive (structural) autoregulatory response to a sustained increase in intra-arterial pressure. Permeability changes, such as an increased transcapillary escape rate of albumin, have also been reported in essential hypertension[172].

In summary, it can be seen that peripheral vascular disease and hypertension can independently lead to altered microvascular function, compounding the effects of microvascular abnormalities due to diabetes itself, influences that need to be appreciated given the high prevalence of hypertension and large vessel disease in NIDDM.

1.8 Aims of Thesis.

Early functional changes in the microcirculation are thought to be important in the pathogenesis of diabetic microangiopathy. In IDDM, there is a large body of evidence supporting a pivotal role for increases in pressure, blood flow and permeability; whereas in NIDDM, there have been few studies of cutaneous microvascular function, with the main abnormality found to date being a reduction in microvascular hyperaemia at an early stage in the disease. Capillary pressure is reported to be normal and there have been no studies of fluid permeability. The aims of this thesis are to further characterise cutaneous microvascular function in NIDDM through the following studies:

1. Assessment of microvascular fluid permeability by venous occlusion plethysmography in patients with NIDDM and control subjects (Chapter 3).

2. Further investigation of the limited microvascular hyperaemia reported in patients with NIDDM:

   a). Reconfirmation of original results in a group of patients with large vessel disease excluded by Doppler sonography (Chapter 4.1).

   b). Examination of the effect of improved glycaemic control on microvascular hyperaemia in recently diagnosed NIDDM patients (Chapter 4.2).

   c). Examination of the effect of hypertension on microvascular hyperaemia in NIDDM (Chapter 4.3).

3. Determination of microvascular hyperaemia in subjects at risk of developing NIDDM who have IGT (Chapter 5.1) and insulin resistant patients with acromegaly (Chapter 5.2). Investigation of the relationships of ß-cell dysfunction, insulin resistance, hyperlipidaemia, microalbuminuria and 24-hour ambulatory blood pressure on microvascular hyperaemia in patients with IGT

4. Investigation of the mechanism of limited hyperaemia in subjects with IGT by measuring endothelial-dependent, endothelial-independent (vascular smooth muscle) and neurogenic vasodilation (Chapter 6).

5. Assessment of capillary density in patients with IGT and NIDDM, and control subjects, to determine whether there is evidence of impaired development of the vasculature resulting in reduced capillary density which could contribute to limited microvascular hyperaemia.

CHAPTER 2

METHODS

2.1 Standard protocol for microvascular studies.

Standardised conditions of measurement are a prerequisite for studying the microcirculation in view of the marked effect that factors such as drugs, temperature and sympathetic nervous system activity can have on measures of microvascular function, especially blood flow. The following standard protocol was adopted to attempt to minimise such sources of variability.

Prior to attendance at the laboratories, subjects were asked to refrain from taking caffeine containing drinks and food for two hours, no alcohol was allowed for a 24 hour period, and no smoking for four hours.

Subjects were allowed to acclimatise for a period of 30 minutes in a controlled temperature laboratory at $22 \pm 0.5^{o}C$, lying supine and relaxed in a quiet environment, with the limb under study supported at heart level. During this time, room and skin temperature were monitored every five minutes using a thermocouple thermometer (Fluke 52, RS Components, Corby, UK), and no studies were started until both temperatures were stable.

In view of the effects of hypertension and large vessel disease on microcirculatory function, subjects with either of these were excluded from study. The only exceptions to this were the study described in Chapter 4.3, which was designed specifically to look at the effects of

hypertension on the microcirculation in patients with NIDDM; and Chapter 5.2, where some hypertensive patients with acromegaly were not excluded due to the small sample size. No subjects were taking any vasoactive medication (except insulin or oral hypoglycaemic agents, and in Chapter 5.2 bromocriptine), and all had a random blood glucose concentration between 4 and 15 $mmol.l^{-1}$ at the time of study. Diabetic subjects were excluded if there was a history of hypoglycaemia in the preceding 24 hours.

All studies were approved by the local Ethical Committee and informed verbal consent was obtained from all participants.

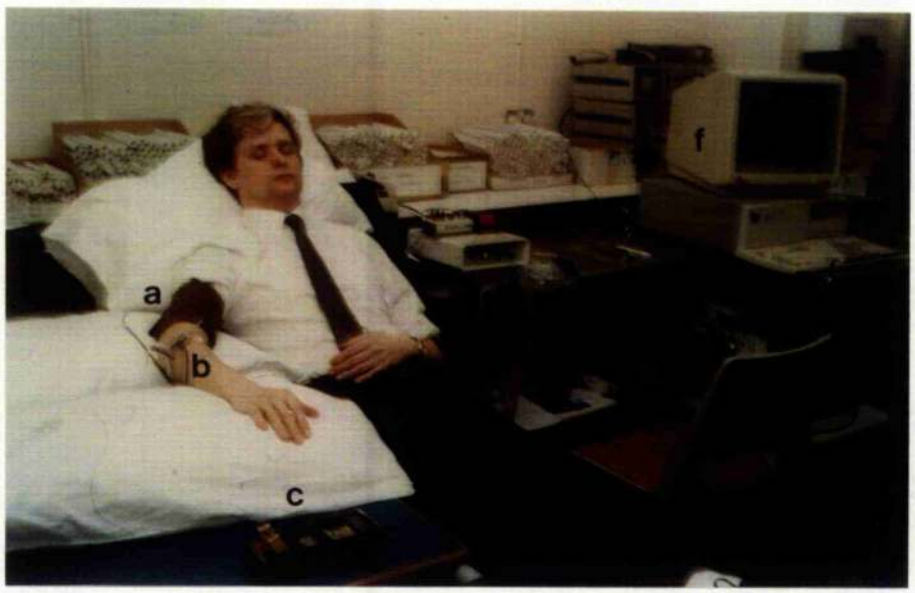
2.2 Venous occlusion plethysmography.

Venous occlusion plethysmography involves the measurement of changes in distal limb volume as a result of increasing venous pressure via a cuff applied to a more proximal part of the limb. The recorded response has a characteristic shape with an initial rapid exponential component attributed to the filling of venous vessels, and a concurrent linear phase thought to be due to fluid filtration (see Fig 2.2.3). Several parameters, including arterial inflow, venous compliance and fluid filtration can be determined from this change in limb volume[173]. The capillary filtration coefficient (CFC), a measure of microvascular fluid permeability, can be derived from measurements of fluid filtration.

Capillary filtration coefficient

A sensitive strain gauge plethysmographic method, employing a computer-based logging and analysis system[174,175], was used in Chapter 3 to compare forearm CFC between patients with NIDDM and control subjects (Fig 2.2.1). Previous studies using plethysmography may have given inaccurate results as CFC calculations were based on the fluid filtration response following a single large increase in venous pressure (>40 mmHg)[80,176], which is known to invoke the veno-arteriolar reflex[177], a protective local axon reflex activated by a rise in venous pressure which normally induces postural vasoconstriction, as previously discussed in section 1.4 (page 14). When the venoarteriolar reflex is activated, resultant arteriolar vasoconstriction leads to slower filling of microvessels in the area of study, thus risking inclusion of part of the vascular filling component of the strain gauge response in the fluid filtration slope[175] (see page 38). This is important as abnormalities of the veno-arteriolar reflex have been demonstrated soon after diagnosis in patients with diabetes[96]. The smaller pressure steps used (<12 mmHg) in the modified plethysmographic technique are known to decrease the time course of this vascular filling component, by avoiding invoking the veno-arteriolar reflex[175], thus excluding any confounding effects of diabetes-related abnormalities in the veno-arteriolar reflex. In addition, basing the calculation of CFC on the responses to several smaller pressure steps in the modified technique[175] may improve reproducibility by minimising any error introduced by measurements from a given step; and by defining at exactly what cuff pressure filtration begins to occur, it avoids the innaccuracy of having to extrapolate back to the origin from a single

Fig 2.2.1 ***The strain gauge plethysmography system used to measure forearm CFC***



a = modified sphygmomanometer cuff

b = strain gauge and gauge holder the forearm

c = thermocouple thermometer

d = air pump

e = amplifiers

f = monitor and microcomputer

point in determining the filtration slope.

Venous occlusion elevates microvascular pressure within the whole microvascular network from the venous end, thereby eliminating any effect of differences in homogeneity of tissue perfusion between diabetic patients and control subjects; however, a potential drawback of the technique is that differences in forearm tissue composition between diabetic and control subjects could influence the results, as the major tissue contributing to fluid filtration in the forearm is skeletal muscle[175]. There are no recognised differences in muscle mass between NIDDM patients and non-diabetic subjects, although body fat content is increased in a proportion of NIDDM patients. Care was therefore taken to ensure that forearm circumference and BMI were similar in NIDDM and control subjects.

In addition to the inherent permeability characteristics of the vessel wall, fluid filtration is dependent on the balance of opposing Starling's forces, that is, intravascular hydrostatic plus tissue oncotic pressures favouring filtration; and plasma oncotic plus tissue hydrostatic pressures resisting filtration[16]. Of these, intravascular hydrostatic pressure is the most predominant[16], and it is this which is raised during venous occlusion. It is assumed that a given rise in venous pressure will lead to a similar rise in microvascular pressure in both diabetic patients and control subjects as there is no evidence that post-capillary venous resistance is different in the two groups. Techniques for measuring tissue oncotic pressure, e.g. implantation of subcutaneous wicks, are open to criticism and have not been used to study NIDDM patients, although reduced tissue oncotic pressure has been demonstrated in IDDM patients using the wick method[178]. Currently, methods for measuring tissue hydrostatic pressure have not been fully developed. Although there is little information on tissue

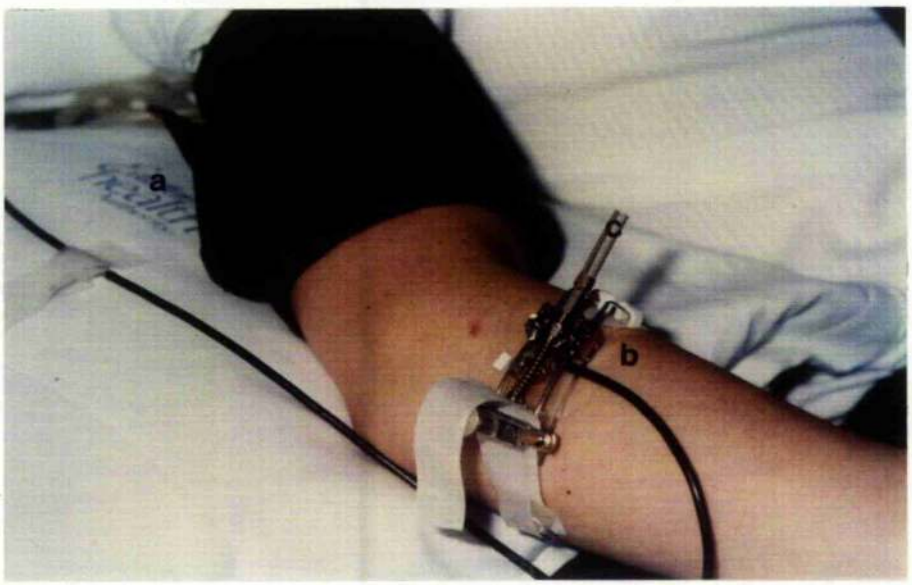
oncotic and hydrostatic pressure in NIDDM patients and control subjects, their contribution to fluid filtration is likely to be small. Lastly, care has been taken to exclude systematic differences in plasma oncotic pressure between diabetic and control subjects by measuring its main determinant[16], serum albumin concentration.

Protocol:

A six-inlet sphygmomanometer cuff (A.C. Cosser, London, UK), which enabled rapid inflation, was wrapped around the upper arm and inflated using an air pump (Compton type 2D/351DM: Dawson McDonald and Dawson Ltd, Ashbourne, Dorset, UK), equipped with a multiple resistance air bleed, which permitted stepped increases in occlusion pressure to be applied[179]. The occlusion pressure was monitored close to the cuff using a pressure transducer (type 256-720: RS Components, Corby, UK) coupled to a modified pressure indicator (type 256-758: RS Components). An additional flexible but inelastic outer cuff was used to minimise cuff compliance and thereby obtain rapid occlusion pressure transmission inwards to the soft tissues of the arm. The mercury-in-silastic strain gauge and gauge holder[175] was applied to the upper one third of the forearm (Fig 2.2.2) and the response to serial small increments in cuff pressure (5-12 mmHg every 5 min), up to a maximum less than diastolic blood pressure, was recorded on a microcomputer for subsequent analysis[174].

Once the ambient venous pressure had been exceeded, each increase in cuff pressure generated a characteristic response (Fig 2.2.3): an initial rapid exponential phase, due to filling of the compliant

Fig 2.2.2 ***The mercury-in-silastic strain gauge and gauge holder in position on the forearm***



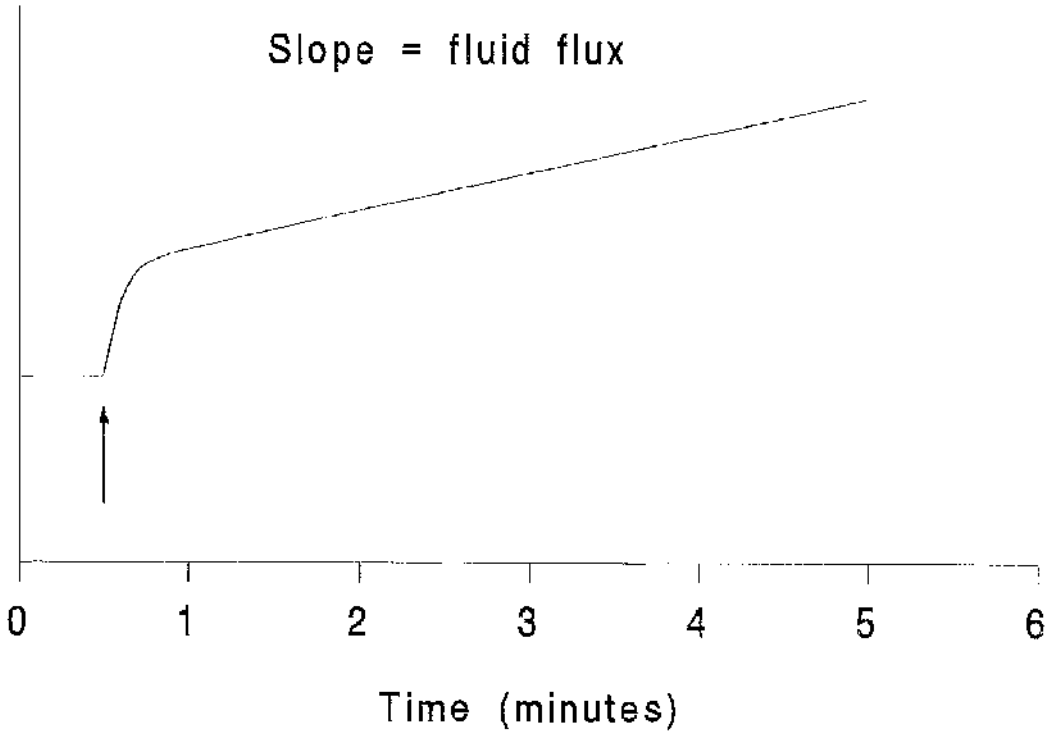
a = modified shygmomanometer cuff

b = strain gauge in gauge holder

c = calibration key for strain gauge

Fig 2.2.3 ***Diagramatic representation of typical recording from the strain gauge in response to an increase in cuff pressure of around 10 mmHg (after ambient venous pressure has been exceeded)***

Arrow indicates onset of increased cuff pressure. Fluid flux is calculated from the slope of the response between the fourth and fifth minute.



venous vessels beneath the gauge, and a concurrent linear phase due to fluid flux from the microcirculation once pressure was high enough to cause filtration. Values for fluid flux were obtained by least squares fitting of the volume response after the vascular compliance component was completed[175]. CFC (in $ml.min^{-1}.100g\ tissue^{-1}.\ mmHg^{-1}$) was calculated from the slope of the linear relationship between the values for fluid flux and corresponding cuff pressure[170] (Fig 2.2.4).

Reproducibility:

CFC determined by this method was found to be highly reproducible with an intra-individual coefficient of variation of 8.5% for five measurements on two subjects on separate occasions over a 6-month period (Table 2.2.1).

2.3 Laser Doppler fluximetry.

Laser Doppler fluximetry is a non-invasive technique for the continuous measurement of microvascular blood flow[180]. A low power laser is used to generate a non-injurious beam of red light which is transmitted to a small area of the skin surface via a flexible fibre optic probe where it penetrates the superficial layers of the dermis. Photons are mostly back scattered by stationary cells, but some undergo a shift in frequency (due to the Doppler effect) after contacting moving red blood cells. These Doppler shifts are photodetected by the fluximeter

Fig 2.2.4 ***The linear relationship between fluid flux and cuff pressure for each pressure step***

The gradient of the line represents CFC

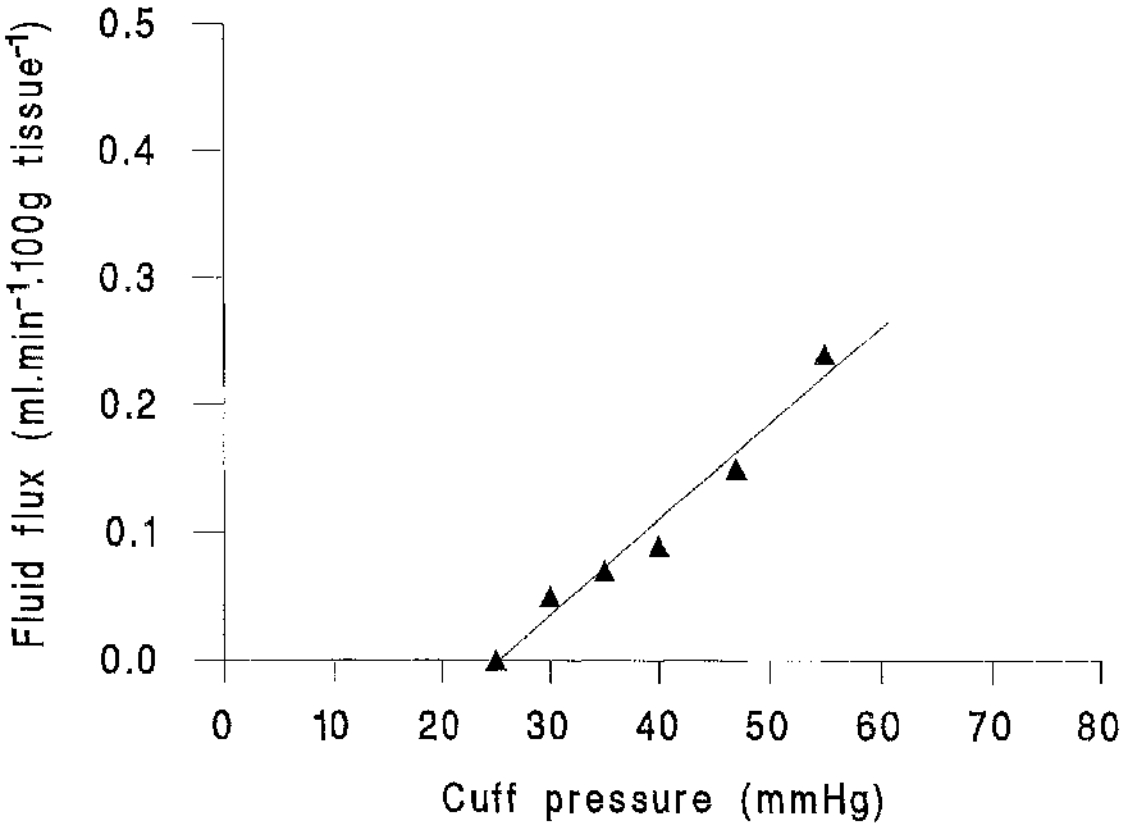


Table 2.2.1 ***Reproducibility of capillary filtration coefficient measurements***

The table shows data for 5 separate measurements over a 6-month period in each of 2 subjects, plus summary statistics.

	CFC ($ml.min^{-1}.100g\ tissue^{-1}.mmHg^{-1}$)							
SUBJECT	1	2	3	4	5	MEAN	SD	CV
M 29yr	4.5	4.8	4.1	4.1	4.2	**4.3**	**0.3**	**7%**
M 32yr	6.9	5.7	6.3	5.4	5.8	**6.0**	**0.6**	**10%**

mean CV = 8.5%

and computed to give a voltage signal which is directly proportional to superficial blood flow[180]. The response measured depends on both the concentration and velocity of red blood cells in the area of measurement[180]. The technique has been validated against several established methods for measuring blood flow and correlates well with microsphere flowmetry[181], ^{133}Xenon clearance[182], plethysmography[183] and thermal clearance[183], despite the fact that some of these do not exclusively measure skin microvascular blood flow. In addition, laser Doppler fluximetry shows broad agreement with direct measurement of skin capillary blood flow[184].

There are, however, several limitations to the technique of laser Doppler fluximetry:

1). Some doubt exists about the exact sampling depth of the instrument[182,183], which is partly determined by the wavelength of laser light employed, e.g. the Periflux Pf2 model produces red laser light of wavelength 632.8nm which is thought to penetrate the skin to a depth of 1mm[185]. This means that arterio-venous shunt and subpapillary plexus flow are measured in addition to capillary flow[186]. There is no evidence, however, to suggest that underlying muscle blood flow is measured[187]. In the current studies, this problem has been partly overcome by choosing areas of skin (dorsum of foot or volar aspect of forearm) where shunt vessels are absent, although this does not exclude plexus flow.

2. Blood flow is measured in terms of multidirectional 'flux' and not in conventional volume flow units[180], with results being expressed in terms of arbitrary units or volts. In view of this, laser Doppler fluximetry is best suited to the study of relative changes in flow or measurement of maximum flow. Both these applications are used in the current studies.

3. There are difficulties with standardisation of flow measurements. This even applies to comparisons of different instruments of the same model, or of readings from an individual fluximeter over time, due to variations in laser output and fibre optic probe performance[180], and currently there is no consensus on standardisation procedures. Recently, however, there has been some movement towards calibration against a 'motility standard', consisting of a suspension of polystyrene microspheres in water, performed at a standard temperature prior to each usage of the instrument[180]. This method of standardisation has been employed for both the Periflux and Moor laser Doppler fluximeters used in the current studies.

4. Readings may be affected by anatomical factors, such as differences in skin thickness, skin pigmentation and capillary density[180]. The possibility of variations in skin thickness has been minimised by the choice of measurement sites used in the current studies, i.e. the volar aspect of the forearm and dorsum of foot, although systematic differences between patients with diabetes and control subjects could only be fully excluded by skin biopsy which was not done. As all participants were Caucasian, variation in skin pigmentation between individuals should not have been a major factor, and would have applied equally to patient and control groups. The possibility of differences in skin capillary density affecting measurement of maximal hyperaemia is addressed in Chapter 7.

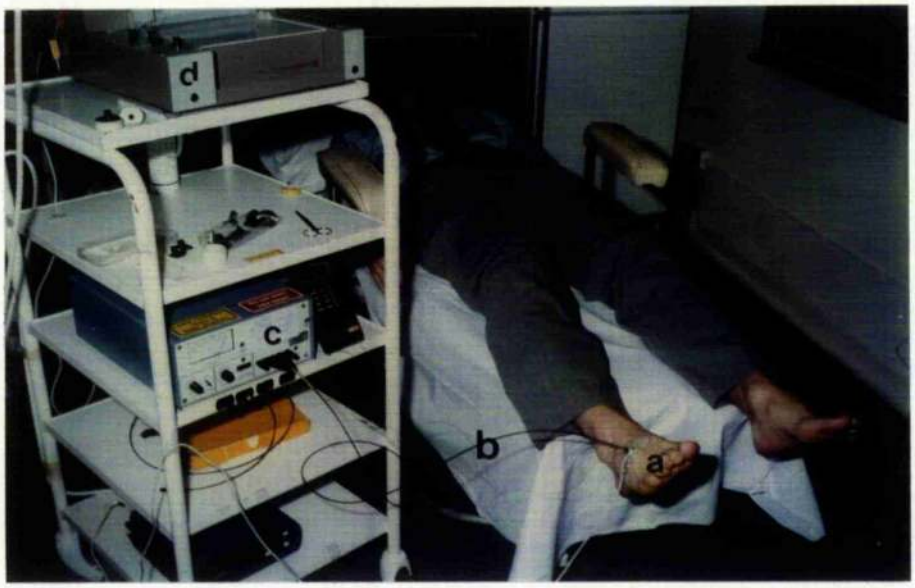
Maximum hyperaemia to local heating

Direct heating of the skin to a temperature of 42-44°C has been shown to induce maximal microvascular vasodilation[188]. The protocol used in the current studies is a modification of that used by Rayman et al[88]. A Periflux Pf2 laser Doppler fluximeter (Perimed, Stockholm, Sweden), which produces low power (2-3mW) red laser light of wavelength 632.8nm from a Helium-Neon source[180], was used to measure the maximum microvascular hyperaemic response and results were expressed arbitrarily in volts (V).

Protocol:

During the acclimatisation period, an area of skin on the dorsum of the right foot was directly heated to 44°C for a period of 30 minutes, using a small brass heater[88] (diameter 9mm) which fitted into a plastic collar secured to the skin surface using a double-sided adhesive disc (Fig 2.3.1 and 2.3.2). The heater had an eccentrically placed hole (diameter 2.5mm) through which the fibre optic laser probe was placed and the heater was rotated within its collar allowing measurement in multiple sites under the heater, with each being heated immediately prior to recording flow[88].

Fig 2.3.1 ***Measurement of maximum microvascular hyperaemia to local heating***



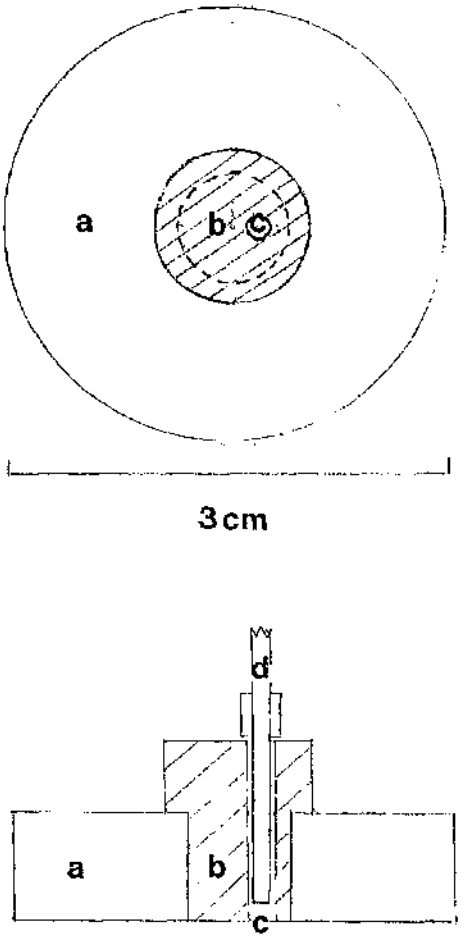
a = brass heater (in collar)

b = fibre optic probe

c = laser Doppler fluximeter

d = chart recorder

Fig 2.3.2 *Diagram of skin heater assembly*



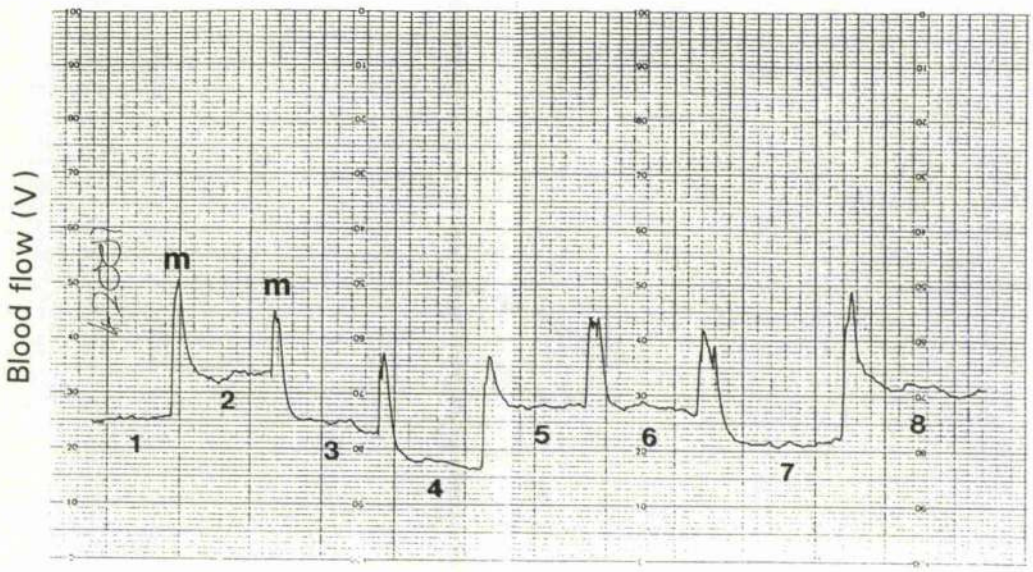
a = heater holder

b = brass heater

c = eccentric hole for probe

d = laser Doppler probe

Fig 2.3.2 ***Section of typical experimental trace showing variability of maximum hyperaemic response recorded from different sites in the heated area***



1-8 = adjacent heated sites

m = movement artefact

Table 2.3.1 ***Reproducibility of maximum hyperaemia measurements***

The table shows data for 5 separate measurements over a 3-month period in each of 2 subjects, plus summary statistics.

	Maximum Hyperaemia (V)							
Subject	1	2	3	4	5	Mean	SD	CV
M 29yr	2.56	2.08	1.96	2.67	2.36	**2.32**	**0.30**	**13%**
M 36yr	2.07	2.25	2.49	2.65	2.64	**2.42**	**0.25**	**10%**

mean CV = 11.5%

Reproducibility:

There was considerable blood flow variation between different sites in the heated area (Fig 2.3.3), as had been previously found[88]; however, the reproducibility of the technique was improved considerably by averaging flow for 30 seconds in each of 8 equally spaced sites to give a single value for maximum hyperaemia (Table 2.3.1)[88].

Iontophoresis

The technique of iontophoresis uses electrical charge to transfer small quantities of pharmacological agents directly across the skin surface. This is particularly suited to studying microcirculatory control mechanisms as it avoids the effects of local trauma and any potential hazards of systemic drug administration. The quantity of drug delivered depends on the charge of the ion, its molecular weight, the strength of the electrical current and the duration of current flow[189]. For a given drug, the amount delivered is directly proportional to the total charge which migrates through the skin surface. This total charge (Q) in millicoulombs (mC) is calculated using Coulomb's law:

$$Q = It$$

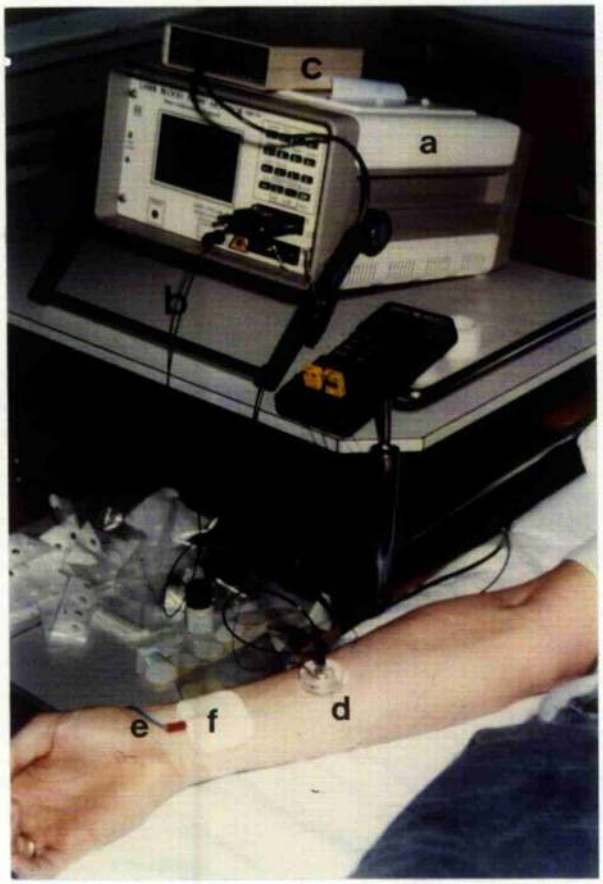
where I is the current in milliamps (mA) and t is the duration of current flow in seconds (s). It can be seen that the total charge can be increased by either increasing current strength or duration of current flow; however, current strengths above 0.2mA may cause significant electrically or thermally induced local axon reflex vasodilation[189]. In

view of this, current strength was set at 0.2mA and total charge was varied by altering the time interval for iontophoresis.

Pharmacological agents can either be iontophoresed directly into the area of measurement to assess their local effect, or at a site adjacent to the area of measurement in which case neurogenic vasodilation can be assessed. In Chapter 6 direct iontophoresis of acetylcholine (ACh) was used to measure endothelium-dependent microvascular vasodilation[189-191], and sodium nitroprusside (NaNP) to measure myogenic vasodilation[189,191], while indirect iontophoresis of ACh, through stimulation of nociceptive C fibres and resultant local axon reflex, allowed assessment of neurogenic vasodilation[189,192,193]. The techniques employed were modifications of those used by Westerman (direct)[189] and Walmsley (indirect)[192].

A complete iontophoresis system (Fig 2.3.4), which had recently been developed by Moor Instruments Ltd, Axminster, Devon, was used for all the iontophoretic studies. Briefly, the system consisted of an active platinum loop electrode contained within an iontophoresis chamber (Fig 2.3.5), and an indifferent electrode, both of which were linked to a constant current generator (iontophoresis controller MIC1). A laser Doppler fluximeter (MBF3D perfusion monitor) was used to measure the response, with a hole through the centre of the iontophoresis chamber acting as the fibre optic probe holder. The solution for iontophoresis was injected into a well inside the chamber. Time intervals and current strength could be set using the Laser Doppler fluximeter software. The MBF3D perfusion monitor differed from that used in previous iontophoresis studies in that it used laser light of longer wavelength (810nm) generated by a semiconductor laser diode. The laser diode is reported to have technical advantages over gas lasers; however, tissue penetration of the MBF3D is thought to be

Fig 2.3.4 ***The Moor iontophoresis system***



a = laser Doppler fluximeter

b = fibre optic probe

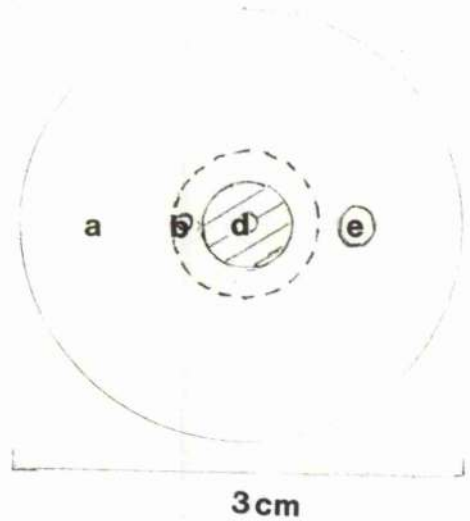
c = iontophoresis controller

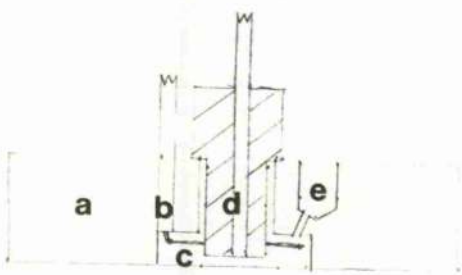
d = iontophoresis chamber on forearm

e = electrode wires

f = indifferent electrode

Fig 2.3.5 *Diagram of direct iontophoresis chamber*





a = iontophoresis chamber

b = active electrode

c = drug well

d = laser Doppler probe

e = air outlet

greater than that of the Periflux Pf2 (Dr R Gush, Moor Instruments, personal communication), leading to increased inclusion of plexus flow. The output of the MBF3D is expressed in arbitrary units (AU) rather than volts.

1% solutions of both ACh chloride (in water with 3% mannitol - 'Miochol' ophthalmic preparation, Cooper Vision Ltd, Southampton) and NaNP (in 5% dextrose - 'Nipride', Roche Ltd, Welwyn Garden City, Hertfordshire) were used for iontophoresis. The ACh solution is reported to be stable for only around two hours (Miochol data sheet), and therefore a fresh solution was made up prior to each study. It was noted in preliminary studies that the ACh solution retained some variable activity for several hours, presumably due to vasoactive breakdown products of ACh. In contrast, NaNP solution is known to remain stable for a 24-hr period when protected from light by foil and kept refrigerated at 4-8^{o}C (Nipride data sheet). Aliquots of NaNP were therefore taken from a stock solution which was made up daily, and heated to room temperature prior to usage.

In preliminary studies using direct iontophoresis, marked intra- and inter-subject variations in the shape and time course of the response curve were observed (Fig 2.3.6). Examination of iontophoresis sites after drug transfer confirmed the heterogeneity of the iontophoretic response, with the majority of sites being uniformly red, although some were non-uniformly red and others contained small blanched areas. The variability in measurement was much higher than previously reported by Westerman et al[189], possibly due to the deeper tissue penetration of the MBF3D. This was not improved by standardising skin temperature to 32-34^{o}C, despite the fact that resting skin temperature is known to influence the magnitude of the blood flow response[194], and a temperature $>32^{o}$C is required to produce a

maximal response[194]. In addition, the use of 2% methylcellulose gel as a drug delivery vehicle[189], instead of aqueous solution, was found to be unhelpful, mainly due to problems with air bubbles in the viscous solution, therefore aqueous solutions of drug were used throughout. In preliminary studies 4mC was the highest charge which avoided an electrically induced response - much lower than the values reported by Westerman for transcutaneous electrical nerve stimulation[189]. Despite this, a few subjects still had a minor electrical component to the blood flow response using 4mC during the studies reported in Chapter 6. As this contributed to less than 5% of the measured response and was present in a similar number of patients and control subjects, it was ignored for the purposes of calculating blood flow responses.

In the light of these various factors, satisfactory reproducibility was obtained by calculating the mean area under the iontophoresis response curve in four adjacent sites, after correcting for basal flow (see Fig 2.3.7).

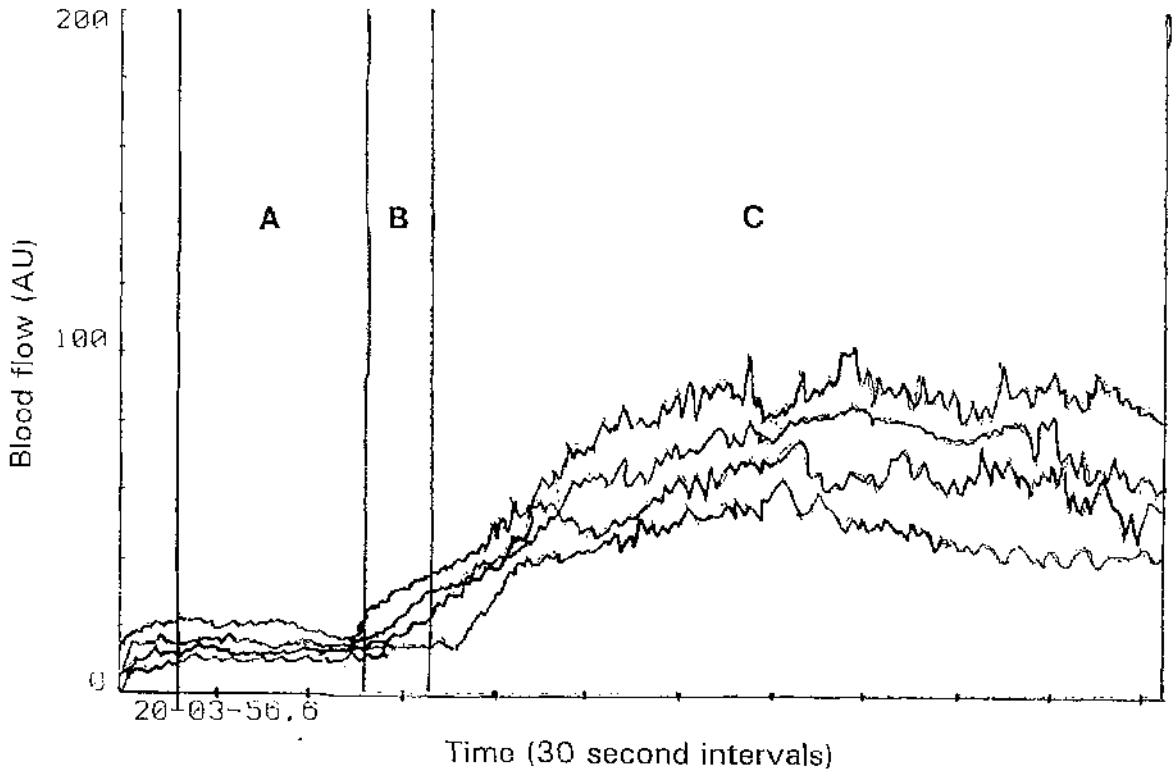
Indirect iontophoresis posed less problems in preliminary studies and satisfactory intra-individual reproducibility was obtained using a current of 0.2mA and a protocol similar to that of Walmsley[192]. In Walmsley's studies, this level of current was found to produce a submaximal flare response; however, others have shown that higher currents produce a significant electrical response[189], which may act by non-neurogenic mechanisms. A maximum current of 0.2mA was therefore used in the current studies to avoid such problems.

After indirect iontophoresis of ACh at 0.2mA for several minutes, a spreading flare response was visible beyond the outer limits of the iontophoresis chamber and at the same time, a reproducible stable blood flow measurement was recordable from the laser Doppler probe positioned in the centre of the chamber. The results of indirect

Fig 2.3.4 *Variability of direct iontophoresis response to acetylcholine*

The figure shows superimposed experimental traces from 4 adjacent sites on the right forearm of one individual recorded sequentially on the same occasion.

A similar variability was observed for responses to sodium nitroprusside



A = baseline period

B = iontophoresis period

C = response period

horizontal lines separate the different periods

iontophoresis were therefore expressed as the mean flow during the last two minutes of iontophoresis, rather than area under the curve.

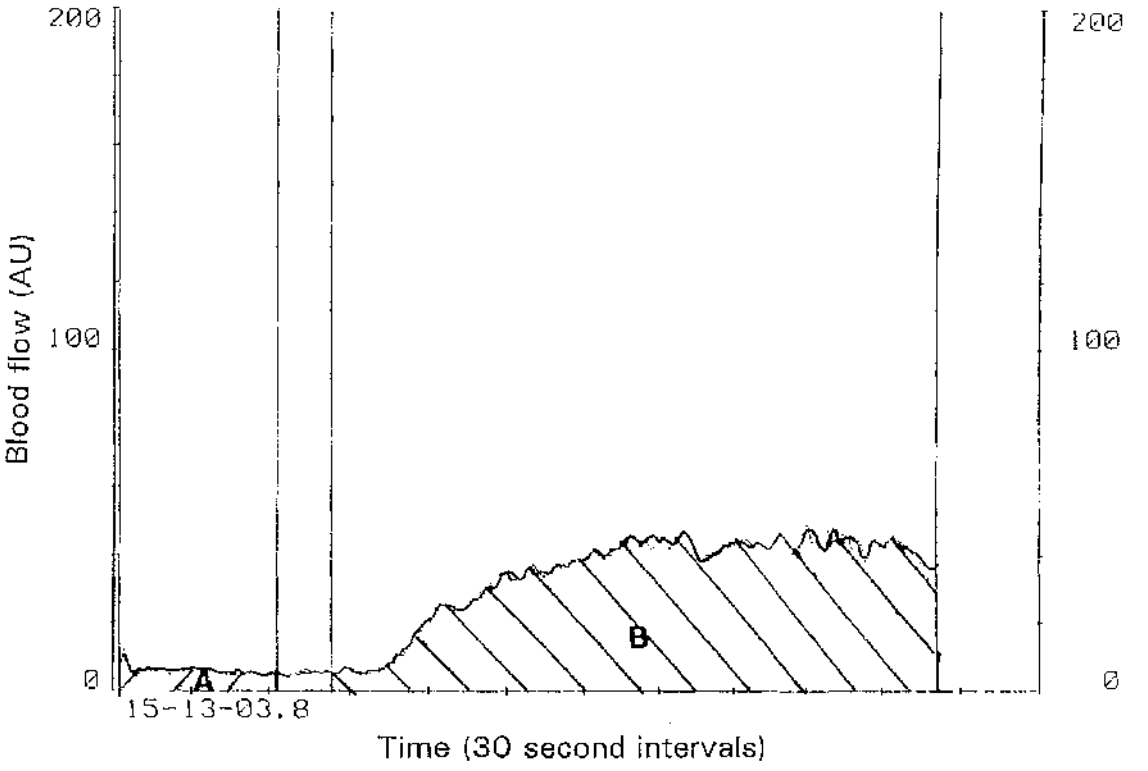
Protocol:

Direct iontophoresis:

At the start of the acclimatisation period, the skin of the forearm was lightly cleansed with 70% v/v isopropyl alcohol to remove sebum and reduce electrical resistance. The indifferent electrode was attached to a rubber electrode receptacle (IER1, Iomed Inc., Salt Lake City, Ut, USA) containing a dispersive gel pad (Hydro Pad, RDG Medical, Croydon, Surrey) and was applied to the skin near the wrist. The perspex iontophoretic chamber (Fig 2.3.5) was attached to the volar skin surface using a double-sided sticky disc, taking care to gain a waterproof seal, and the fibre optic laser probe was inserted through the centre of the iontophoresis chamber. The solution for iontophoresis was injected into a well inside the chamber, taking care to avoid the introduction of air bubbles which could interfere with the laser signal or charge transfer. The active electrode was made either anodal (ACh) or cathodal (NaNP) depending on the polarity of the drug being iontophoresed.

Following one minute's recording of basal blood flow, a total charge of 4mC (0.2mA for 20s) was applied and blood flow response measured for four minutes (ACh) or 6 minutes (NaNP). A longer response period was recorded for NaNP as preliminary experiments had shown that the response to NaNP was of slower onset and longer duration than that to ACh. The area under the curve for basal and

Fig 2.3.5 *Calculation of area of response following direct iontophoresis of acetylcholine*



A = area of basal flow

B = total area of response

The iontophoresis response is calculated by subtracting the predicted area due to basal flow (based on area A) from the total area of response.

response periods was recorded automatically by the instrument using system software. Data was downloaded directly onto an IBM compatible PC using the Moorsoft software package (Moor Instruments, Axminster, Devon), and a hard copy of the results obtained via the instrument's printer.

This whole process was repeated for a total of four sites on the right arm for ACh and four sites on the left arm for NaNP. A fifth iontophoresis site was used on each arm for a control solution (3% mannitol in de-ionised water for ACh and 5% dextrose for NaNP). The responses were subsequently corrected for basal flow and then expressed in terms of $AU.min^{-1}$ (Fig 2.3.7). The mean response for four sites was then calculated for each drug.

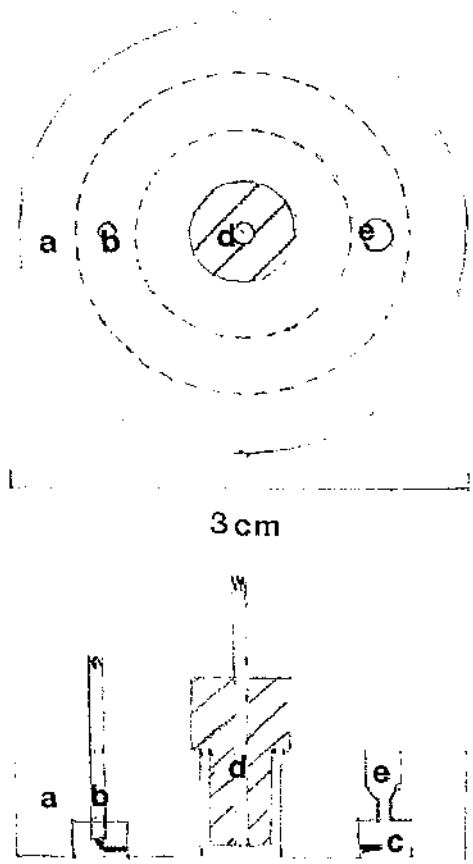
Indirect iontophoresis:

Following direct iontophoresis of ACh plus control solution, a sixth site on the right forearm was used for indirect iontophoresis of ACh 1% solution. The chamber used for indirect iontophoresis is illustrated in Fig 2.3.8. Basal flow was again recorded for one minute followed by iontophoresis at 0.2mA for ten minutes (Fig 2.3.9). Using the Moorsoft package, mean basal flow was determined and maximal flow was calculated as the mean flow over the last two minutes of the iontophoresis period.

Reproducibility:

Using the above protocols, reproducibility of the responses to direct iontophoresis of Ach and NaNP were both 14% (Tables 2.3.3 and

Fig 2.3.8 *Diagram of indirect iontophoresis chamber*



a = iontophoresis chamber

b = active electrode

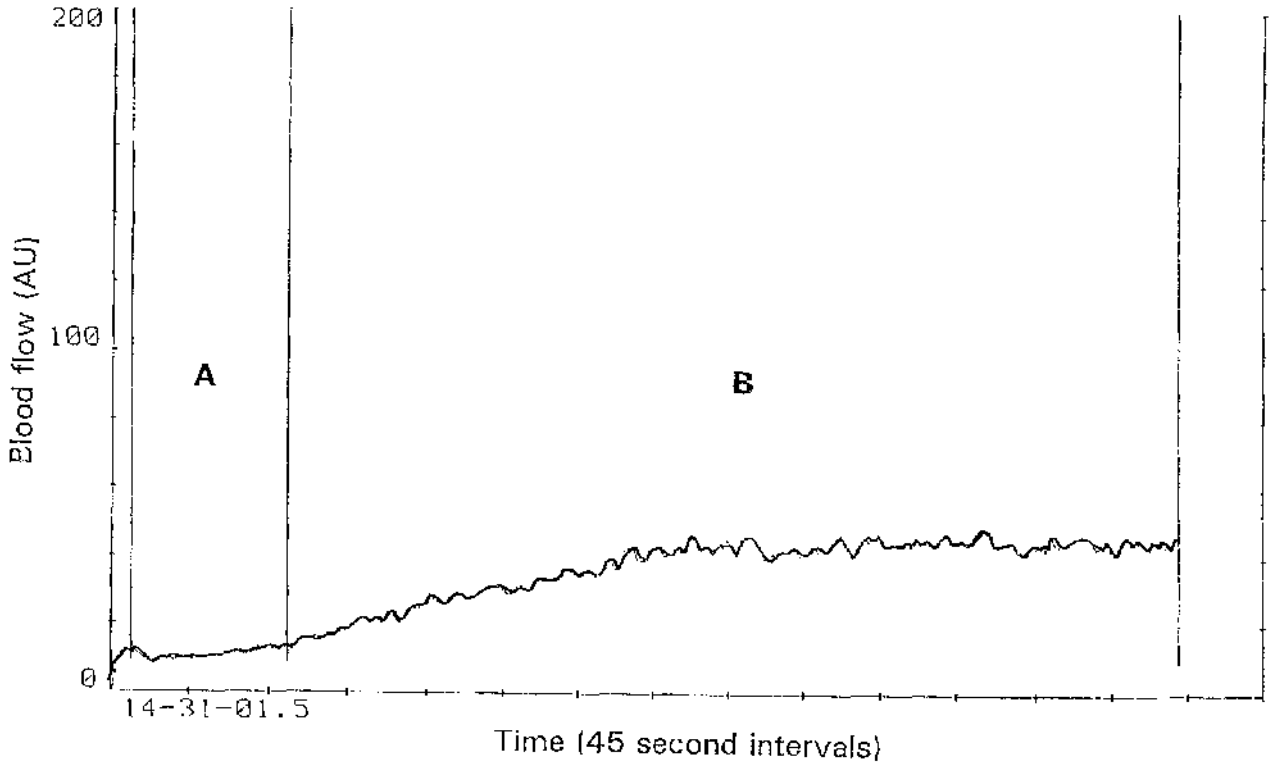
c = drug well

d = laser Doppler probe

e = air outlet

Fig 2.3.6 *Experimental trace of response to indirect iontophoresis of acetylcholine*

The figure shows that a stable response is reached after 3-4 minutes of iontophoresis.



A = baseline period

B = iontophoresis period

Table 2.3.2 ***Reproducibility of direct iontophoresis response to acetylcholine***

The table shows data for 4 separate measurements over a 2-month period in each of 2 subjects, plus summary statistics.

	IONTOPHORESIS RESPONSE ($AU.min^{-1}$)						
SUBJECT	1	2	3	4	MEAN	SD	CV
M 29yr	2311	2370	1976	1962	**2155**	**216**	**10%**
M 29yr	1560	1050	1621	1357	**1397**	**257**	**18%**

mean CV = 14%

Table 2.3.3 ***Reproducibility of direct iontophoresis response to sodium nitroprusside***

The table shows data for 4 separate measurements over a 2-month period in each of 2 subjects, plus summary statistics.

	IONTOPHORESIS RESPONSE ($AU.min^{-1}$)						
SUBJECT	1	2	3	4	MEAN	SD	CV
M 29yr	632	696	787	834	**737**	**91**	**12%**
M 29yr	928	1204	1175	865	**1043**	**172**	**16%**

mean CV = 14%

Table 2.3.4 ***Reproducibility of indirect iontophoresis response to acetylcholine***

The table shows data for 4 separate measurements over a 2-month period in each of 2 subjects, plus summary statistics.

	IONTOPHORESIS RESPONSE (AU)						
SUBJECT	1	2	3	4	MEAN	SD	CV
M 29yr	28	31	27	33	**29.8**	**2.7**	**9%**
M 29yr	33	38	42	36	**37.2**	**3.7**	**10%**

mean CV = 9.5%

2.3.4), while that to indirect iontophoresis of Ach was 9.5% (Table 2.3.5).

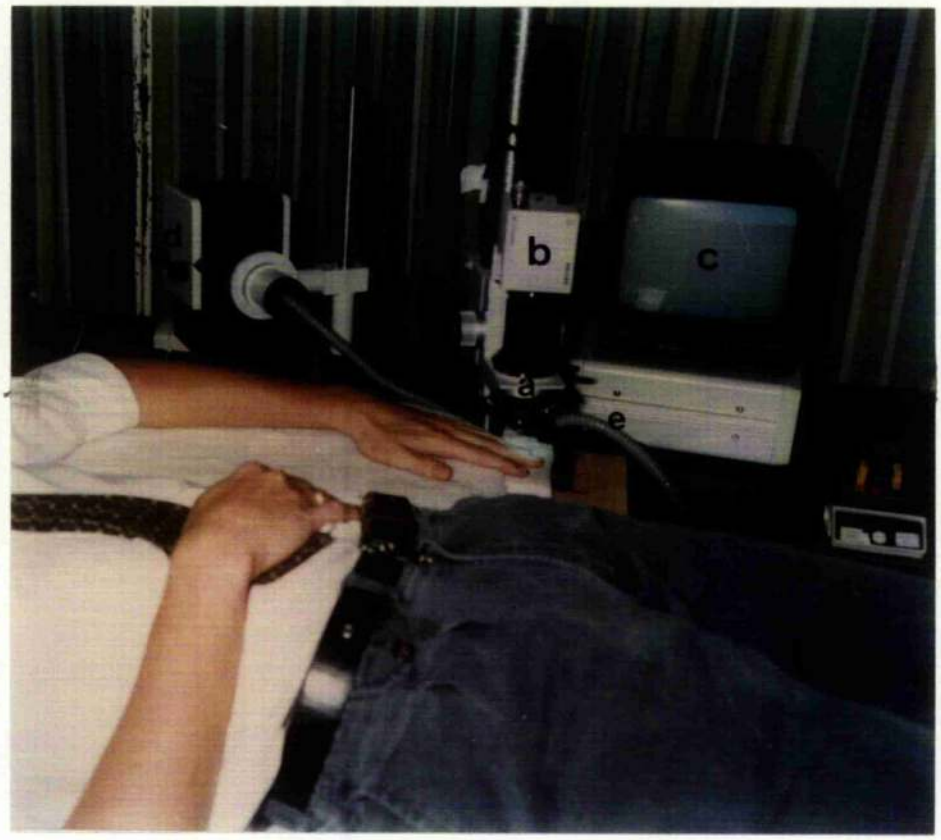
2.4 Capillary videomicroscopy.

The technique of capillary videomicroscopy[195] allows direct visualisation and recording of blood flow in the superficial dermal capillaries through the relatively transparent epidermis. Capillary videomicroscopy was used to determine skin capillary density in patients with acromegaly and control subjects in Chapter 5.2; and patients with NIDDM, pre-diabetic and control subjects in Chapter 7.

The equipment required for capillary videomicroscopy is illustrated in Fig 2.4.1. A custom made Leitz system was used. The finger under study was illuminated under an objective lens (Leitz, Leica, London, UK; final magnification X200) using a mercury vapour lamp and fibreoptic cable (Leitz 100W type 307-072.042, Leica, London, UK). The mercury vapour lamp has an emission spectrum of the same wavelength as the absorption spectrum of haemoglobin (370-450nm), producing maximum contrast between the moving column of red blood cells which can be seen as dark areas against the neutral background produced by surrounding structures[196]. The capillary wall itself is indistinguishable from other structures and so no estimate of capillary wall dimensions can be made using this technique.

Images are recorded directly onto video tape via a video camera (CCD camera model LDH0703/30, Philips, London, UK) and recorder (model AG7350-B SVHS, Panasonic, London, UK), with a time-date

Fig 2.4.1 ***Equipment used for capillary videomicroscopy***



Picture shows subject lying supine with dorsum of middle finger illuminated under the capillary microscope.

a = capillary microscope

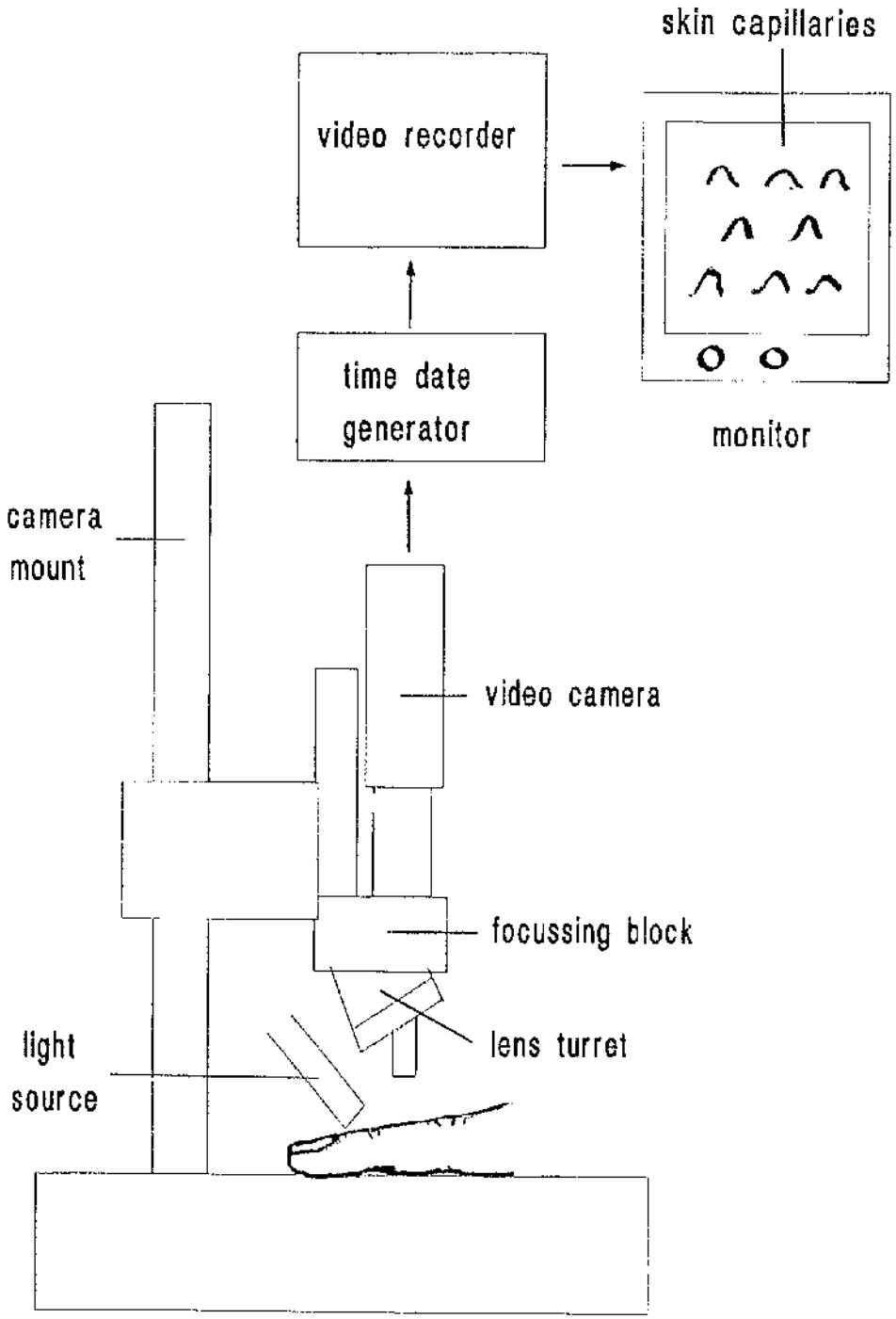
b = video camera

c = monitor

d = mercury vapour lamp

e = fibre optic cable

Fig 2.4.2 *Diagramatic representation of capillary videomicroscopy system*



generator (For-A model VTG, Video South Ltd., Bath, Avon, UK) being incorporated in the circuit (Fig 2.4.2). A monitor (Hitachi 9" monochrome monitor, model VM-920K, Leica UK, London, UK) linked to the video system allows real time and play back viewing of the images being recorded on video tape (Fig 2.4.3).

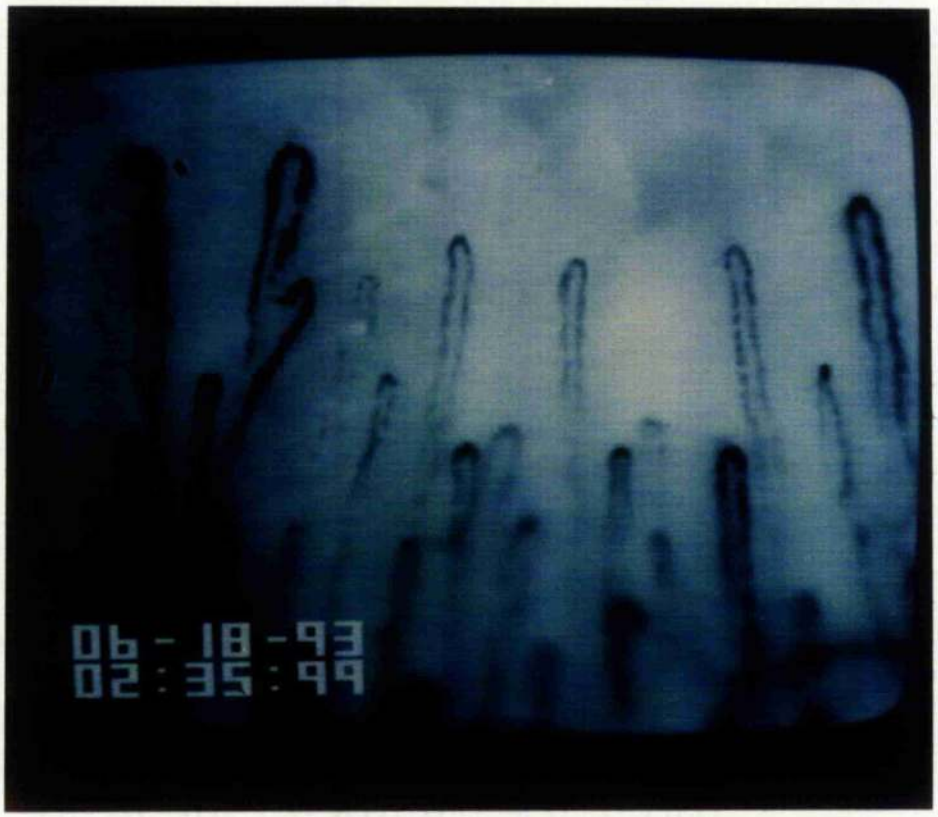
Capillary density

Both basal capillary density and capillary recruitment were measured in case there were any differences in basal capillary perfusion between the different groups investigated. Initial studies to assess reactive hyperaemia following arterial occlusion using a finger cuff gave poor reproducibility; however, local venous occlusion provided a reproducible measure of capillary recruitment, with capillary density values approaching the maximum observed following arterial occlusion.

Protocol:

A small inflatable cuff (width 1.6cm, Mini-Penile pressure cuff DC1.6, PMS Instruments, Maidenhead, UK) was positioned loosely around the base of the left middle finger. Following this, the middle phalanx was positioned under the microscope objective lens, and the dorsal skin surface coated with a thin layer of nail varnish to reduce light scattering[195]. One minute video recordings were made in each of six adjacent sites following a grid pattern. Venous occlusion was induced by inflating the finger cuff to 35mmHg, using a standard

Fig 2.4.3 ***Photograph of monitor screen showing skin nailfold capillaries***



Capillaries lie perpendicular to the skin surface in areas other than the nailfold, so that only the apices of the capillary loops are visible.

mercury shygmomanometer, and after a period of 5 minutes video recordings were repeated in the same six sites.

Capillary number was determined by counting the total number of visible capillaries in each one minute period, which lay within a grid attached to the front of the television monitor that covered the major part of the screen surface and represented an area of 0.25mm^2. Capillary density was expressed as capillaries per mm^2.

Reproducibility:

There was some variability in the number of capillaries between each of the six sites (mean CV = 21%); however, reproducibility of the measurement was improved considerably by taking the mean value for the six sites as the capillary density (basal CV = 5% (Table 2.4.1) and post venous occlusion CV = 7% (Table 2.4.2)).

2.5 Statistics

As small numbers of subjects were used in each study, it could not be assumed that data were normally distributed. Summary statistics have therefore been expressed as median and range and non-parametric tests have been used throughout. The Mann-Whitney U Test or Kruskal-Wallace Test were used for unrelated samples; and Wilcoxon Rank Sum Test for two related samples, as appropriate. Associations between different variables were determined using two-

Table 2.4.1 ***Reproducibility of basal capillary density measurements***

The table shows data for 3 separate measurements over a 2-month period in each of 2 subjects, plus summary statistics.

	CAPILLARY DENSITY (caps.mm^{-2})					
SUBJECT	1	2	3	MEAN	SD	CV
M 29yr	105	103	102	**103**	**1.6**	**2%**
F 35yr	86	91	101	**93**	**7.6**	**8%**

mean CV = 5%

Table 2.4.2 ***Reproducibility of capillary density measurements after venous occlusion***

The table shows data for three separate measurements over a 2-month period in each of two subjects, plus summary statistics.

	CAPILLARY DENSITY (caps.mm^{-2})					
SUBJECT	1	2	3	MEAN	SD	CV
M 29yr	117	106	114	**112**	**5.7**	**5%**
F 35yr	95	103	113	**104**	**9.0**	**9%**

mean CV = 7%

tailed Spearman Rank Correlations or multiple regression analysis. All statistical analyses were carried out on an IBM compatible microcomputer using the SPSS/PC+ statistical package (SPSS Inc, Chicago, Ill., USA).

CHAPTER 3

MICROVASCULAR FLUID PERMEABILITY IN NON-INSULIN-DEPENDENT DIABETES

As discussed in Chapter 1, altered microvascular permeability may be one of the functional changes associated with the development of diabetic microangiopathy. In patients with IDDM, increased microvascular permeability to fluid and a variety of solutes is present from an early stage in a variety of tissues (Chapter 1.4, page 14); however in NIDDM there have been less studies on microvascular permeability and results have been conflicting (Chapter 1.5, page 18). In addition, there have been no studies looking specifically at microvascular fluid permeability in NIDDM.

In this chapter, forearm capillary filtration coefficient (CFC) has been determined, as a measure of microvascular fluid permeability, in NIDDM patients and control subjects using a sensitive strain gauge plethysmographic method, employing a computer based logging and analysis system[174].

Subjects and Methods:

Subjects

Twenty four NIDDM patients (15 M : 9 F, age 64 (39-78) years, duration of diabetes 6 (0.5-30) years, median and range) and 24 age- and sex-matched control subjects recruited from the local community

were studied (Table 3.1). With this sample size the study had a 90% power at the 5% level (2-tailed) to detect a difference in CFC of 1.5 x 10^{-3} ml.min^{-1}.100g tissue^{-1}.mmHg^{-1}. A previous study using the same technique to compare CFC in young IDDM patients and control subjects had shown a median difference of around 4.0 x 10^{-3} ml.min^{-1}.100g tissue^{-1}.mmHg^{-1} between the two groups[79].

NIDDM patients were recruited consecutively from the diabetic clinic of the Royal Devon and Exeter hospital. The diabetic patients were treated with diet alone (n = 3), or diet plus oral hypoglycaemic agents (n = 9) or insulin (n = 12), and were on no other potentially vasoactive medication. There were seven ex-smokers and three current smokers in the NIDDM group, and eight ex-smokers amongst the control subjects.

Each patient was assessed for the presence of microvascular complications (Table 3.2). The retinae were examined by direct ophthalmoscopy through dilated pupils and appearances categorised as no retinopathy, background retinopathy, maculopathy or proliferative retinopathy. Vibration perception threshold (VPT) was measured on the tip of the great toe using a biothesiometer (Biomedical Instrument Co, Newbury Oh, USA) to assess large fibre function (readings off scale recorded as 50); and thermal sensory thresholds for warming and cooling were determined on the sole of the foot using a computerised thermal testing system (developed and supplied by the Middlesex Hospital, London, UK[197]) to assess small fibre function (warm threshold off scale recorded as 8). Patients were classed as having neuropathy if they had an age-adjusted VPT greater than the 95th centile for a normal population[198] and/or thermal sensory threshold for warming or cooling greater than 2SD above the age-

Table 3.1 ***Clinical Characteristics of diabetic patients and non-diabetic control subjects***

	NIDDM	CONTROL
N (M/F)	15/9	15/9
AGE (yr)	64 (39-78)	61 (37-79)
DURATION OF DIABETES (yr)	6 (0.5-30)	-
BODY MASS INDEX ($kg.m^{-2}$)	27.5 (21-36)	26 (21-31)
PLASMA GLUCOSE ($mmol.l^{-1}$)	10.1 (6.2-14.9)	5.0 (3.9-5.8)
SERUM FRUCTOSAMINE ($\mu mol.l^{-1}$)	316 (258-441)	-
SERUM ALBUMIN ($g.l^{-1}$)	41 (38-47)	42 (37-46)
BLOOD PRESSURE (mmHg)		
SYSTOLIC	143 (111-160)	138 (119-158)
DIASTOLIC	81.5 (70-94)	83 (72-90)
FOREARM CIRCUMFERENCE (mm)	280 (210-308)	265 (235-315)

Data are shown as median and range

Table 3.2 *Microvascular complications in the NIDDM patients*

	N	MEDIAN (RANGE)
RETINOPATHY		
NONE	17	-
BACKGROUND	5	-
MACULOPATHY	1	-
PROLIFERATIVE	1	-
NEPHROPATHY[a]		
ACR < 2.5	10	1.8 (1.0-2.4) $mg.mmol^{-1}$
ACR > 2.5	6	7.0 (3.8-32.5) $mg.mmol^{-1}$
NEUROPATHY		
ABSENT	14	-
VPT		15 (8-20) V
TT (COOL)		0.4 (0.2-1.3) ^{o}C
TT (WARM)		1.3 (0.5-5.0) ^{o}C
PRESENT	10	-
VPT		38 (25-50) V
TT (COOL)		1.1 (0.4-3.1) ^{o}C
TT (WARM)		4.3 (0.5-8.0) ^{o}C

[a] data only available for 16 patients

(ACR = albumin creatinine ratio; VPT = vibration perception threshold; TT = thermal threshold)

adjusted mean[197]. An estimation of urinary albumin excretion was obtained by measurement of the albumin : creatinine ratio (urinary albumin $mg.l^{-1}$ divided by urinary creatinine $mmol.l^{-1}$) in an early morning urine sample. In all subjects with a raised ACR, urinary tract infection was excluded by culture of a mid-stream specimen of urine.

None of the subjects had hypertension (brachial blood pressure > 160/100 mmHg), significant renal impairment (serum creatinine outwith normal range, i.e. > 120 $\mu mol.l^{-1}$), or a history of ischaemic heart disease, cerebrovascular disease or peripheral vascular disease.

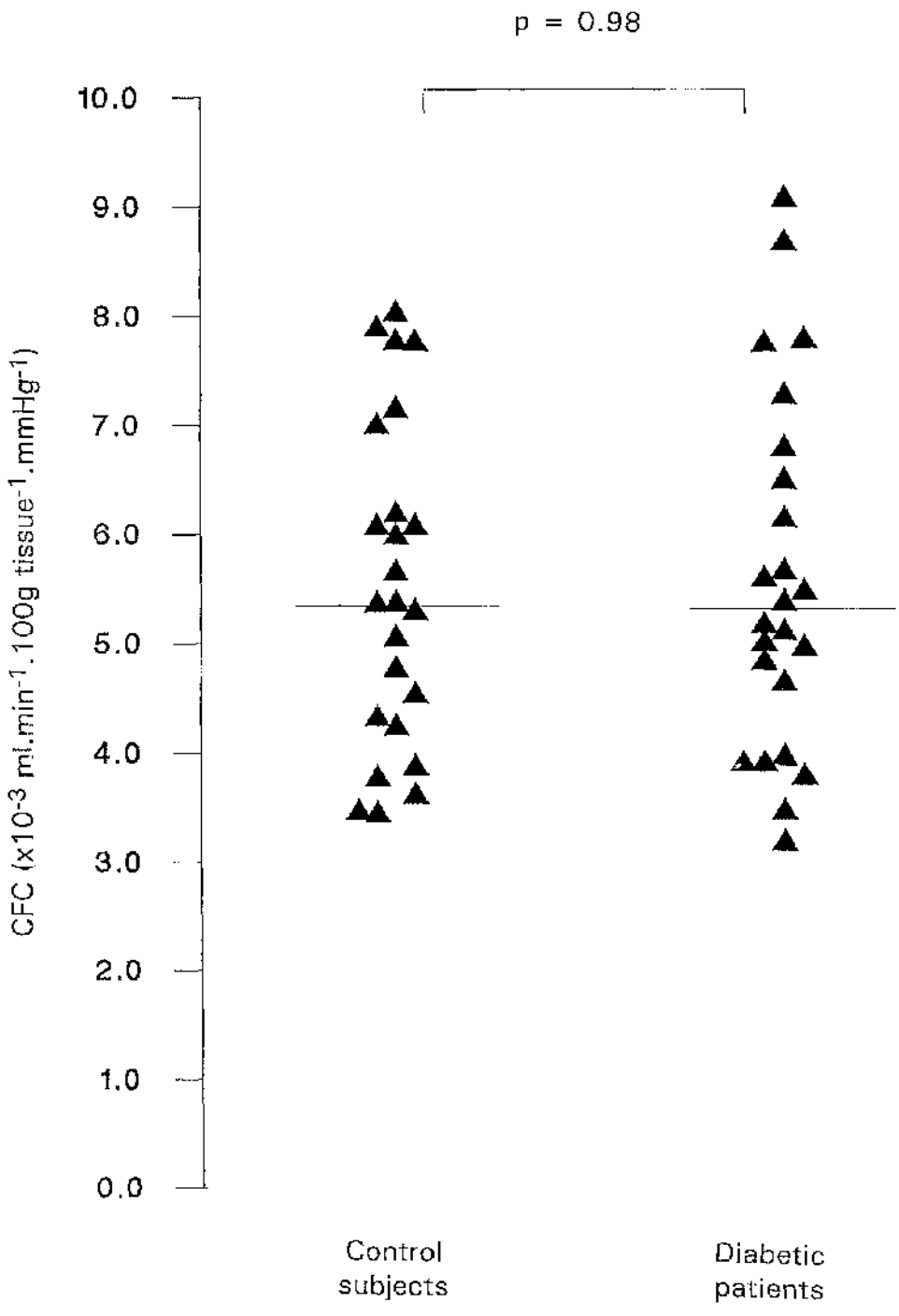
Methods

During the 30 minute acclimatisation period average brachial blood pressure was determined from the mean of 3 readings using an automated blood pressure recorder (Dynamap 845: Critikon Inc, Tampa, Fl., USA). Forearm CFC was derived from measurements of fluid filtration using venous occlusion plethysmography with stepped increases in venous pressure as described in Chapter 2.2 (page 32). At the end of each study, a venous blood sample was taken for estimation of plasma glucose concentration, serum albumin and fructosamine (colorimetric method, Fructosamine Test Plus, Roche Diagnostics, Welwyn Garden City, Herts., UK) concentrations.

Results:

There was no difference in median CFC between NIDDM patients (5.3 (3.2-9.1) x 10^{-3} $ml.min^{-1}.100g\ tissue^{-1}.mmHg^{-1}$) and control subjects (5.4 (3.5-8.0) x 10^{-3} $ml.min^{-1}.100g\ tissue^{-1}.mmHg^{-1}$;

Fig 3.1 *Capillary filtration coefficient in NIDDM patients and control subjects (horizontal bars represent median values)*



p = 0.98, Mann-Whitney) (Fig 3.1). CFC did not correlate with age in either the control or diabetic subjects. In the diabetic patients, there was no correlation of CFC with plasma glucose (p = 0.3) or fructosamine (p = 0.4) concentrations, BMI (p = 0.1), blood pressure (p = 0.1), vibration (p = 0.4) or thermal (p = 0.3) sensory thresholds, or ACR (p = 0.7) (Spearman Rank Correlation); and there were no relationships between CFC and the degree of retinopathy (p = 0.8), type of diabetic treatment (p = 0.9), or duration of diabetes (p = 0.9) (Kruskal-Wallis).

Diabetic and control subjects were well matched for baseline characteristics (Table 3.1), although VPT was significantly higher in the NIDDM patients (19 (8-50) V vs 13 (6-32) V control subjects, p < 0.03). In particular, there were no significant differences in serum albumin concentration (p = 0.6), BMI (p = 0.1), or forearm circumference (p = 0.4).

Discussion:

This study has demonstrated that capillary filtration coefficient is not increased in patients with NIDDM of short to long disease duration, in marked contrast to the situation in IDDM where CFC is elevated at an early stage[79]. In the present study there was no relationship of CFC with glycaemic control, which accords with the findings in a group of young IDDM patients who had good glycaemic control[79]. There are some potential confounding factors which could have obscured real differences in microvascular fluid permeability between the diabetic patients and control subjects. As the major tissue contributing to fluid filtration in the forearm is skeletal muscle, differences in tissue

composition (e.g. increased adipose tissue in the diabetic patients) may have affected the results; however, this is unlikely in view of the similarities in BMI and forearm circumference in the two groups (Table 3.1). In addition, it does not seem likely that any differences in plasma oncotic pressure could be contributing to the observed results, as serum albumin concentrations are similar in the two groups. As discussed in Chapter 1.2 (page 7), there is currently much interest in the role of impaired early development of the vasculature in the pathogenesis of vascular disease[46], and as CFC is dependent on the surface area available for fluid exchange and the permeability per unit surface area[199], it is possible that reduced skeletal muscle capillary density could have led to an apparently normal CFC by reducing surface area in the presence of increased permeability per unit surface area. There are, however, no data to suggest a reduction in skeletal muscle or skin capillary density in NIDDM[117]. The issue of skin capillary density is further investigated in Chapter 7. It therefore seems probable that the results of this study reflect a true similarity in microvascular fluid permeability in patients with NIDDM and control subjects, which contrasts with the increases in permeability to sodium fluorescein and radio-labelled albumin reported in previous studies[116,118]. As protein movement across the capillary wall is thought to accompany that of fluid by the process of solvent drag[19], the increased capillary permeability to albumin appears to be inconsistent with the results of the present study; however, this was most often related to a slow disappearance of radioactive albumin from the tissues, reflecting reduced lymphatic washout rather than true increased permeability[118]. In addition, the abnormality was most marked in hypertensive NIDDM patients[118], and was only found in a minority of normotensive patients, all of whom had neuropathy and

poor glycaemic control[119].

A smaller difference in CFC than 1.5 x 10^{-3} ml.min^{-1}.100g $tissue^{-1}$.$mmHg^{-1}$ between diabetic and control subjects may not have been detected with the sample size used; however, as a previous study showed a median difference of around 4.0 x 10^{-3} ml.min^{-1}.100g $tissue^{-1}$.$mmHg^{-1}$ between young IDDM patients and control subjects[79], it seems unlikely that any important increase in CFC in the NIDDM patients was missed. As the patients in the present study were normotensive, relatively free of microvascular complications and had acceptable glycaemic control, the results do not preclude significant changes in microvascular fluid permeability in NIDDM patients with hypertension, more advanced microangiopathy, or marked hyperglycaemia.

In summary, capillary filtration coefficient is not increased in normotensive NIDDM patients with reasonable glycaemic control, in contrast to findings in patients with IDDM, making it unlikely that alterations in fluid permeability are of primary importance in the pathogenesis of microvascular disease in NIDDM.

CHAPTER 4

MAXIMUM MICROVASCULAR HYPERAEMIA IN NON-INSULIN-DEPENDENT DIABETES

As mentioned in Chapter 1.5 (page 19), the major abnormality of skin microvascular function identified to date in patients with NIDDM has been a reduction in maximum vasodilation in response to a variety of stimuli. In this chapter the microvascular hyperaemic response to local heating is further explored in NIDDM patients with no evidence of large vessel disease, and the influence of glycaemic control and hypertension on microvascular vasodilation is investigated.

4.1 Microvascular hyperaemia in NIDDM patients with large vessel disease excluded by Doppler sonography

The major abnormality of skin microvascular function identified to date in patients with NIDDM is a reduction in hyperaemic responses[121,122]. A major criticism of previous studies was that the criteria used to exclude large vessel disease, namely, the ankle systolic pressure index, may have been inadequate[200], leading to the possibility that reduced hyperaemia in some NIDDM patients may have been due to undetected peripheral vascular disease. Difficulties arise in interpreting ankle systolic blood pressure in diabetic patients, as this can be falsely elevated in a minority of patients due to decreased

vascular compliance[201] secondary to sclerosis or calcification of the vessel wall[202]. Several other techniques can be employed to overcome this difficulty, such as oscillotonometry[203], Doppler waveform analysis or pulse-wave velocity[204], or arteriography. In the present study measurements of maximal hyperaemic response to local heating of the foot skin were repeated in a group of NIDDM patients in whom large vessel disease had been rigorously excluded using Doppler waveform analysis.

Subjects and methods:

Subjects

Eleven NIDDM patients (7M : 4F, age 62 (50-70) years, duration 8 (5-24) years, median and range) (Table 4.1), were recruited consecutively from a larger cohort of NIDDM patients attending the Royal Southants Hospital, Southampton, who were taking part in a prospective study of cardiovascular risk factors and vascular disease. None of the subjects studied had clinically evident large vessel disease as determined by a history suggestive of vascular disease, absent pedal pulses, or ankle : brachial systolic pressure index < 1.0. Large vessel disease was then more rigorously excluded by continuous wave Doppler assessment (Sonicaid Vasoview scanner, Sonicaid Medical Inc, Virginia, USA) of arterial pulse waveform shape, turbulence and damping in the femoral, popliteal, posterior tibial and dorsalis pedis arteries.

The NIDDM patients were treated with diet alone ($n = 1$), or oral hypoglycaemic agents ($n = 10$). Eleven age- and sex-matched

Table 4.1.1 ***Clinical Characteristics of diabetic patients and non-diabetic control subjects***

	NIDDM	CONTROL
N (M/F)	7/4	7/4
AGE (yr)	62 (50-70)	61 (50-70)
DURATION OF DIABETES (yr)	8 (5-24)	-
BODY MASS INDEX ($kg.m^{-2}$)	25 (22-30)	25 (21-29)
PLASMA GLUCOSE ($mmol.l^{-1}$)	9.6 (5.1-12.3)	4.6 (3.8-5.8)
HbA_{1C} (%)	7.7 (5.3-8.6)	-
BLOOD PRESSURE (mmHg)		
SYSTOLIC	138 (116-158)	138 (114-159)
DIASTOLIC	82 (62-88)	80 (64-89)
PLASMA INSULIN ($\mu U.ml^{-1}$)	35.8 (13.6-67.1)	-

Data are shown as median and range

control subjects were also studied. None of the participants had evidence of significant renal impairment (serum creatinine > 120 μmol.l^{-1}, or proteinuria), or hypertension (brachial blood pressure > 160/90 mmHg). There were three ex-smokers and one current smoker in the diabetic group, and three ex-smokers amongst the control subjects.

The NIDDM patients were screened for the presence of microvascular complications using dilated fundoscopy, biothesiometry and ACR determination as defined in Chapter 3 (pages 71 and 74). Three had background retinopathy and one had maculopathy; two had a raised VPT (35 and 41V respectively); and two had an elevated ACR (4.1and 6.5 mg.mmol^{-1}), but none dipstick positive proteinuria.

Methods

During the acclimatisation period, a small area of skin on the dorsum of the right foot was heated to 44^{o}C using a brass heater attached to the skin surface as described in Chapter 2.3 (page 43), and brachial blood pressure was recorded as the mean of 3 readings using an automated device (Dynamap 850, Critikon Inc., Tampa, Fl., USA). Maximum blood flow was determined by laser Doppler fluximetry (Perimed Pf2; Perimed , Stockholm, Sweden) as the mean reading of 8 sites in the heated area[88], expressed arbitrarily in volts (V).

At the end of the study a venous blood sample was obtained for assessment of ambient plasma glucose concentration, and in the diabetic subjects HbA_{1c} (affinity column chromatography; normal range 4-6%). Fasting plasma insulin was also measured in the NIDDM

patients using a specific 2-site ELISA[204] which had minimal cross-reactivity with pro-insulin and other insulin-like molecules (normal range 1-15 $\mu U.ml^{-1}$).

Results:

Maximum hyperaemia was significantly reduced in the NIDDM patients (0.82 (0.42-1.41) V, vs 1.40 (0.89-2.13) V control subjects; $p<0.005$, Mann-Whitney) (Fig 4.1.1). There was no significant correlation of maximum hyperaemia with diabetes duration ($p = 0.8$), blood pressure ($p = 0.6$) or glycaemic control ($p = 0.8$ for both glucose and HbA_{1c}, Spearman Rank). In contrast, there was a negative correlation between maximum hyperaemia and fasting plasma insulin ($R_s = -0.63$, $p<0.04$) (Fig 4.1.2).

There were no significant differences between NIDDM patients and control subjects with respect to BMI ($p = 0.8$) or blood pressure ($p = 0.6$) (Table 4.1.1).

Discussion:

This study confirms the finding of reduced maximal microvascular hyperaemia in patients with NIDDM, as has been demonstrated previously[120,121]. On this occassion large vessel disease was excluded by Doppler waveform analysis, so that impaired vasodilation must be due to an intrinsic microvascular defect. Microvascular function in non-diabetic subjects with peripheral vascular disease has been found to remain normal until the atheromatous process is at an advanced

Fig 4.1.1 *Maximum microvascular hyperaemia in NIDDM patients and control subjects (horizontal bars represent median values)*

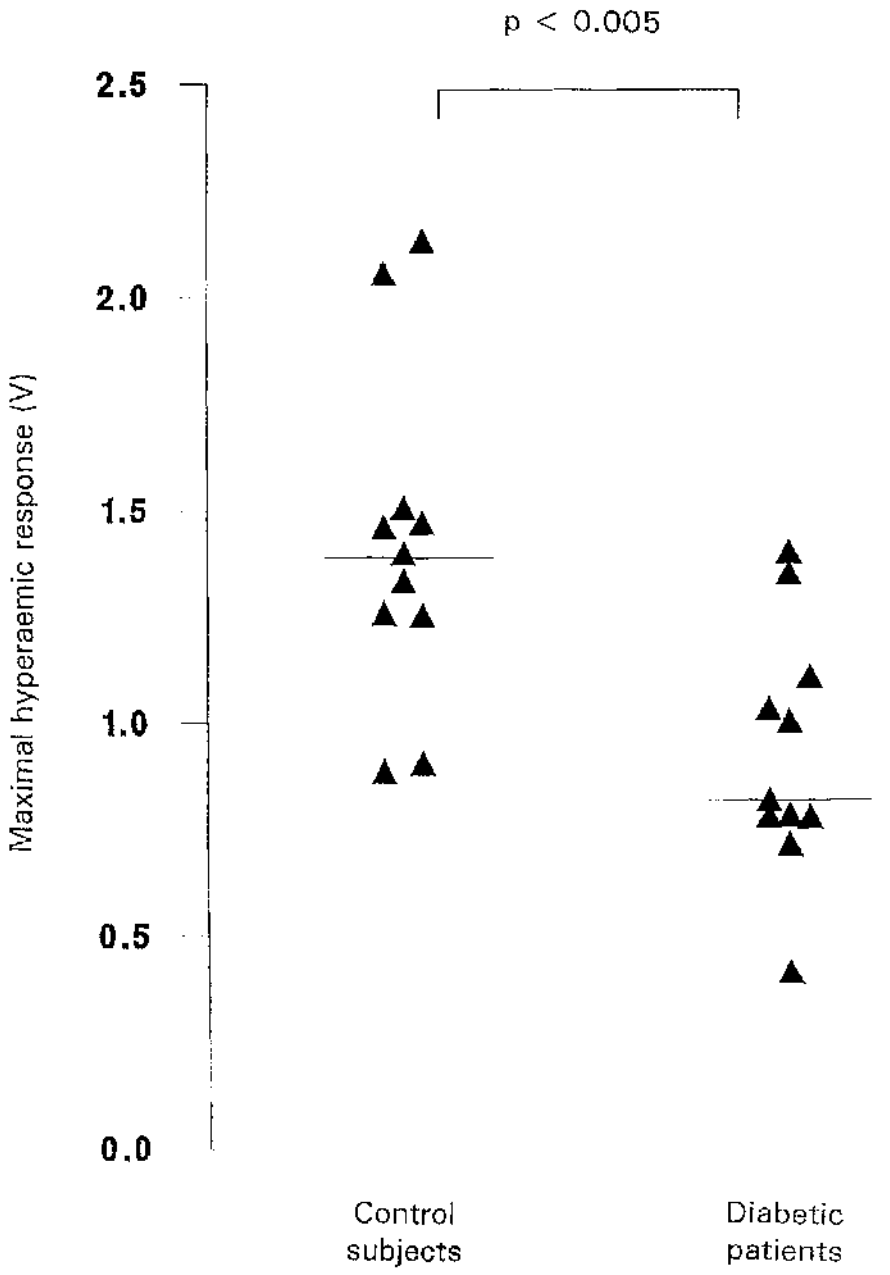
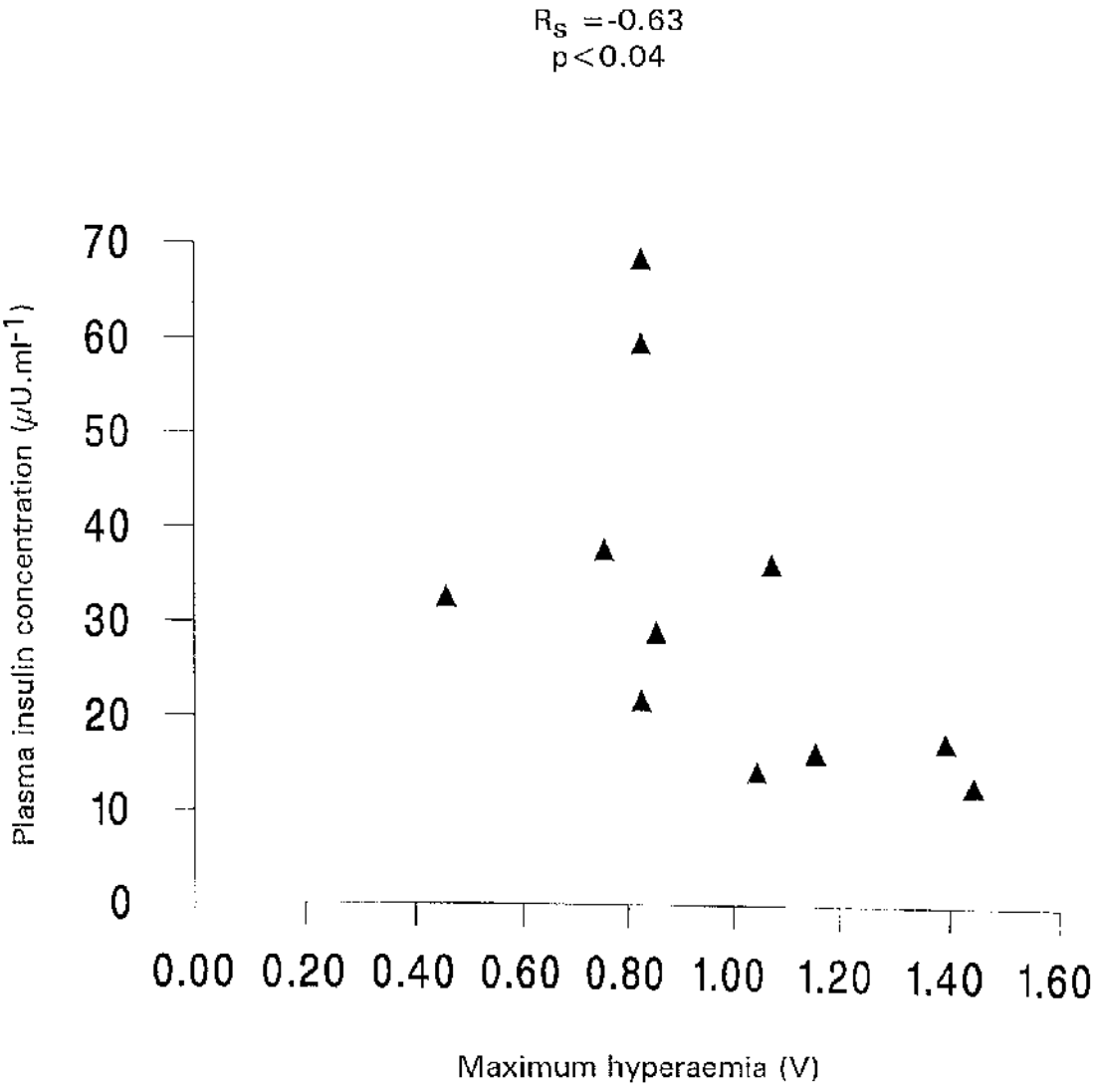


Fig 4.1.2 *Correlation between maximum hyperaemia and fasting plasma insulin concentration in NIDDM patients*



stage[160,161,165], and it is interesting to note that the actual values for hyperaemia in the current study were not dissimilar from those found previously in NIDDM patients selected on the basis of a normal ankle systolic pressure index[122]. Doppler sonography is time consuming, requires specialised equipment and a skilled operator. In addition, all subjects who were thought to be free from large vessel disease on the basis of clinical criteria were subsequently found to have normal Doppler waveform assessments. In view of these points it was decided that ankle systolic pressure index was a simple and adequate method to exclude significant large vessel disease in the absence of symptoms, when taken in conjunction with intact peripheral pulses, and was the method employed in the remainder of studies in this thesis.

No significant correlation could be found between glycaemic control and microvascular hyperaemia in this small number of subjects. The associations between microvascular vasodilation and glycaemic control and hypertension are further explored in the other studies in this chapter. In contrast, there was a significant negative association between fasting plasma insulin concentration and maximal hyperaemia and this theme is further explored in Chapter 5.3.

In summary, these results confirm the previous findings of reduced microvascular hyperaemia in NIDDM, in a group of patients in which large vessel disease had been rigorously excluded by Doppler sonography.

4.2 Effect of glycaemic control in recently diagnosed NIDDM patients

Reduced microvascular hyperaemia in response to local heating of the foot skin has been shown to be already present at the time of diagnosis in patients with NIDDM[122], with the degree of impaired vasodilation being similar to that found in IDDM patients of a similar age, but with a disease duration of several years[88]. Recently much attention has focused on the role of improved glycaemic control in reducing the rate of microvascular complications in IDDM with publication of the results of the Diabetes Control and Complications Trial[205]. Whether improved glycaemic control will produce similar benefits in patients with NIDDM remains uncertain, and the observed differences in the epidemiology of microvascular complications in NIDDM compared with IDDM argue against extrapolating directly from the results of the Diabetes Control and Complications Trial to NIDDM patients. Hopefully this question will be clarified when the United Kingdom Prospective Diabetes Study[206] results are published in the near future.

Short term improvement in glycaemic control has been shown to influence the distribution of peripheral blood flow in poorly controlled NIDDM patients[76] and those with neuropathy[114]; and to reduce total limb blood flow in newly diagnosed patients[113]. There are, however, no studies of the effects of sustained improvements in glycaemic control on microvascular function in NIDDM. In this section, therefore, the effects of one year of improved glycaemic control on microvascular hyperaemia in response to local heating of the foot skin, have been determined in a group of recently diagnosed NIDDM patients.

Subjects and methods:

Subjects

Twelve NIDDM patients (6M : 6F, age 51.5 (27-66) years, median and range) from the Exeter cohort of the United Kingdom Prospective Diabetes Study were followed from diagnosis (Table 4.2.1). This is a multicentre study looking at the effects of glycaemic control on the development of vascular complications in NIDDM, and whether this process is influenced by different treatment modalities[206]. Patients were recruited consecutively from referrals to the clinic who had no evidence of hypertension or significant renal impairment as previously defined (page 82). Large vessel disease was excluded on the basis of absence of symptoms suggestive of vascular disease, palpable pedal pulses, and ankle : brachial systolic pressure index $>$ 1.0. Nine participants were non-smokers and two were ex-smokers. Patients were treated with diet and if necessary sulphonylurea and/or insulin therapy according to the study protocol[206]. Six patients had been randomised to an 'active control policy'[206] aiming for a fasting plasma glucose concentration $<$ 6 mmol.l^{-1}, and the others to 'less strict control' (fasting plasma glucose concentration $<$ 15 mmol.l^{-1})[206].

At entry to the study, the presence of diabetic retinopathy was determined by a combination of ophthalmoscopy and retinal photography[35]; and ACR was measured in an early morning urine sample to screen for microalbuminuria as previously described (page 74). To detect asymptomatic neuropathy, VPT was measured using a biothesiometer (page 71), while autonomic nerve function was assessed using standard cardiovascular reflex tests[207].

Using the above criteria, there was minimal clinical evidence of microangiopathy, with three subjects having minimal background retinopathy, one having microalbuminuria (ACR = 4.0 $mg.mmol^{-1}$), one having mild large fibre neuropathy (VPT = 20 V), and none having cardiovascular autonomic neuropathy.

Methods

Subjects were seen every 3 months in a special UKPDS clinic. Glycaemic control was assessed by measurement of fasting venous plasma glucose and HbA1c concentrations (High performance liquid chromatography; Biorad Laboratories Ltd, Hemel Hempstead, Herts., UK; normal range 4.3-6.1%) and treatment altered to meet the study targets as previously defined.

Maximal microvascular hyperaemia was measured (as described in Chapter 2.3, page 43) within a few weeks of diagnosis, at the end of a 3 month period of therapy with diet alone, and after one year of treatment. A record was taken of the exact positioning of the heated site on the foot for each subject, so that the same site could be used for sequential measurements. During the acclimatisation period brachial blood pressure was calculated as the mean of 3 measurements using an automated device (Dynamap 845; Critikon Inc, Tampa, Fl., USA).

Results:

The low maximum microvascular hyperaemic response present at diagnosis in the NIDDM patients was unchanged after the initial three month period of dietary treatment (0.97 (0.22-2.17) V baseline vs 0.99

(0.38-2.74) V at three months; $p=0.24$, Wilcoxon Rank Sum) (Fig 4.2.1) despite a significant improvement in glycaemic control (9.6 (6.6-17.3) mmol l^{-1} baseline vs 8.1 (5.4-13.1) mmol l^{-1} at three months; $p<0.005$) and BMI (28 (23-47) kg m^{-2} baseline vs 25 (23-44) kg m^{-2} at three months; $p<0.05$) (Table 4.2.1). At one year however, there was a significant improvement in the maximum microvascular hyperaemic response (1.20 (0.51-3.29) V; $p<0.05$ vs baseline) (Fig 4.2.2), and there had also been a further improvement in glycaemic control (Table 4.2.1). Despite this, there was only a borderline change in BMI ($p=0.06$) and no significant change in blood pressure ($p=0.6$) after one year of treatment (Table 4.2.1).

Although the number of subjects in each group was small, there was no significant difference in the improvement in microvascular hyperaemia achieved in those allocated to the 'active' control policy (% change relative to baseline value +11 (-21 to +53) %) compared with those allocated to less strict control (% change relative +30 (-10 to +129) %, $p=0.11$). Surprisingly, glycaemic control in the latter group did not differ significantly from that in the group allocated to active control (fasting plasma glucose concentration 6.7 (5.2-13.0) mmol l^{-1} vs 7.1 (5.6-9.4) mmol l^{-1} active control, $p=0.8$; HbA_{1c} 6.0 (5.0-7.2)% vs 5.8 (5.3-7.7)% active, $p=0.9$).

There was no significant correlation of maximum microvascular vasodilation with fasting plasma glucose concentration, plasma glucose concentration at the time of measurement of hyperaemic response, HbA_{1c}, blood pressure or BMI, at baseline or one year. There was however an association between the improvement in maximum hyperaemia over one year and the improvement in glycaemic control ($R_s = 0.53$, $p < 0.05$) (Fig 4.2.3), whereas there was no

Fig 4.2.1 *Maximum microvascular hyperaemia at diagnosis and after 3 months therapy in NIDDM patients*

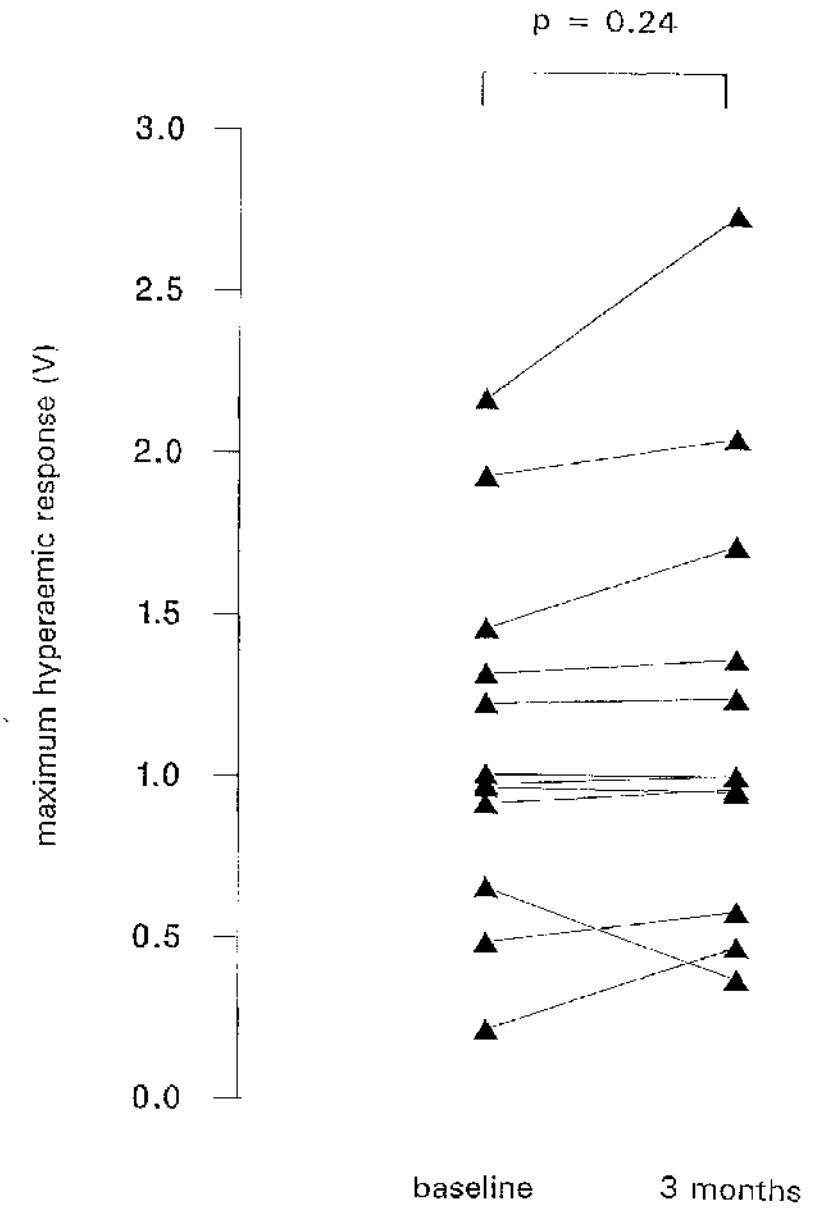


Fig 4.2.2 *Maximum microvascular hyperaemia at diagnosis and after 1 year of treatment in NIDDM patients*

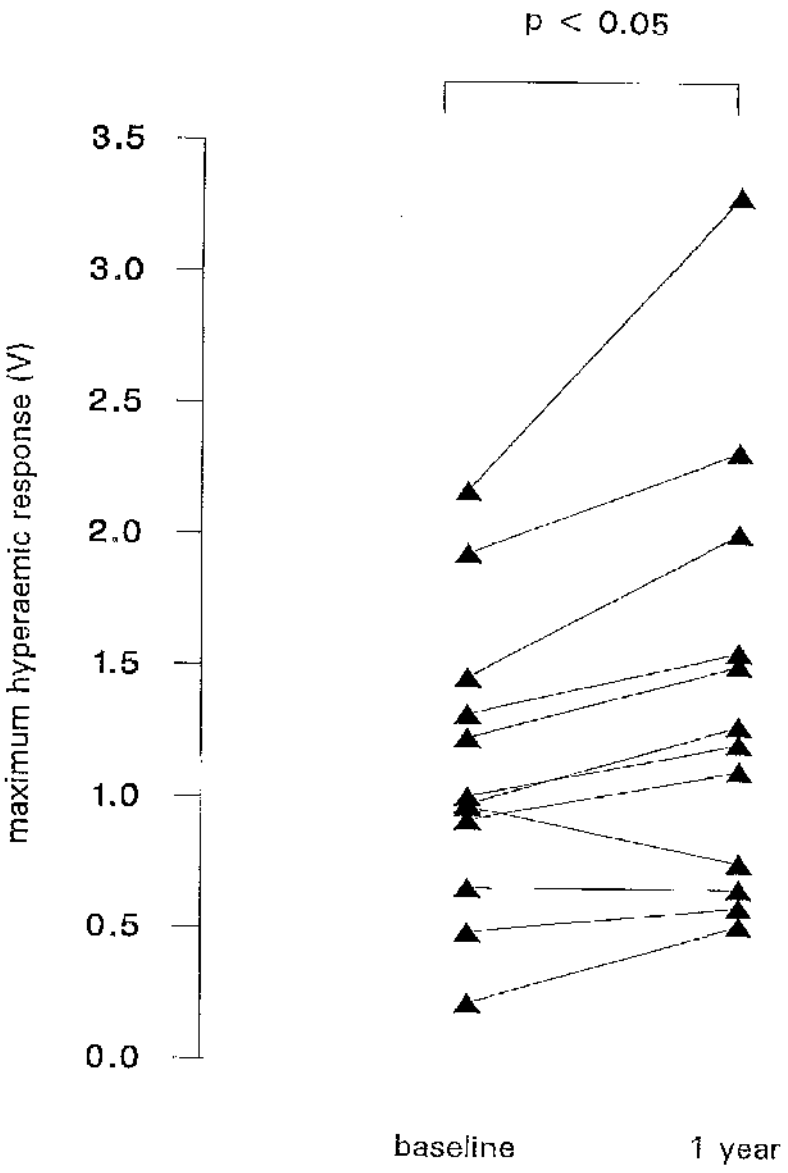


Fig 4.2.3 *Correlation between improvement in maximum hyperaemia over one year and improvement in glycaemic control*

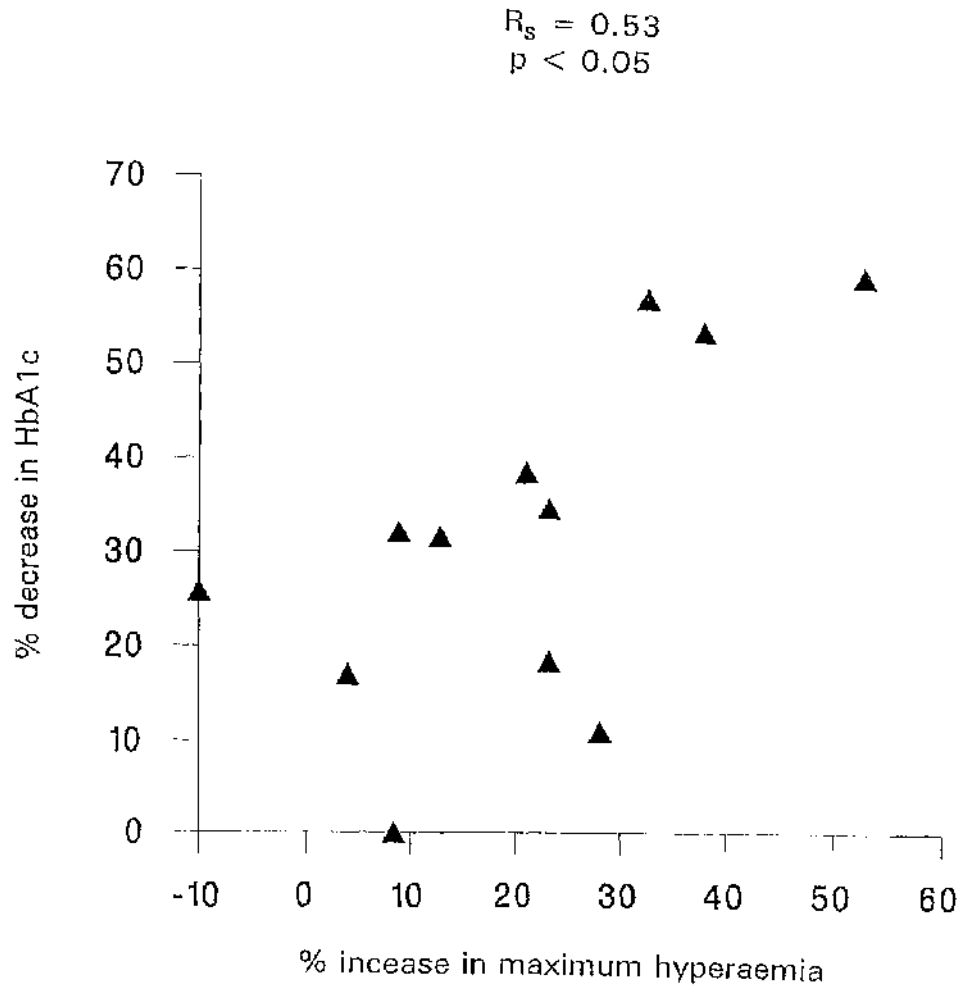


Table 4.2.1 ***Changes in glycaemic control, BMI and blood pressure over 1 year of improved diabetic control***

	Diagnosis	3 months	1 year
Fasting Plasma glucose (mmol l^{-1})	9.6 (6.6-17.3)	8.1 (5.4-13.1)[a]	6.4 (5.9-10.8)[b]
Study plasma glucose (mmol l^{-1})	7.4 (5.5-13.5)	6.3 (4.0-14.4)[c]	6.5 (4.6-10.6)
HbA1c (%)	8.6 (6.1-13.3)	6.1 (5.9-10.8)[a]	5.7 (5.3-7.7)[d]
BMI (kg m^{-2})	28 (23-47)	25 (23-44)[c]	26 (23-42)
Systolic BP (mmHg)	125 (99-138)	126 (98-142)	123 (94-148)
Diastolic BP (mmHg)	75 (59-85)	73 (59-84)	73 (58-87)

Data shown as median and range

[a] $p<0.005$ vs value at diagnosis

[b] $p<0.005$ vs value at 3 months

[c] $p<0.05$ vs value at diagnosis

[d] $p<0.05$ vs value at 3 months

correlation with the decrement in either fasting plasma insulin level (excluding those that were insulin treated) ($p = 0.4$) or BMI ($p = 0.9$).

Discussion:

This study has demonstrated that the limited microvascular vasodilation present at diagnosis in patients with NIDDM may improve over the course of one year of improved glycaemic control. Whether this improvement is sustained over a longer period remains to be determined. There was no difference in the improvement achieved between those allocated to tight control and those to less strict control, although both groups achieved a similar level of glycaemic control at one year. Improvement in maximum microvascular hyperaemia was found with all the therapeutic strategies employed in this study, although the numbers in each group were too small to determine whether any one type of diabetes treatment was superior to the others.

Improvement in microvascular vasodilation was associated with improved glycaemic control but not decrement in fasting plasma insulin concentration and BMI. No change in hyperaemic response was apparent after three months of improved glycaemic control, suggesting a slowly reversible process which would favour a structural change in the vessel wall. The exact nature of this remains to be determined, but possible candidates are vascular smooth muscle cell hypertrophy and basement membrane thickening. The latter of these was previously thought to be a largely irreversible process; however, some studies do show a reduction in basement membrane thickness with improved glycaemic control[208,209], despite some debate about the methodologies used[1]. Future studies combining measurement of

microvascular hyperaemia with skin biopsy and microvascular histology will be required to establish the relationship between improved diabetic control and changes in microvascular structure and function.

In summary, there is an increase in microvascular vasodilation in recently diagnosed NIDDM patients who improve their glycaemic control.

4.3 Effect of hypertension

The preceding studies have demonstrated that microvascular hyperaemic responses are reduced in patients with NIDDM, confirming the findings of previous studies[121,122]. In contrast, studies using laser Doppler fluximetry in non-diabetic patients with essential hypertension yield inconsistent results, with some showing no reduction in microvascular hyperaemic responses[169,170], while others do[171]. This is despite the fact that increased structurally-based resistance to blood flow has been demonstrated in essential hypertension[169]. As hypertension and NIDDM commonly co-exist, this study looks at the effects of hypertension on maximal hyperaemia and resistance to blood flow in the skin microcirculation of patients with NIDDM.

Subjects and Methods:

Subjects

Nine untreated hypertensive NIDDM patients (brachial blood pressure consistently > 160/90 mmHg), 9 normotensive NIDDM patients and 9 normotensive non-diabetic control subjects were studied. The groups were age- and sex-matched and summary details are shown in Table 4.3.1. Patients were recruited consecutively from the diabetic clinic of the Royal Devon and Exeter hospital and control subjects from a panel of healthy volunteers. None of the participants had evidence of significant renal impairment or large vessel disease as previously defined (pages 82 and 88). The NIDDM patients were screened for the presence of microvascular complications (pages 71 and 74) Five of the hypertensive NIDDM patients had retinopathy (3 background, 2 treated maculopathy), three had large fibre neuropathy (VPT > 30V, no history of foot ulceration) and four had microalbuminuria (ACR > 2.5 $mg.mmol^{-1}$); whereas three of the normotensive NIDDM patients had retinopathy (2 background, 1 maculopathy), three had large fibre neuropathy and three had microalbuminuria.

Methods

The protocol and equipment used for skin heating, brachial blood pressure measurement and determination of maximal hyperaemia was as previously described (page 43). As blood pressure depends on cardiac outout and total peripheral resistance, it is possible to get an estimate of resistance to flow in a maximally vasodilated

Table 4.3.1 ***Clinical characteristics of hypertensive and normotensive diabetic patients and non-diabetic control subjects***

	NIDDM		CONTROL
	HYPERTENSIVE	NORMOTENSIVE	
N (M/F)	6/3	6/3	6/3
AGE (yr)	62 (48-65)	60 (47-66)	59 (45-68)
DURATION OF DIABETES (yr)	9 (4-17)	7 (4-19)	-
DIABETES TREATMENT (N)			
DIET ALONE	0	3	-
ORAL HYPOGLYCAEMIC	4	6	-
INSULIN	5	0	-
BODY MASS INDEX ($kg.m^{-2}$)	28 (24-36)	26 (21-34)	27 (23-30)
PLASMA GLUCOSE ($mmol.l^{-1}$)	6.9 (4.0-13.1)	8.7 (5.1-14.9)	4.8 (3.9-5.8)
HbA_{1C} (%)	7.5 (6.5-11.1)	7.5 (5.3-12.2)	-
BLOOD PRESSURE (mmHg)			
SYSTOLIC	182 (162-228)	136 (116-158)	138 (126-148)
DIASTOLIC	98 (94-114)	78 (58-84)	76 (66-86)
MAP	125 (118-149)	99 (84-109)	99 (88-105)

Data are shown as median and range

MAP = mean arterial pressure

microcirculatory bed by dividing mean arterial pressure (in mmHg) by maximal hyperaemia (in V), results being expressed in arbitrary units of mmHg.V^{-1} [169].

At the end of the study a venous blood sample was obtained for assessment of ambient plasma glucose concentration, and in the diabetic subjects HbA_{1c} (determined by affinity column chromatography; normal range 4-6%).

Results:

The maximal microvascular hyperaemic response was reduced in both the hypertensive (1.05 (0.70-1.42) V) and normotensive (1.04 (0.79-1.63) V) NIDDM patients when compared with control subjects (1.40 (1.26-2.13) V; $p < 0.01$ for hypertensive and $p < 0.05$ for normotensive patients respectively, Mann-Whitney) (Fig 4.3.1); however, maximal hyperaemia was similar in both groups of diabetic patients ($p = 0.82$). In contrast, resistance to blood flow was significantly greater in the diabetic patients with hypertension (127.2 (87.5-181.3) mmHg.V^{-1} vs 84.7 (61.9-123.0) mmHg.V^{-1} normotensive diabetic patients; $p < 0.02$) (Fig 4.3.2). In addition, resistance to blood flow was greater in the normotensive NIDDM patients than in the control subjects (70.7 (44.7-79.9) mmHg.V^{-1}; $p < 0.05$) (Fig 4.3.2).

BMI was similar in the different groups ($p = 0.2$) (Table 4.3.1). There were no significant differences in ambient plasma glucose concentration at the time of study ($p = 0.4$), or longer term glycaemic control, as reflected by HbA_{1c} measurements ($p = 0.9$), between the hypertensive and normotensive NIDDM patients (Table 4.3.1); however, there were significant differences in diabetes treatment

Fig 4.3.1 *Maximum microvascular hyperaemia in hypertensive NIDDM patients, normotensive NIDDM patients and normotensive non-diabetic control subjects (horizontal bars represent median values)*

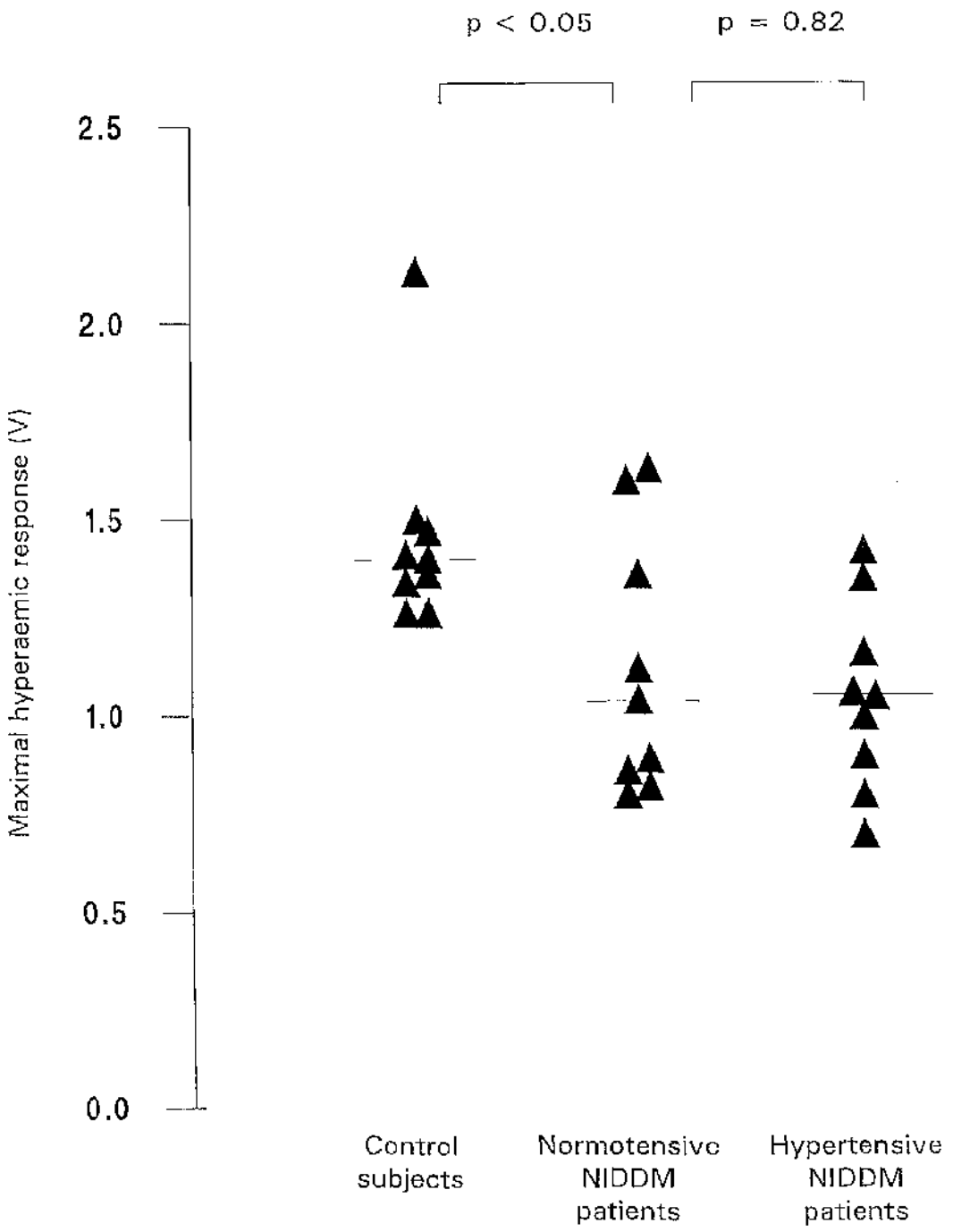
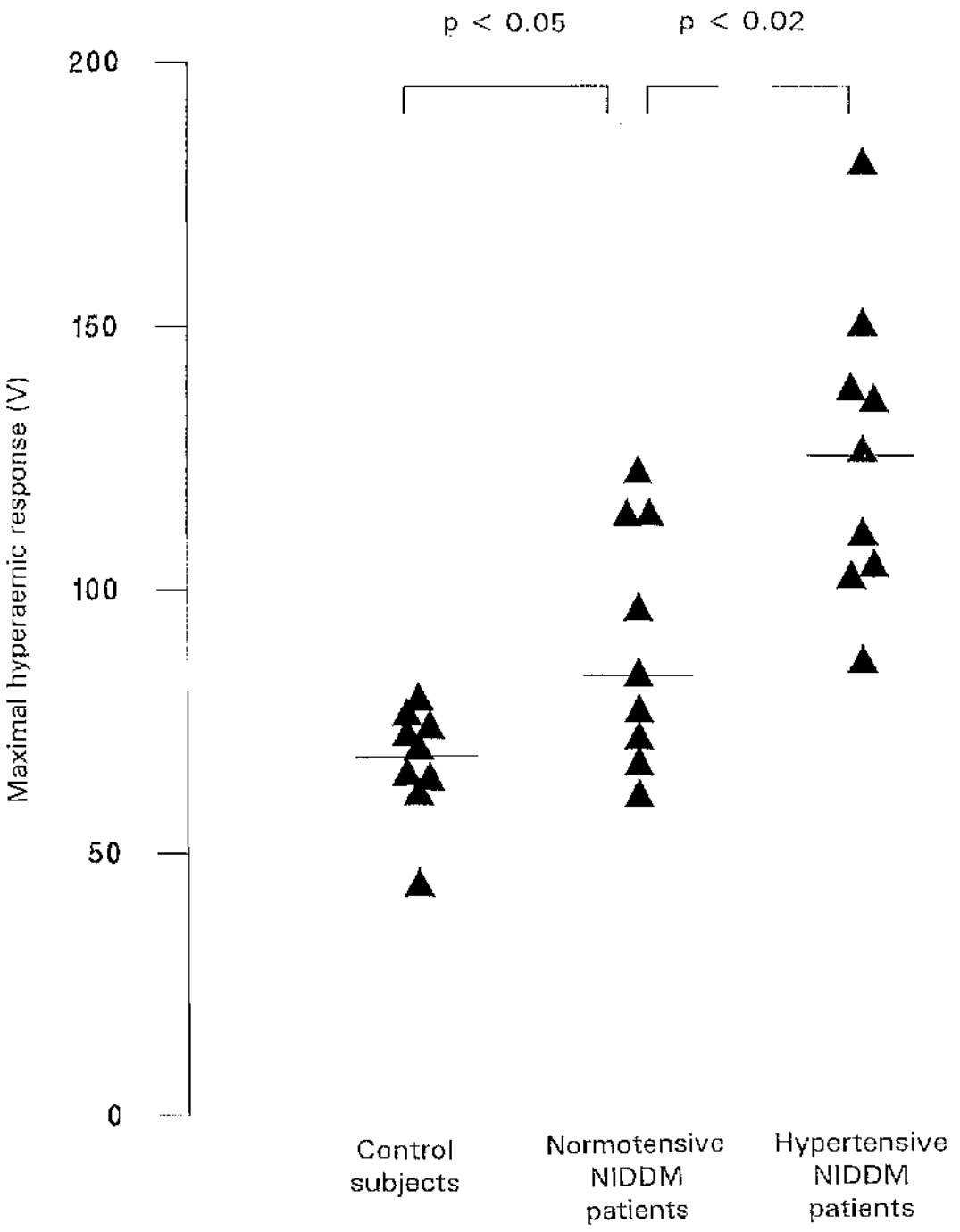


Fig 4.3.2 *Calculated resistance to blood flow in hypertensive NIDDM patients, normotensive NIDDM patients and normotensive non-diabetic control subjects (horizontal bars represent median values)*



between the two groups with insulin being used in the majority of hypertensive patients but none of the normotensive patients (Table 4.3.1).

Discussion:

This study has demonstrated that patients with NIDDM with and without hypertension have similar values for maximal hyperaemia in response to local heating of the skin. In both cases maximal hyperaemia was reduced in comparison to values obtained in control subjects. Resistance to blood flow, however, was increased in hypertensive when compared to the normotensive NIDDM patients.
Heating the skin to a temperature of 44°C has been shown to induce maximum microvascular vasodilation[188]. Under such conditions, blood flow is entirely dependent on the pressure gradient and structural vascular resistance[169]. The resistance calculation used in this study assumes that venous pressure was zero and that brachial artery pressure was equal to arteriolar pressure in the area of study. The former assumption leads to a slight overestimate of the pressure gradient as venous pressure is likely to be around 10 mmHg[18]; whereas, in the supine position, the latter assumption is likely to hold. These errors are small and systematic and are therefore unlikely to influence the observed results. The site of increased resistance to flow is likely to lie on the arteriolar side of the capillary bed, although there is some evidence that the post-capillary segment contributes in essential hypertension[210].

The majority of the hypertensive NIDDM patients were treated with insulin therapy, whereas none of the normotensive NIDDM patients

was. It is interesting to speculate that peripheral hyperinsulinaemia may relate to increased vascular resistance, especially in view of the correlation observed between hyperinsulinaemia and maximal hyperaemia in Chapter 4.1. It is also possible that insulin treatment equated with poorer past glycaemic control in the hypertensive group and this could have influenced the results, although in previous studies of NIDDM patients, no correlation has been found between past glycaemic control and reduced hyperaemic responses[121,122], despite the improvements observed in hyperaemic responses associated with improved glycaemic control in recently diagnosed NIDDM patients observed in Chapter 4.2. Alternatively, arterial hypertension per se may have induced secondary structural changes in the microcirculation as previously described[169]. As the prevalence of different microvascular complications was similar in the hypertensive and normotensive NIDDM patients, it is unlikely that the observed differences in resistance to flow reflect differences in the severity of microangiopathy in the two groups.

In summary, these results suggest that hypertension is associated with an additional rise in pre-capillary vascular resistance in NIDDM which, while protecting the microcirculation from the effects of increased arterial pressure, may further diminish protective hyperaemic responses.

Summary

The studies in this chapter have confirmed that there is a reduction in microvascular hyperaemia in response to local heating in patients

with NIDDM. This limited vasodilation is not due to occult large vessel disease, is present at the time of diagnosis of diabetes, and improves in recently diagnosed patients who achieve good glycaemic control, providing a further reason for striving for optimal control. In NIDDM patients with hypertension, hyperaemic responses are not further impaired, but there is a rise in estimated resistance to blood flow. Such a rise in vascular resistance is of clinical relevance in the diabetic foot, where reduced hyperaemia is likely to contribute to impaired inflammatory responses to infection and tissue injury, and also to a slow healing rate[211]. In patients with NIDDM, therefore, the effect of hypertension may add to the burden of intrinsic microvascular disease, increasing the chances of microvascular failure and tissue necrosis.

CHAPTER 5

MAXIMUM MICROVASCULAR HYPERAEMIA IN INSULIN RESISTANT STATES

The microvascular hyperaemic response to local heating of the foot skin is already reduced in newly diagnosed NIDDM patients[122]. This could arguably reflect a prolonged phase of undiagnosed diabetes and/or the effects of metabolic abnormalities other than hyperglycaemia (e.g. hyperinsulinaemia) on the microcirculation. The work in this chapter attempts to clarify this question by studying microvascular hyperaemia in subjects at high risk of developing NIDDM who have IGT; by exploring the relationship of hyperinsulinaemia to microvascular vasodilation in patients with acromegaly - a disease state associated with insulin resistance; and by relating blood pressure and metabolic abnormalities during the pre-diabetic phase to maximal hyperaemic responses in subjects with IGT.

5.1 Impaired glucose tolerance.

Both structural microangiopathy[35] and abnormal microvascular function[122] are detectable at the time of diagnosis in patients with NIDDM. Prior to the development of NIDDM, it is possible to define a 'pre-diabetic' phase, otherwise known as IGT (Chapter 1.2, page 7), on the basis of an abnormal blood glucose profile following a standard

75g oral glucose test (OGTT)[212]. Although the OGTT remains the 'gold standard' for defining IGT, it shows poor intra-individual reproducibility[213], and recently it has been argued that glucose tolerance can be characterised as reliably, and more simply, on the basis of a fasting plasma glucose level[214]. Regardless of this, there is little evidence of structural microangiopathy in subjects with IGT[64,65], and little information on microvascular function (Chapter 1.5, page 21). As reduced microvascular hyperaemia is present at diagnosis in NIDDM[122], the aim of this study was to determine whether abnormal vasodilation is already present in subjects with IGT.

Subjects and Methods:

Subjects

Subjects at risk of developing NIDDM were recruited from the Oxford cohort of the Fasting Hyperglycaemia Study, a multicentre prospective randomised study comparing the effects of diet and exercise, with or without sulphonylurea treatment, on IGT. This group had been recruited from a high risk population who all had one or more risk factors for the development of NIDDM (affected first degree relative, previous gestational diabetes, marked obesity, known IGT). All had a fasting venous plasma glucose concentration between 5.5 and 7.8 $mmol.l^{-1}$ (i.e. greater than 95th centile for the normal population and less than WHO definition for diabetes[212]) on two occasions prior to entry to the study, and all had normal fasting serum cholesterol and triglyceride levels.

Microvascular function was assessed in all suitable subjects with fasting hyperglycaemia attending the study clinic in a 1 month period. This amounted to 11 subjects (5 male : 6 female; age 52 (40-73) years, median and range) (Table 5.1.1), of whom six had been randomised 1 to 3 months beforehand to treatment with placebo tablets, or the sulphonylurea gliclazide, in a dose of 80-160 mg daily, plus a diet and exercise program. The remaining five subjects were all studied at their first clinic visit prior to any therapy randomisation. Eleven age- and sex-matched control subjects with no family history of NIDDM were studied for comparison (Table 5.1.1). None of the participants had hypertension, renal impairment or large vessel disease as previously defined (pages 82 and 88), and none were taking vasoactive medication. Two of the subjects with IGT were ex-smokers and two were current smokers, while three of the control subjects were ex-smokers.

All subjects with fasting hyperglycaemia were screened for microvascular disease as previously described (pages 71 and 74). None of those with IGT had evidence of retinopathy, two had mild large fibre neuropathy (VPT = 22 and 30 V), and none had microalbuminuria.

Methods:

During the acclimatisation period, brachial blood pressure was calculated as the mean of 3 measurements using an automated device (Takeda UA-751, A&D Engineering Inc., Milpitas, Ca, USA). Subsequently, measurements of maximum microvascular hyperaemia were carried out on the dorsum of the right foot (page 43).

Table 5.1.1 ***Clinical characteristics of subjects with impaired glucose tolerance and non-diabetic control subjects***

	IGT	CONTROL
N (M/F)	5/6	5/6
AGE (yr)	52 (40-71)	53 (40-71)
BMI ($kg.m^{-2}$)	27 (24-37)	25 (19-31)
PLASMA GLUCOSE ($mmol.l^{-1}$)	6.4 (5.2-7.1)	4.5 (4.0-5.2)
SERUM CHOLESTEROL ($mmol.l^{-1}$)	5.6 (4.0-5.8)	5.2 (4.3-5.5)
SERUM TRIGLYCERIDES ($mmol.l^{-1}$)	1.2 (0.8-1.3)	1.1 (0.7-1.5)
BLOOD PRESSURE (mmHg)		
SYSTOLIC	132 (117-154)	132 (122-148)
DIASTOLIC	76 (68-90)	72 (66-88)

Data are shown as median and range

Results:

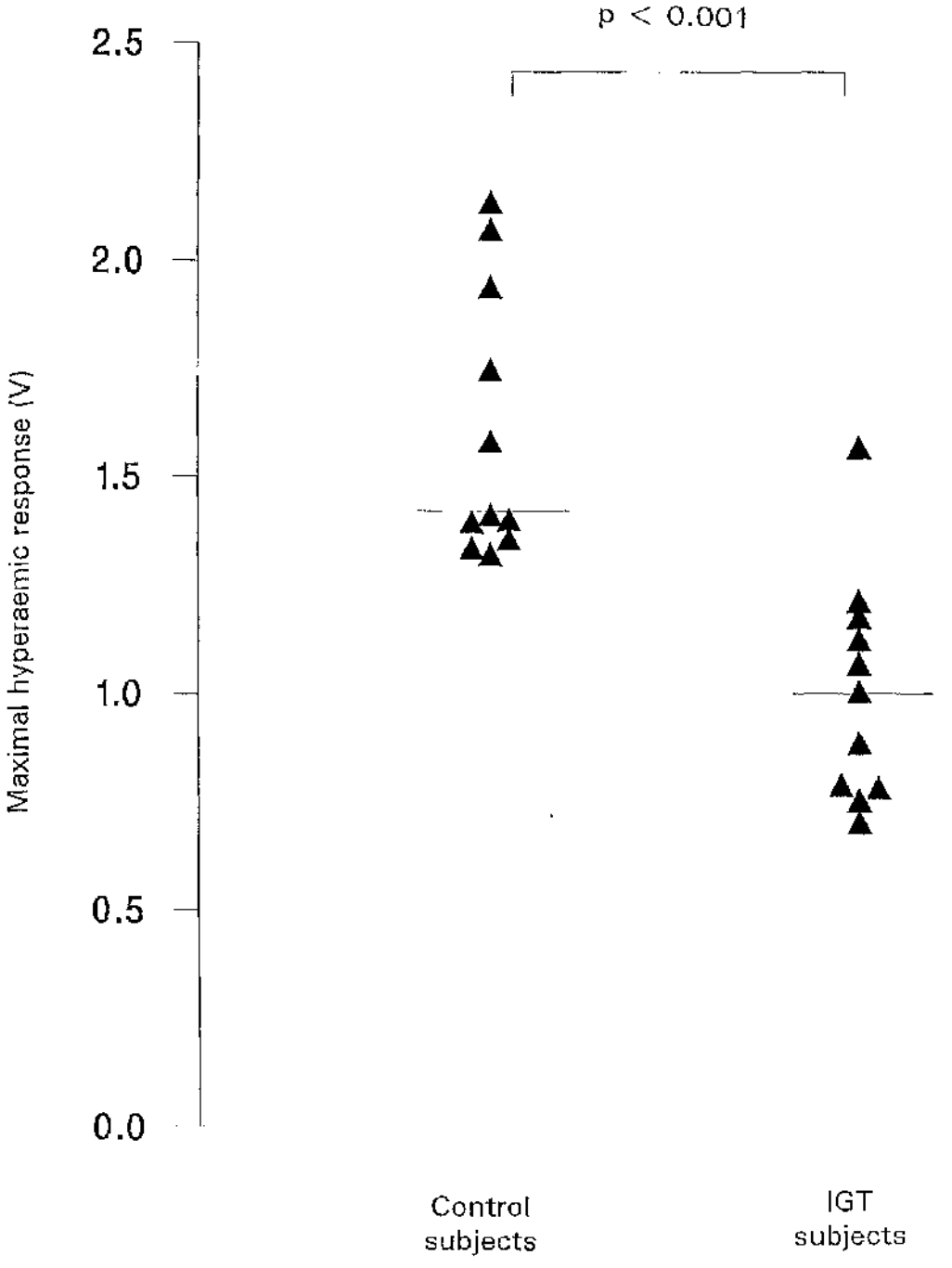
The maximum hyperaemic response was significantly lower in the subjects with IGT than in control subjects (1.01 (0.71-1.57) V vs 1.41 (1.32-2.13) V, $p < 0.001$, Mann-Whitney) (Fig 5.1.1). Values for maximum hyperaemia were similar in those already randomised to treatment and those who were not (0.98 (0.76-1.18) V vs 1.01 (0.71-1.57)V; $p = 0.4$). Median fasting plasma glucose concentration on the day of study was 6.4 (5.2-7.1) $mmol.l^{-1}$ in the group with IGT (vs 4.5 (4.0-5.2) $mmol.l^{-1}$ in control subjects; $p < 0.0002$), with two subjects having improved on therapy to fasting normoglycaemia. There were no significant differences in blood pressure ($p = 0.7$), BMI ($p = 0.1$), serum cholesterol ($p = 0.5$), or triglycerides ($p = 0.6$) between the subjects with fasting hyperglycaemia and control subjects (Table 5.1.1).

Discussion:

This study has demonstrated that functional microvascular abnormalities, such as reduced maximal hyperaemia, may already be present in subjects with IGT, who are at risk of developing NIDDM. The observed degree of impairment is similar to that seen in newly diagnosed NIDDM patients[122].

The mechanism for limited vasodilation seems unlikely to be related to the modest elevations in plasma glucose present in these subjects with IGT, which are similar to optimal treatment levels for both IDDM and NIDDM patients. This view is supported by the results of Chapter 4.2, in which sustained improvement in glycaemic control to

Fig 5.1.1 *Maximum microvascular hyperaemia in subjects with fasting hyperglycaemia and non-diabetic control subjects (horizontal bars represent median values)*



similar levels in newly diagnosed NIDDM patients was associated with marked improvement in microvascular vasodilation. Once more this raises the possibility that reduced microvascular hyperaemia may relate to the effects of other metabolic abnormalities occurring in the pre-diabetic state, such as hyperlipidaemia, or hyperinsulinaemia as a result of insulin resistance. In the current study, hyperlipidaemia did not seem to be of much importance as there was no significant elevation in either serum cholesterol or triglycerides in the subjects with IGT who had reduced microvascular vasodilation. The interactions of hyperinsulinaemia and insulin resistance with microvascular vasodilation are further explored in Chapters 5.2 and 5.3.

5.2 Acromegaly

Acromegaly is a disease caused by excess circulating growth hormone (GH) levels, usually as a result of hypersecretion from a pituitary adenoma[215]. GH excess results in metabolic effects mediated by various growth factors, in particular insulin-like growth factor-1 (IGF-1)[215]. One feature of this is the development of insulin resistance and hyperinsulinaemia, with the result that around 25% of patients with acromegaly develop IGT and 15% frank diabetes[216]. Acromegaly is also associated with a high prevalence of hypertension and increased cardiovascular mortality[217].

As similar metabolic abnormalities are present in acromegaly and the pre-diabetic state, maximum microvascular hyperaemia was determined in patients with acromegaly to investigate the influence of

hyperinsulinaemia on the microcirculation. In view of possible differences in dermal structure between patients with acromegaly and control subjects, basal skin capillary density was also determined.

Subjects and Methods:

Subjects

All acromegalic patients (n = 19) attending the Endocrine clinic at the Royal Devon and Exeter Hospital were identified and invited to participate in the study. Three patients with known diabetes mellitus were excluded, along with two who had evidence of large vessel disease and three who were taking potentially vasoactive medication for other reasons. Two further patients were unwilling to participate, leaving nine patients with acromegaly (3M : 6F; age 68 (52-70) years, median and range) who were suitable for study (Table 5.2.1). Nine age- and sex-matched control subjects selected from a panel of healthy volunteers were studied for comparison (Table 5.2.1). Acromegaly had been treated in a variety of ways (Table 5.2.2). Four of the selected acromegalic patients were being concurrently treated with bromocriptine and five had mild hypertension, but were included in the study to obtain an adequate sample size.

The acromegalic patients were screened for the presence of microvascular complications (pages 71 and 74). None had evidence of retinopathy; three had a raised VPT (25, 28 and 36 V); and one had elevated ACR (6.1 $mg.mmol^{-1}$). Two of the acromegalic patients were current smokers and two of the control subjects were ex-smokers.

Table 5.2.1 ***Clinical characteristics of patients with acromegaly and control subjects***

	ACROMEGALIC	CONTROL
N (M/F)	3/6	3/6
AGE (yr)	68 (52-70)	65 (50-69)
BMI ($kg.m^{-2}$)	28 (22-35)	25 (19-29)
PLASMA GLUCOSE ($mmol.l^{-1}$)	4.8 (4.1-5.8)	4.6 (3.2-5.6)
SERUM CHOLESTEROL ($mmol.l^{-1}$)	5.5 (4.1-5.9)	5.2 (3.9-5.7)
SERUM TRIGLYCERIDES ($mmol.l^{-1}$)	1.1 (0.8-1.3)	0.9 (0.7-1.2)
PLASMA INSULIN ($\mu U.ml^{-1}$)	10.2 (3.1-33.0)	-
SERUM IGF-1 ($U.ml^{-1}$)	1.72 (0.86-4.24)	-
BLOOD PRESSURE (mmHg)		
SYSTOLIC	148 (113-156)	135 (130-142)
DIASTOLIC	88 (65-104)	78 (66-89)

Data are shown as median and range

Table 5.2.2 ***Details of main form of treatment for acromegaly in patients studied***

TREATMENT	*N*
Bromocriptine	4
Transphenoidal hypophysectomy[a]	2
Radiotherapy	1
Hypophysectomy plus radiotherapy[b]	1
Untreated	1

[a]One male subject additionally on testosterone replacement therapy

[b]Subject additionally on thyroxine replacement therapy

Methods:

Following an overnight fast, the acromegalic patients had a standard 75g OGTT to assess glucose tolerance status[212], with basal blood samples being collected for measurement of plasma insulin (using a sensitive 2-site ELISA method[204]; normal fasting range 1-15 $\mu U.ml^{-1}$), plasma glucose, and serum lipid concentrations, and a 2-hour sample for plasma glucose concentration. In the patients with acromegaly, serum IGF-1 levels were measured by RIA[218] (normal range varies with age and sex, upper limit of normal around 1.5 $U.ml^{-1}$ for the subjects in this study) to determine disease activity[219]. IGF-1 was measured in preference to GH itself, as serum levels are relatively constant[219] in contrast to the pulsatile nature of GH secretion, thus avoiding the need to measure GH profiles over the course of 12 or 24 hours.

On a separate occasion, maximum microvascular hyperaemia was measured on the dorsum of the right foot (page 43), and basal skin capillary density determined on the dorsal surface of the middle phalanx of the left middle finger (page 65). Bromocriptine was witheld for a period of 24 hours, and antihypertensive medication for 48 hours before the study. During the acclimatisation period brachial blood pressure was determined as the mean of 3 readings using an automated device (Dynamap 845; Critikon Inc, Tampa, Fl., USA).

Results:

Maximum microvascular hyperaemia was significantly reduced in the acromegalic patients (0.96 (0.56-1.70) V vs 1.46 (1.24-2.13) V

control subjects; $p < 0.05$, Mann-Whitney) (Fig 5.2.1), although there was no difference in basal skin capillary density (92 (76-120) caps.mm^{-2} acromegalic patients vs 90 (74-136) caps.mm^{-2} control subjects; $p = 0.8$) (Fig 5.2.2). In the acromegalic patients there was no correlation between maximum hyperaemia and IGF-1 concentration ($p = 0.4$), but there was a tendency towards a negative association between maximum hyperaemia and fasting plasma insulin concentration ($Rs = -0.63$; $p = 0.06$, Spearman Rank) (Fig 5.2.3).

There were no significant differences between acromegalic patients and control subjects with respect to BMI ($p = 0.2$), serum cholesterol ($p = 0.2$), serum triglycerides ($p = 0.3$), or blood pressure ($p = 0.1$). There was no correlation between maximum hyperaemia and blood pressure ($p = 0.2$) or plasma glucose ($p = 0.5$), serum cholesterol ($p = 0.4$) and serum triglyceride ($p = 0.4$) concentrations.

Two acromegalic patients had IGT (fasting plasma glucose concentration < 7.8 mmol.l^{-1} and 2-hour glucose concentration 7.8-11.1 mmol.l^{-1})[212], while the others had normal glucose tolerance (Table 5.2.2). These two, plus another one patient, had fasting hyperinsulinaemia (defined as a fasting plasma insulin concentration greater than the upper limit of the normal range, i.e. > 15 μU.ml^{-1}, with a normal fasting plasma glucose concentration) (Table 5.2.3). Despite treatment, six patients had evidence of active acromegaly on the basis of elevated IGF-1 levels (Table 5.2.1).

Discussion:

Maximum microvascular hyperaemia is reduced in patients with acromegaly, a disease which may be associated with insulin resistance

Fig 5.2.1 *Maximum microvascular hyperaemia in patients with acromegaly and control subjects (horizontal bars represent median values)*

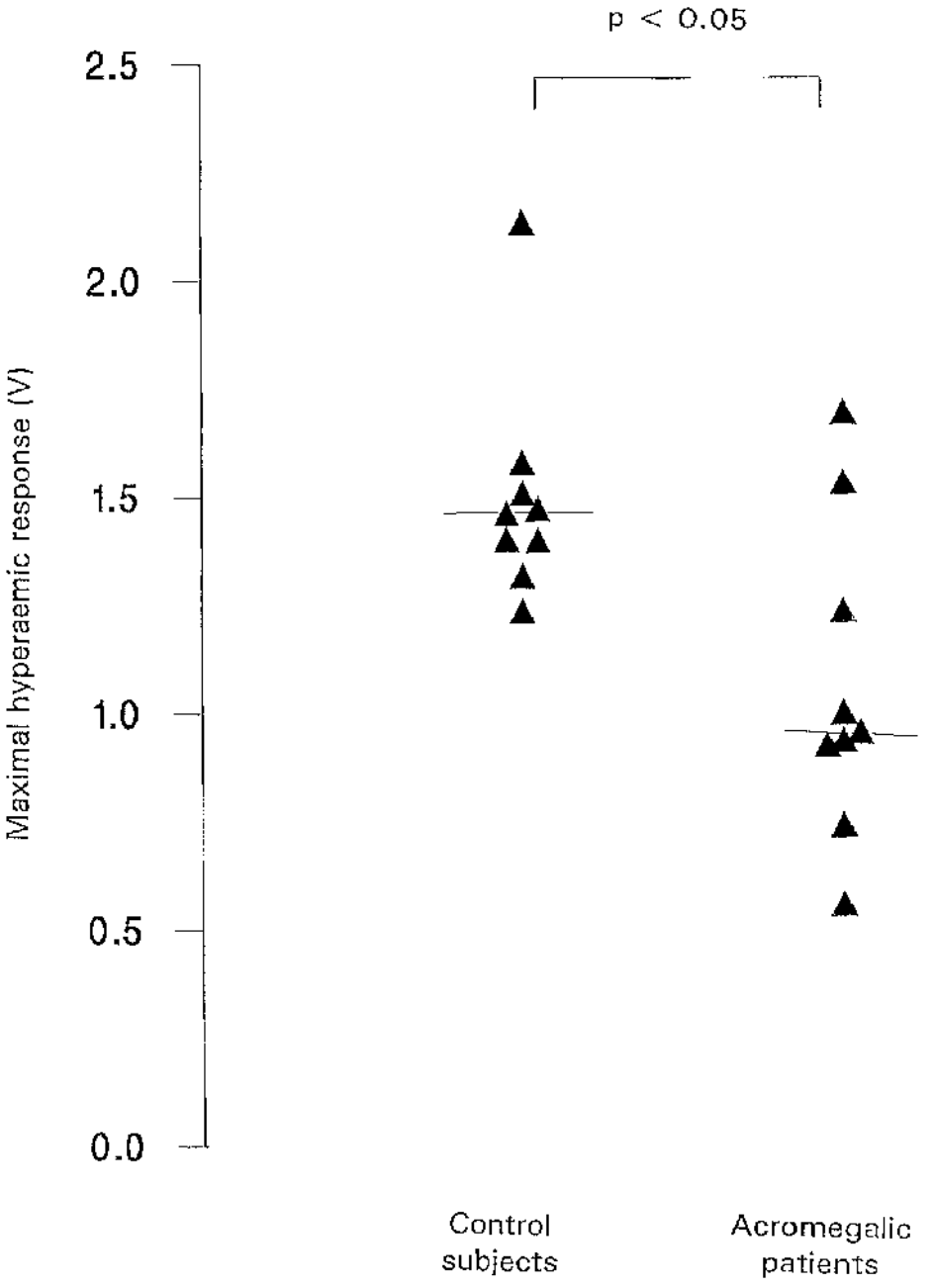


Fig 5.2.2 *Basal skin capillary density in patients with acromegaly and control subjects (horizontal bars represent median values)*

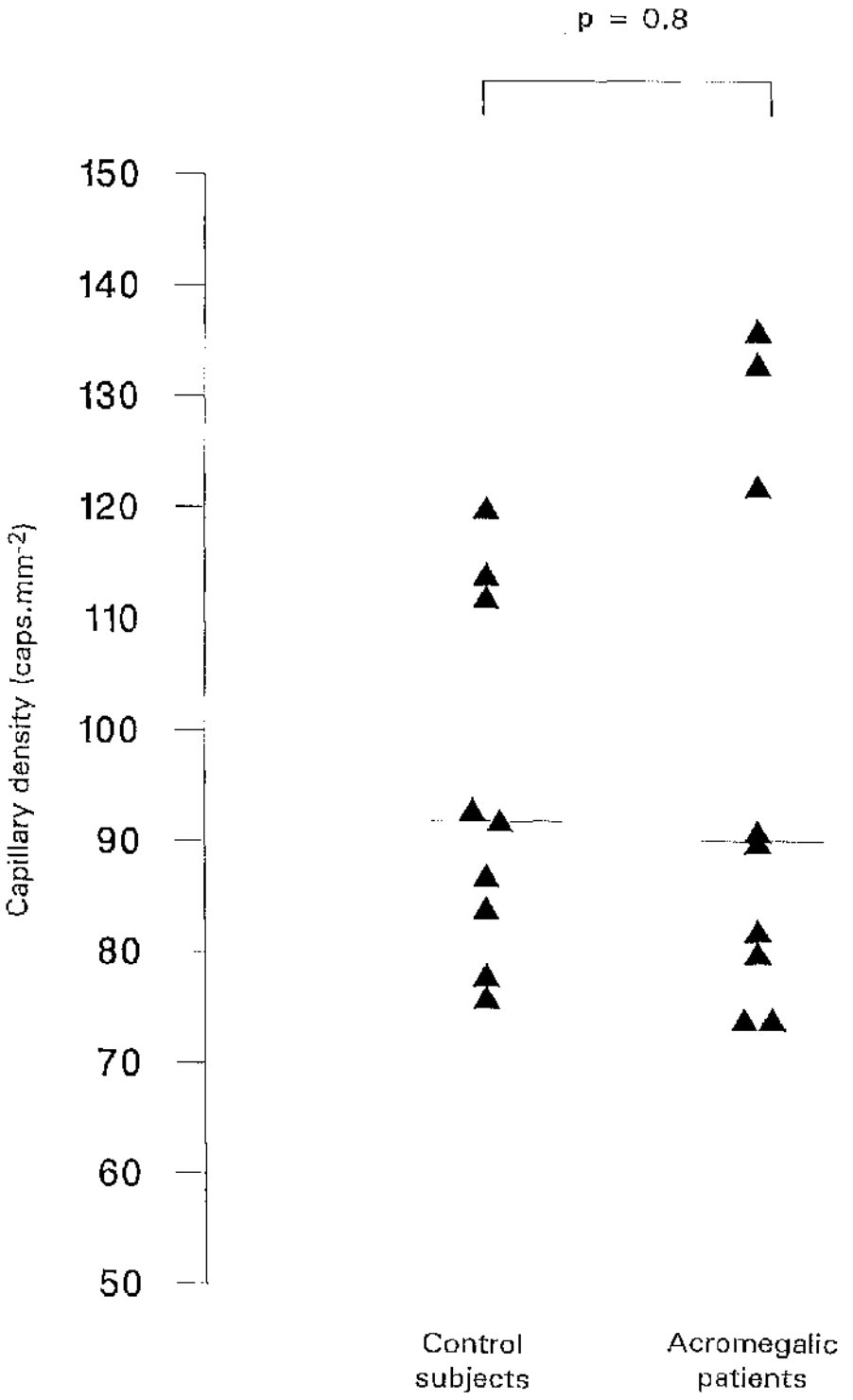


Fig 5.2.3 *Correlation of maximum hyperaemia with fasting plasma insulin concentration in the acromegalic patients*

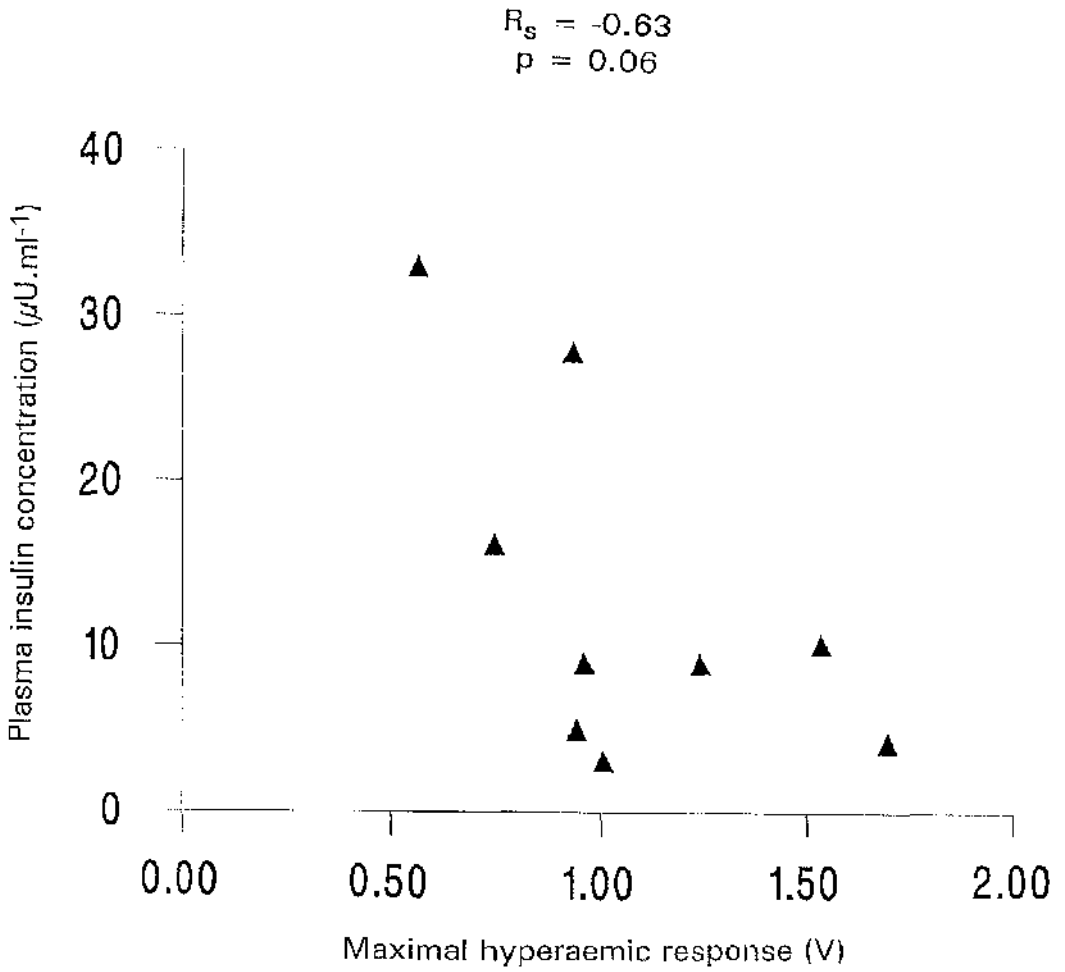


Table 5.2.3 *Details of hyperinsulinaemic acromegalic patients*

Subject	OGTT plasma glucose ($mmol.l^{-1}$)		Plasma insulin	BMI	Blood pressure
	Fasting	2-Hour	($\mu U.l^{-1}$)	($kg.m^{-2}$)	(mmHg)
F 69yr	5.8	6.8	33.0	29	154/100
F 68yr	4.7	10.4	16.2	33	156/84
F 68yr	4.1	11.0	27.8	28	148/90

and hyperinsulinaemia due to the antagonistic effects of growth hormone on insulin action[216]. Three of the acromegalic patients included in this study had fasting hyperinsulinaemia in the face of a normal fasting plasma glucose concentration, and two patients had IGT. There was a tendency towards a negative association between fasting plasma insulin level and maximum hyperaemic response, similar to the relationship observed in Chapter 4.1.

The metabolic abnormalities of insulin resistance, impaired glucose tolerance, hypertension, hyperlipidaemia and obesity often occur in combination[37], making it difficult to assess the individual contribution of each to limited microvascular vasodilation. There was no significant difference between acromegalic patients and control subjects with respect to blood pressure, despite the fact that five of the acromegalic patients were known to have mild hypertension. This may reflect a type 2 statistical error due to small sample size; however, the inclusion of some subjects with mild hypertension is unlikely to have biased the results as most studies in patients with essential hypertension have shown no reduction in maximal hyperaemia when compared with matched control subjects[169,170]. Similarly in Chapter 4.3 no further reduction in hyperaemic response was observed in NIDDM patients with hypertension when compared to normotensive patients, although hypertension is associated with an increased resistance to blood flow.

In keeping with the results in patients with IGT (Chapter 5.1), hyperlipidaemia does not seem to be an important determinant of limited microvascular vasodilation in patients with acromegaly, as there was no difference in either plasma cholesterol or triglyceride concentrations compared to control subjects, and no significant correlation of lipid levels with maximum hyperaemia.

Although BMI appears similar in the acromegalic patients and control subjects, BMI and body weight may not be reliable indicators of the extent or distribution of obesity in patients with acromegaly, as much of their increased weight is derived from hypertrophy of internal organs and body tissues such as liver and skeletal muscle[215], and not from excess adipose tissue. In addition, some acromegalic patients have tall stature due to onset of the disease prior to fusion of the epiphyses of long bones[215]. It is therefore difficult to exclude differences in body composition, with respect to adipose tissue, between patients with acromegaly and control subjects. It is possible that waist-hip ratio would be a more meaningful indicator of body composition in patients with acromegaly, although it was not measured in the current study and may be open to the same criticisms as above.

Six of the acromegalic patients had evidence of active disease and it is possible that this may have influenced microvascular hyperaemia due to effects of GH or IGF-1 on the microcirculation, although it is noteworthy that there was no correlation between serum IGF-1 concentration and maximal hyperaemia.

Marked soft tissue hypertrophy is a feature of acromegaly[215], suggesting that differences in dermal tissue structure between patients with acromegaly and control subjects could be contributing to the observed results. This possibility is made less likely by the fact that no difference was found in skin capillary density between patients with acromegaly and control subjects. The relationship between skin capillary density and maximum hyperaemia is further explored in Chapter 7.

It was not possible to be as rigorous with selection criteria in the current study as in the other studies in this thesis. Acromegaly is a very rare disease and after exclusion of diabetes and large vessel

disease, the majority of patients left suitable for study were taking bromocriptine or antihypertensive medication, both of which could affect microvascular reactivity and although these were discontinued for a period before measurement of maximum hyperaemia, a prolonged effect of either drug on the microcirculation cannot be ruled out. Ideally repeating the study using normotensive newly diagnosed acromegalic patients would eliminate many of these problems, although this would be impracticable outwith a specialist centre with a particular interest in the management of acromegaly.

In summary, despite the difficulties with patient selection, maximum microvascular hyperaemia is reduced in patients with acromegaly, and may be related to fasting insulin concentration.

5.3 Relationship of insulin resistance, ß-cell dysfunction and blood pressure to microvascular hyperaemia in IGT

The studies in Chapters 4.1 and 5.2 have suggested an association between increased plasma insulin concentration and reduced microvascular hyperaemia in patients with NIDDM and acromegaly respectively. This chapter further explores the interactions of hyperinsulinaemia, insulin resistance and ß-cell dysfunction with limited microvascular vasodilation in a larger group of subjects with IGT.

Insulin sensitivity and ß-cell function may be studied in vivo using a variety of techniques. It is possible to gain some information regarding insulin secretion and action using an intravenous glucose infusion with measurement of glucose and insulin levels during the infusion, and

mathematical modelling of the results. An example of such a technique is Continuous Infusion of Glucose with Model Assessment (CIGMA)[220], which has been used in this chapter. Such relatively simple techniques give results which are comparable to those of more complex euglycaemic hyperinsulinaemic, and hyperglycaemic clamp studies[220].

Subjects participating in the majority of studies in this thesis were deemed to be normotensive on the basis of discrete blood pressure recordings taken using a standard sphygmomanometer or automated device. Such discrete measures may lead to more subtle abnormalities of blood pressure being missed and recently 24-hour ambulatory blood pressure monitoring has been used to detect unrecognised hypertension or loss of the normal diurnal rhythm of blood pressure, whereby lower values are found during sleep. An abnormal diurnal rhythm has been found in some patients with NIDDM[221] and has been related to the presence of microvascular complications[221]. Loss of diurnal rhythm could therefore be associated with altered microvascular function. 24-hour ambulatory monitoring has therefore been employed to assess whether abnormal blood pressure profiles are related to reduced microvascular hyperaemia.

Subjects and Methods:

Subjects

Twenty four subjects with IGT (13M : 11F; age 46.5 (36-64) years, median and range) (Table 5.3.1) were recruited consecutively from the Exeter cohort of the Fasting Hyperglycaemia Study. The details of this study and patient selection are described in Chapter 5.1

Table 5.3.1 ***Clinical characteristics of subjects with impaired glucose tolerance***

	IGT
N (M/F)	13/11
AGE (yr)	46.5 (36-64)
BMI ($kg.m^{-2}$)	28 (22-34)
PLASMA GLUCOSE ($mmol.l^{-1}$)	5.8 (4.8-7.1)
HBA_{1C} (%)	5.7 (5.0-7.3)
TOTAL CHOLESTEROL ($mmol.l^{-1}$)	4.98 (3.31-7.83)
HDL CHOLESTEROL ($mmol.l^{-1}$)	1.09 (0,77-1.98)
LDL CHOLESTEROL ($mmol.l^{-1}$)	3.10 (1.45-4.77)
TRIGLYCERIDES ($mmol.l^{-1}$)	1.06 (0.46-5.48)
BLOOD PRESSURE (mmHg)	
SYSTOLIC	123 (97-146)
DIASTOLIC	70.5 (51-83)

Data are shown as median and range

(page 106). Fifteen were allocated to tablet therapy with placebo or gliclazide 80-160 mg daily and the others to a diet and exercise program alone. Three subjects were current and four subjects ex-smokers.

All subjects were screened for microvascular disease as previously described (pages 71 and 74). None had retinopathy, one had mild and one marked large fibre neuropathy (VPT 23 and 45 V respectively) and two had microalbuminuria.

Methods

Prior to therapy randomisation and following an overnight fast, an intravenous glucose tolerance test was carried out on each subject using CIGMA to determine ß-cell function and insulin resistance[220]. Basal values for plasma glucose, C-peptide and insulin were determined from the mean of three samples taken at five minute intervals before an infusion of 10% Dextrose was started. Dextrose was infused at a dose of 5 mg.kg ideal body weight^{-1}.min^{-1} over 60 minutes, with further blood samples taken for glucose, C-peptide and insulin measurement at 50, 55 and 60 minutes. Blood samples were collected from an indwelling venous cannula in the forearm which was flushed with 0.9% Sodium Chloride between samples, a heating blanket around the forearm ensuring arterialisation of venous blood prior to collection. Plasma insulin was measured using a sensitive 2-site ELISA method[204] (normal fasting range 1-15 μU.ml^{-1}), and C-peptide by RIA[222]. CIGMA data for ß-cell function and insulin resistance were then derived from the plasma glucose, C-peptide and insulin concentrations using computer based mathematical modelling[220], with

results expressed as a percentage of predicted normal. Basal blood samples were also collected to measure fasting serum lipids.

24-hour ambulatory blood pressure monitoring was carried out on a separate occasion. Ambulatory blood pressure recordings were made on the left arm using the Takeda TM-2420 device (A&D Engineering Inc., Milpitas, Ca, USA). After careful positioning and taping of the blood pressure cuff and tubing, three test readings were taken using the TM-2420 device and compared with three simultaneous readings from the right arm taken using an automated device (Takeda UA-751, A&D Engineering Inc., Milpitas, Ca, USA). The positioning was deemed satisfactory if both systolic and diastolic blood pressures differed by less than 5mmHg between the two devices[223]. This procedure was repeated at the end of the 24-hour recording to check the reliability of the data, which was only accepted if the difference between the two devices remained less than 5mmHg. Subjects kept a diary in which they recorded their daily activities including time of going to sleep and wakening. Mean 24-hour blood pressure, and mean daytime and nocturnal blood pressures were calculated for each subject.

At a final visit, by which time subjects had been randomised to treatment, microvascular hyperaemia in response to local heating was measured on the dorsum of the right foot as previously described (page 43).

Results:

A spectrum of values was observed for insulin sensitivity and ß-cell function in the subjects with IGT (Table 5.3.2). Maximum

microvascular hyperaemia showed a significant negative correlation with fasting plasma insulin concentration (R_s = -0.70; $p < 0.001$) (Fig 5.3.1) and positive correlation with insulin sensitivity (R_s = 0.52; $p < 0.02$) (Fig 5.3.2). Multiple regression analysis with maximum hyperaemia as the dependent variable and fasting plasma insulin concentration and insulin sensitivity as the independent variables confirmed a significant relationship between limited vasodilation and insulin level (t = -4.53, $p<0.0005$), whereas insulin sensitivity no longer showed a significant association (t = 1.021, p = 0.3), probably as a result of the relationship between fasting plasma insulin and insulin sensitivity (R_s = -0.56, $p < 0.05$). There was no association of maximum hyperaemia with ß-cell function (p = 0.5), plasma glucose concentration (p = 0.8), waist-hip ratio (0.9), BMI (0.9) or serum lipid concentrations (p = 0.1 for total cholesterol and HDL cholesterol; p = 0.4 for LDL cholesterol; and p = 0.8 for triglycerides).

None of the subjects with IGT had undetected hypertension on 24-hour ambulatory monitoring and all had a normal diurnal rhythm for arterial blood pressure, with lower values during sleep (Table 5.3.2).

Discussion:

This study has further explored the interactions of circulating insulin with microvascular function in subjects with IGT. Hyperinsulinaemia was associated with reduced microvascular hyperaemia, in agreement with the results of Chapters 4.1 and 5.2. Furthermore estimated insulin sensitivity has an inverse relationship with limited vasodilation.

Table 5.3.2 ***Insulin sensitivity, ß-cell function and blood pressure in subjects with impaired glucose tolerance***

FASTING PLASMA INSULIN ($\mu U.ml^{-1}$)	9.3 (4.6-19.3)
INSULIN SENSITIVITY (%)	80.7 (19.5-178.8)
ß-CELL FUNCTION (%)	87.1 (38.5-169.4)
DAYTIME MEAN BLOOD PRESSURE (mmHg)	97.3 (81.6-110.2)
NOCTURNAL MEAN BLOOD PRESSURE (mmHg)	77.1 (67.2-97.3)

Data are shown as median and range

Fig 5.3.1 ***Correlation between maximum hyperaemia and fasting plasma insulin concentration in subjects with impaired glucose tolerance***

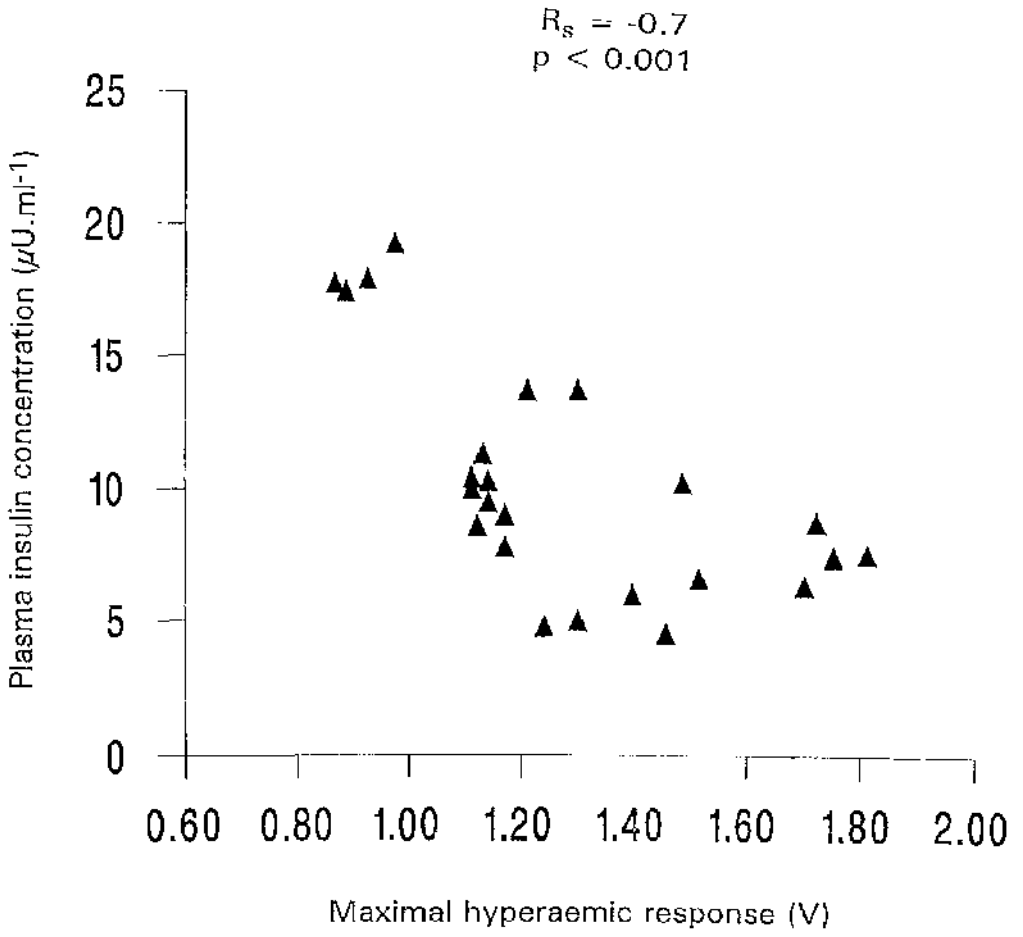
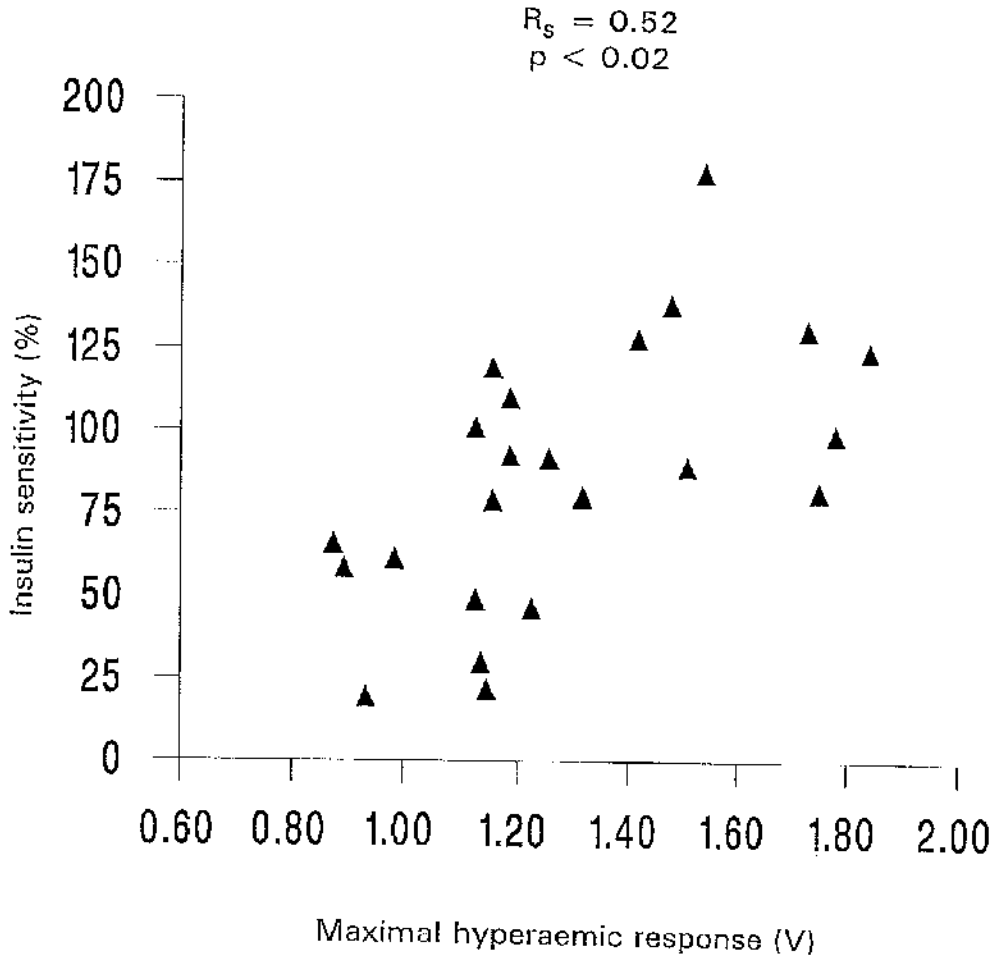


Fig 5.3.2 *Correlation between maximum hyperaemia and calculated insulin sensitivity in subjects with impaired glucose tolerance*



In contrast ß-cell dysfunction does not seem to be an important determinant of reduced microvascular hyperaemia.

In the current study, there was little evidence that other factors associated with the insulin resistant state had any relationship to impaired microvascular function. There was no evidence of undetected hypertension or loss of diurnal rhythm of arterial blood pressure on 24-hour ambulatory monitoring, making it unlikely that such factors contribute to limited microvascular vasodilation. Plasma glucose levels were only slightly elevated (Table 5.3.1) and were below the treatment target levels proposed for both IDDM and NIDDM patients. In view of this and also the lack of association between plasma glucose concentration and reduced microvascular hyperaemia, this would not seem to be a major determinant of limited vasodilation in subjects with IGT. Similarly, there was no association between serum lipid levels and reduced hyperaemia, although only a few subjects in the current study had elevated cholesterol or triglyceride levels (Table 5.3.1). The subjects with IGT tended to be overweight, although again there was no association with limited vasodilation and BMI or waist-hip ratio (Table 5.3.1).

These results lend suggest that raised circulating levels of insulin, as a result of insulin resistance, may be having a detrimental effect on microvascular cell function in subjects with IGT. A similar association between plasma insulin and microvascular hyperaemia was observed in NIDDM patients in Chapter 4.1 and in insulin resistant patients with acromegally in Chapter 5.2. The microcirculatory effects of insulin may be mediated through stimulation of microvascular cell proliferation, paralleling the effects observed in cell culture experiments[43,143-5]; or via other pathways such as disturbances of normal vasodilator

mechanisms at a biochemical level[25-28]. The mechanism of impaired microvascular vasodilation is further examined in Chapter 6.

Summary

The studies in this chapter have demonstrated that abnormal microvascular function, in the form of limited vasodilation, can be demonstrated in the pre-diabetic phase in subjects with IGT who are at risk of developing NIDDM, and also in insulin resistant patients with acromegaly. There was no evidence of abnormal 24-hour blood pressure profiles which could potentially be related to altered microvascular function in the subjects with IGT. The major factors related to reduced microvascular hyperaemia at this stage are hyperinsulinaemia and insulin resistance, whereas mild hyperglycaemia and lipid abnormalities do not seem to play a major role.

CHAPTER 6

MECHANISM OF REDUCED MICROVASCULAR HYPERAEMIA

The preceding chapters have demonstrated that the major microvascular functional abnormality present in patients with NIDDM, and in subjects at risk of developing NIDDM who have IGT, is reduced microvascular hyperaemia. Impaired vasodilation may arguably be due to abnormal neurovascular cell function and/or structural changes in the microcirculation. This chapter explores the role of abnormal neurovascular cell function in causing reduced maximum microvascular hyperaemia.

The mechanism of microvascular vasodilation in response to local heating of the skin in normal subjects is unknown. Theoretically, it could be due to one or more of endothelium-dependent, endothelium-independent (myogenic), or neurogenic vasodilator mechanisms[189]. It is possible to look at the integrity of these mechanisms using the technique of iontophoresis[189], which allows the transfer of vasoactive substances directly across the skin surface. This has advantages over other methods of administration of pharmacological mediators in the study of the microcirculation, such as local injection which may itself cause hyperaemia due to local trauma, and intra-arterial administration which may have effects on proximal larger blood vessels. As previously discussed (Chapter 1.6, page 23), endothelium-dependent vasodilation occurs due to the release of EDRF, the major component of which has now been identified as nitric oxide[25]. This acts on vascular smooth muscle cells to cause vasodilation[25]. Acetylcholine (ACh) is known to stimulate EDRF release from endothelial cells, and iontophoresis of ACh was therefore used to assess endothelial

dependent vasodilation[189-191]. Sodium nitroprusside (NaNP) acts directly on vascular smooth muscle cells to cause vasodilation and can therefore be used to study endothelium-independent (or myogenic) vasodilation[189,191]. Finally, iontophoresis of ACh at a distance from the site of measurement leads to an axon flare response and this can be used to assess neurogenic vasodilation[191-193].

In order to try and determine the earliest changes underlying impaired microvascular vasodilation, subjects with IGT but not frank NIDDM were used in the studies in this chapter.

Subjects and Methods:

Subjects

Seven subjects with IGT (3M : 4F, age 62 (42-67) years, median and range) (Table 6.1) recruited consecutively from the Exeter cohort of the Fasting Hyperglycaemia Study (see Chapter 5.1) were studied. Six of these had been randomised 1 to 3 months beforehand to treatment with placebo tablets, or the sulphonylurea gliclazide, in a dose of 80-160 mg daily, plus a diet and exercise program. The remaining subject had been randomised to diet and exercise therapy alone. Seven age- and sex-matched non-diabetic control subjects with no family history of diabetes were selected from a panel of healthy volunteers and studied for comparison (Table 6.1).

None of the participants had hypertension, renal impairment, or large vessel disease as previously defined (pages 82 and 88), and none were taking vasoactive medication. Three of the subjects with IGT

Table 6.1 *Clinical characteristics of subjects with impaired glucose tolerance and non-diabetic control subjects*

	IGT	CONTROL
N (M/F)	3/4	3/4
AGE (yr)	62 (42-67)	61 (45-66)
BMI ($kg.m^{-2}$)	30 (24-32)	27(25-30)
PLASMA GLUCOSE ($mmol.l^{-1}$)	6.0 (5.1-6.8)[a]	5.1(4.8-5.4)
BLOOD PRESSURE (mmHg)		
SYSTOLIC	124 (112-146)	122 (114-150)
DIASTOLIC	72 (62-82)	70 (60-84)
BASAL SKIN BLOOD FLOW (AU)	13.1 (6.2-23.5)	10.0 (5.1-20.8)

Data are shown as median and range

[a] $p < 0.02$ vs control subjects

were ex-smokers, while one of the control subjects was a current smoker and one an ex-smoker.

All subjects with IGT were screened for microvascular disease, as previously described (pages 71 and 74) and in addition had retinal photography carried out[35]. None had retinopathy or microalbuminuria, but one had evidence of mild (VPT = 23 V) and another moderate (VPT = 30 V) large fibre neuropathy.

Methods

Iontophoresis was carried out on both forearms as described in Chapter 2.3 (page 55) using the Moor iontophoresis system and MBF3D perfusion monitor (Moor Instruments Ltd, Axminster, Devon). During the 30 minute acclimatisation period average brachial blood pressure was determined from the mean of three readings using an automated blood pressure recorder (Dynamap 845: Critikon Inc, Tampa, Fl., USA). Data on mean basal blood flow (averaged from all measurement sites for each subject) is given in Table 6.1. At the end of the study a venous blood sample was taken to measure ambient plasma glucose concentration.

Results:

The iontophoretic response to ACh was significantly reduced in the subjects with IGT (518 (410-905) AU.min^{-1} vs 1236 (875-1588) AU.min^{-1} control subjects, median and range; $p < 0.003$ Mann-Whitney) (Fig 6.1). In contrast there was no significant difference in the iontophoretic response to NaNP (683 (301-1175) AU.min^{-1} vs 898

Fig 6.1 *Iontophoretic response to acetylcholine in subjects with IGT and control subjects (horizontal bars represent median values)*

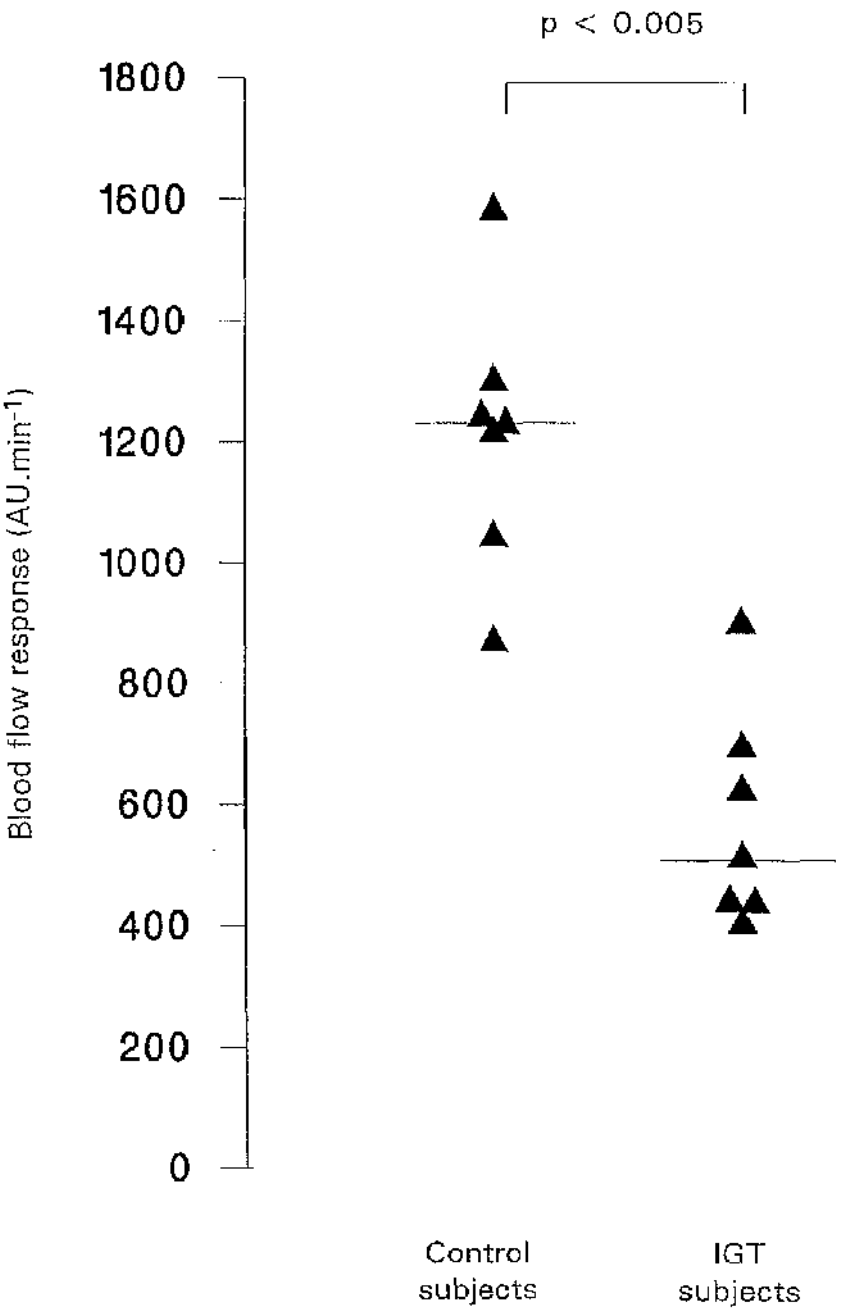


Fig 6.2 *Iontophoretic response to sodium nitroprusside in subjects with IGT and control subjects (horizontal bars represent median values)*

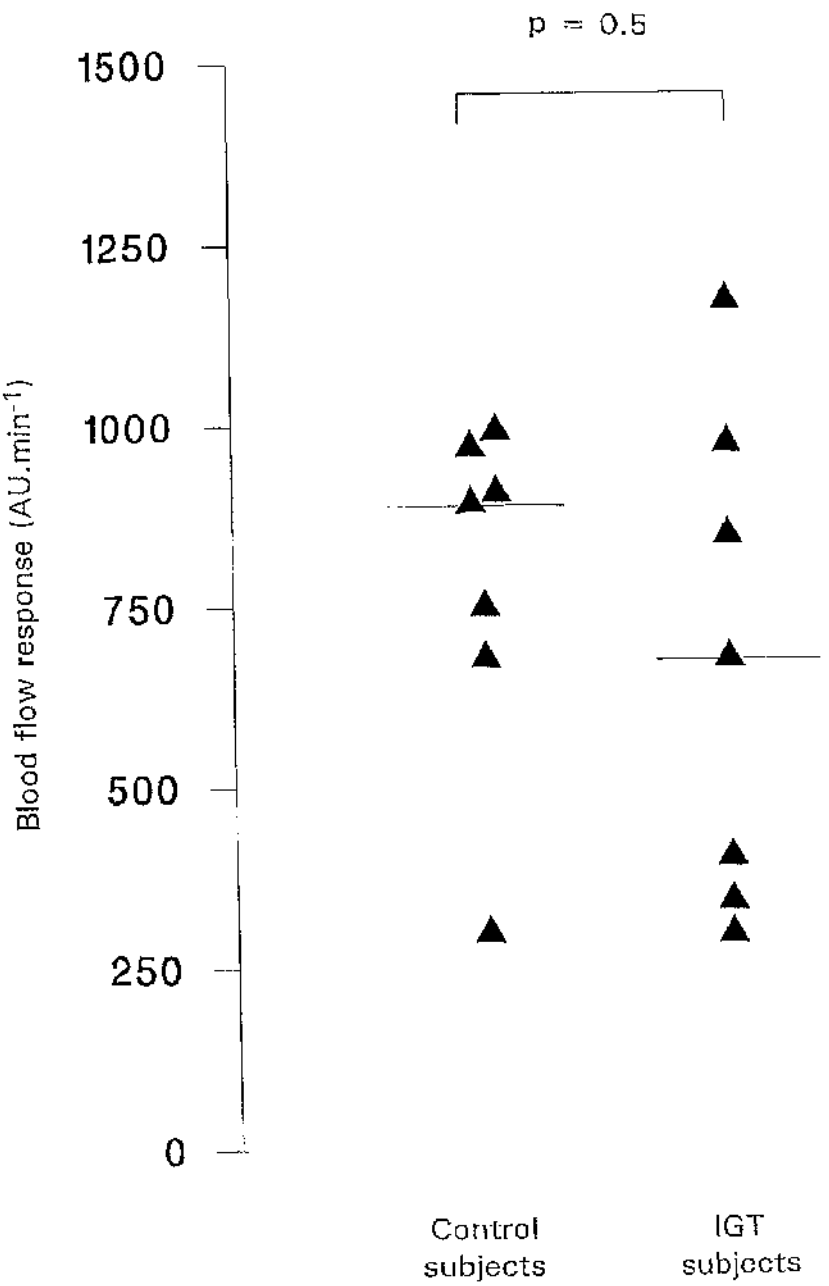


Fig 6.3 *Neurogenic vasodilation in response to Acetylcholine in subjects with IGT and control subjects (horizontal bars represent median values)*

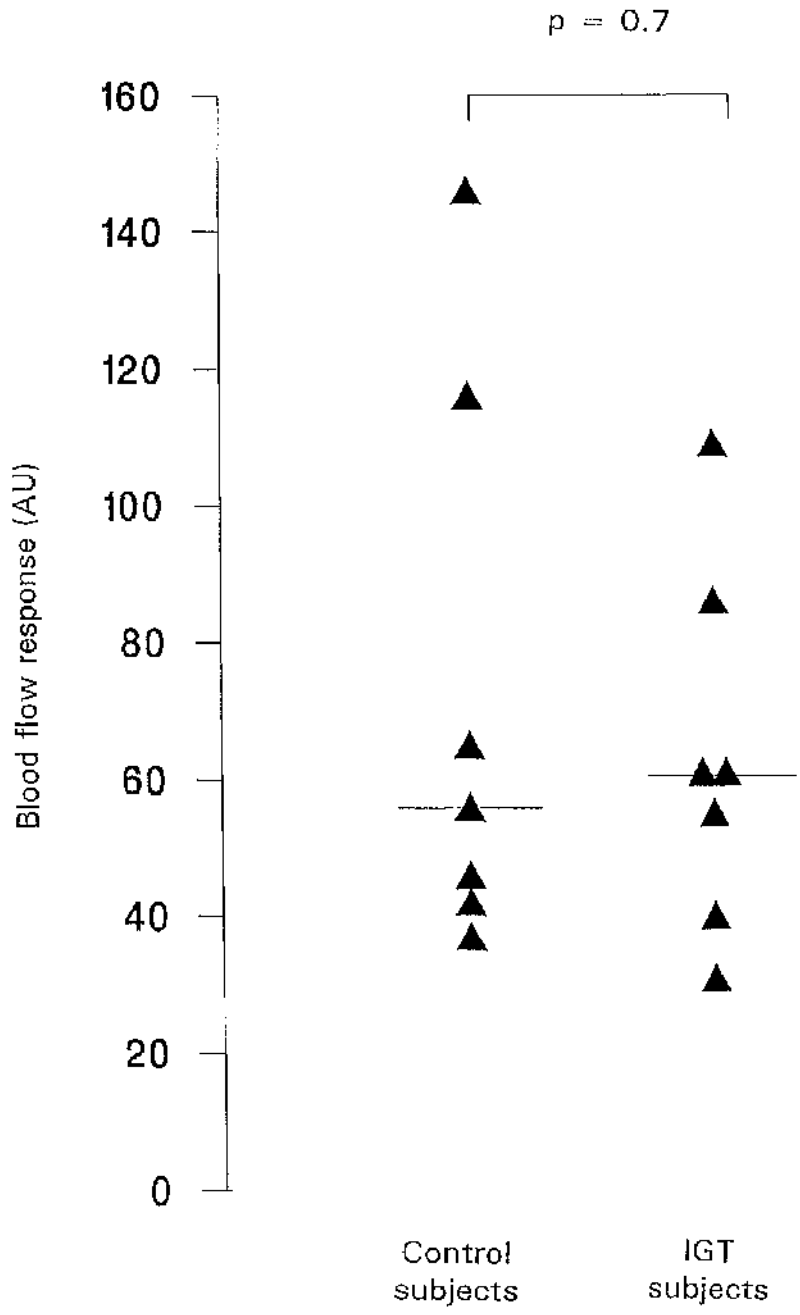
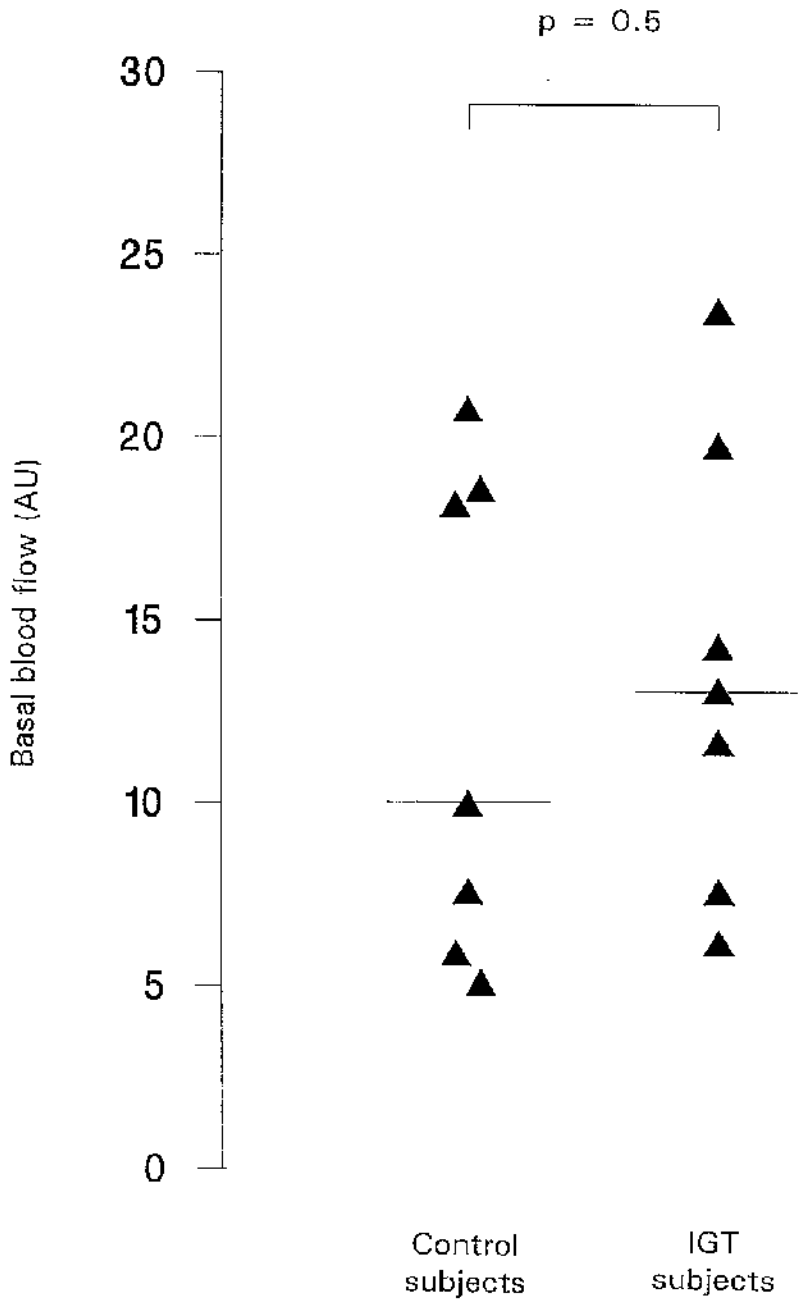


Fig 6.4 *Basal blood flow in subjects with IGT and control subjects (horizontal bars represent median values)*



(303-998) AU.min^{-1} control subjects; p = 0.5) (Fig 6.2) or the neurogenic flare response to ACh (61 (31-109) AU vs 46 (37-146) control subjects; p = 0.8) (Fig 6.3).

There was no difference in blood pressure (p = 0.7), BMI (p = 0.4) or basal blood flow (p = 0.5) (Fig 6.4) between subjects with IGT and control subjects, although plasma glucose concentration was significantly higher in those with IGT (6.0 (5.1-6.8) mmol.l^{-1} vs 5.1 (4.8-5.4) mmol.l^{-1}; p < 0.02) (Table 6.1).

Discussion:

Endothelium-dependent vasodilation was reduced in subjects with IGT compared to age- and sex-matched control subjects, suggesting the presence of endothelial cell dysfunction. This is in agreement with the results of previous studies looking at larger forearm vessels in patients with NIDDM using the technique of plethysmography[126]. In contrast, there was no reduction in myogenic or neurogenic vasodilation, although studies in larger vessels have shown reduced myogenic vasodilation in NIDDM[126]. In view of the small sample size used, it is not possible to exclude small differences in myogenic and neurogenic vasodilation between subjects with IGT and control subjects, however, such changes would not be likely to have a significant pathophysiological effect in view of the major reduction in endothelium-dependent vasodilation.

There was no difference in mean basal blood flow between subjects with IGT and control subjects, suggesting that the results reflect a true reduction in endothelium-dependent vasodilation, rather than an

apparent reduction due to overperfusion under resting conditions in subjects with IGT.

The molecular mechanisms underlying this endothelial dysfunction are difficult to elucidate further in vivo. Hyperglycaemia-induced cell damage would not seem likely to be a major factor, for although plasma glucose was significantly higher in the IGT subjects, this was only elevated to a minor degree and was within the range associated with an improvement in maximum microvascular hyperaemia in patients with NIDDM described in Chapter 4.2. Alternatively, it is possible that minor degrees of hyperglycaemia could lead to more subtle changes in endothelial cell function by activating or inhibiting various intracellular enzyme systems leading perhaps to an imbalance of vasodilator and vasoconstrictor mediators. The ability of insulin to cause microvascular cell hyperplasia has been previously discussed and it is possible that hyperinsulinaemia could lead to reduced endothelium-dependent vasodilation by driving cells down a path towards cell division at the expense of secretory function. These potential mechanisms at the cellular level will require further investigation in vitro using cell culture techniques.

CHAPTER 7

SKIN CAPILLARY DENSITY

In addition to defective neurovascular cell function, differences in skin microvascular density could potentially contribute to diminished hyperaemic responses, as an increased flow through a reduced number of capillaries could lead to an underestimate of the blood flow response measured by laser Doppler fluximetry due to there being less change in total flux. As mentioned in Chapter 1.2 (page 7), there is currently much interest in the role of impaired early vascular development in the pathogenesis of adult cardiovascular disease, and low birth weight has been associated with hypertension[44] and premature death from ischaemic heart disease[45]. Low birth weight has also been associated with an increased prevalence of NIDDM and IGT in adult life[46], possibly due to maldevelopment of the pancreatic ß-cells or islet vasculature.

The studies in this chapter look at whether there is decreased skin capillary density in subjects with IGT and NIDDM. In addition to determining capillary density under basal conditions, measurements were repeated after a period of venous occlusion in an attempt to achieve a measure of maximum capillary density, in order to avoid confounding effects of potential differences in basal tissue perfusion in the different groups.

Subjects and Methods:

Subjects

Fifteen patients with NIDDM (7M : 8F, age 57 (35-70) years, duration 5 (0.5-14) years, median and range), 15 subjects with IGT, and 15 non-diabetic control subjects were studied (Table 7.1). The groups were age- and sex- matched. With this sample size the study had a 90% power at the 5% level of detecting a difference in capillary density of 12 caps.mm^{-2}. Patients with NIDDM were recruited consecutively from the Diabetic Clinic at the Royal Devon and Exeter Hospital, while subjects with IGT were recruited from the Fasting Hyperglycaemia Study clinic, and control subjects from a panel of healthy volunteers.

None of the participants had hypertension renal impairment or large vessel disease as previously defined (pages 82 and 88). There were four ex-smokers and two current smokers in the NIDDM group; five ex-smokers and one current smokers in the IGT group; and four ex-smokers and one current smoker amongst the control subjects. Four NIDDM patients were treated with diet alone, five with oral hypoglycaemic agents and six with insulin; while eleven of the subjects with IGT were being treated with gliclazide or placebo tablets and the remainder with a diet and exercise program as described previously (page 106 and 107).

All of the NIDDM patients and subjects with IGT were assessed for the presence of microvascular complications (pages 71 and 74). Five NIDDM patients had retinopathy (3 background, 2 treated maculopathy), three had large fibre neuropathy (VPT > 30, without previous foot ulceration) and four had microalbuminuria; whereas none

Table 7.1 ***Clinical characteristics of NIDDM patients, subjects with impaired glucose tolerance and non-diabetic control subjects***

	NIDDM	IGT	CONTROL
N (M/F)	7/8	7/8	7/8
AGE (yr)	57 (35-70)	54 (36-64)	57 (35-66)
BMI ($kg.m^{-2}$)	27 (22-33)	27 (23-33)	25 (23-30)
PLASMA GLUCOSE ($mmol.l^{-1}$)	7.6 (4.8-11.5)	5.7 (4.8-6.7)	4.9 (4.2-5.5)
BLOOD PRESSURE (mmHg)			
SYSTOLIC	136 (108-159)	123 (97-147)	128 (104-158)
DIASTOLIC	74 (62-89)	69 (51-82)	74 (64-88)
SKIN TEMPERATURE (^{o}C)	32.1 (29.8-33.6)	32.0 (28.4-34.8)	32.2 (31.7-34.1)

Data are shown as median and range

of the subjects with IGT had retinopathy or microalbuminuria, and one had mild large fibre neuropathy (VPT = 25).

Methods

Capillary videomicroscopy was used to record images of the capillaries in the dorsal skin overlying the middle phalanx of the left middle finger. Recordings were made under basal conditions and following 10 minutes venous occlusion at 35 mmHg using an inflatable cuff positioned around the proximal phalanx (Chapter 2.4, page 65). Mean capillary density (number of capillaries in $1mm^2$) was determined from measurements in six adjacent sites as described in Chapter 2.4 (pages 65 and 67).

At the end of the study a venous blood sample was obtained for assessment of ambient plasma glucose concentration, and in the diabetic subjects HbA_{1c} (determined by affinity column chromatography; normal range 4-6%).

Results:

There were no significant differences between the three groups of subjects in either basal capillary density (112 (71-144) caps.mm^{-2} NIDDM patients vs 107 (76-140) caps.mm^{-2} IGT subjects vs 112 (76-138) caps.mm^{-2} control subjects; p= 0.9 Kruskal Wallis) (Fig 7.1), or capillary density after venous occlusion (122 (87-157) caps.mm^{-2} NIDDM patients vs 121 (90-143) caps.mm^{-2} IGT subjects vs 123 (81-147) caps.mm^{-2} control subjects; p= 0.9) (Fig 7.2). In addition, there were no differences in intra-individual variability in basal capillary

Fig 7.1 ***Basal capillary density in NIDDM patients, subjects with IGT, and control subjects (horizontal bars represent median values)***

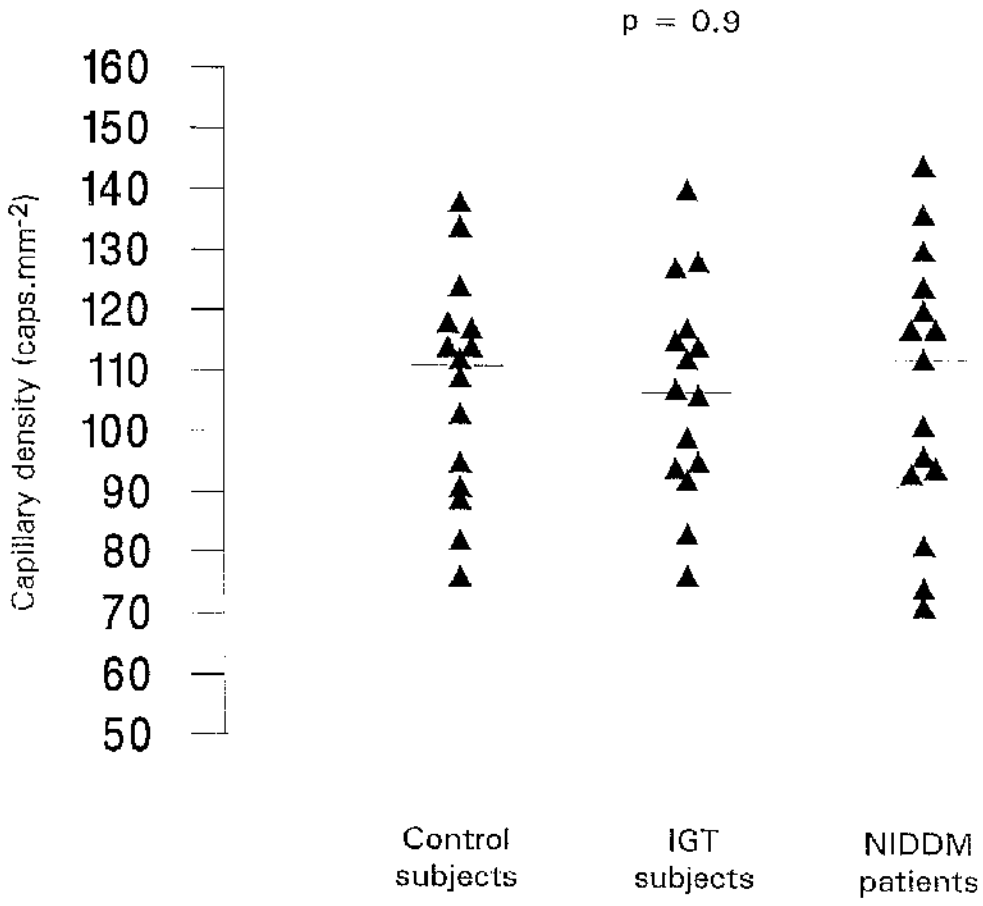


Fig 7.2 *Capillary density following venous occlusion in NIDDM patients, subjects with IGT, and control subjects (horizontal bars represent median values)*

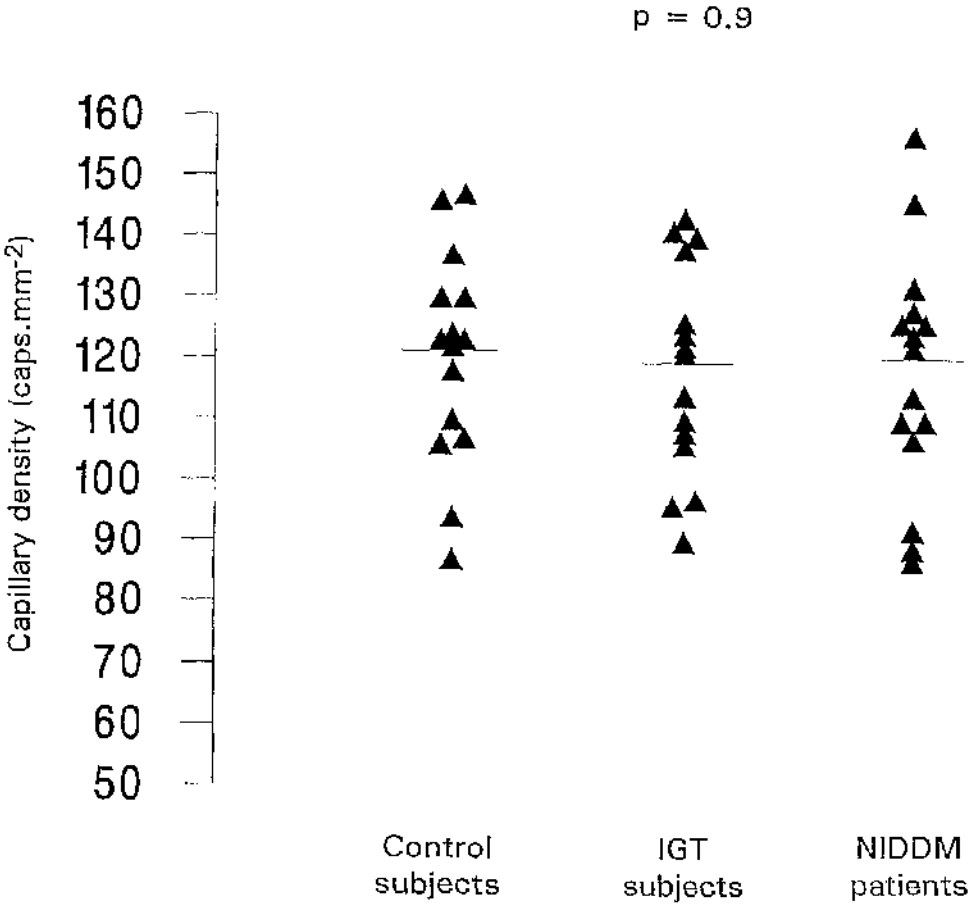
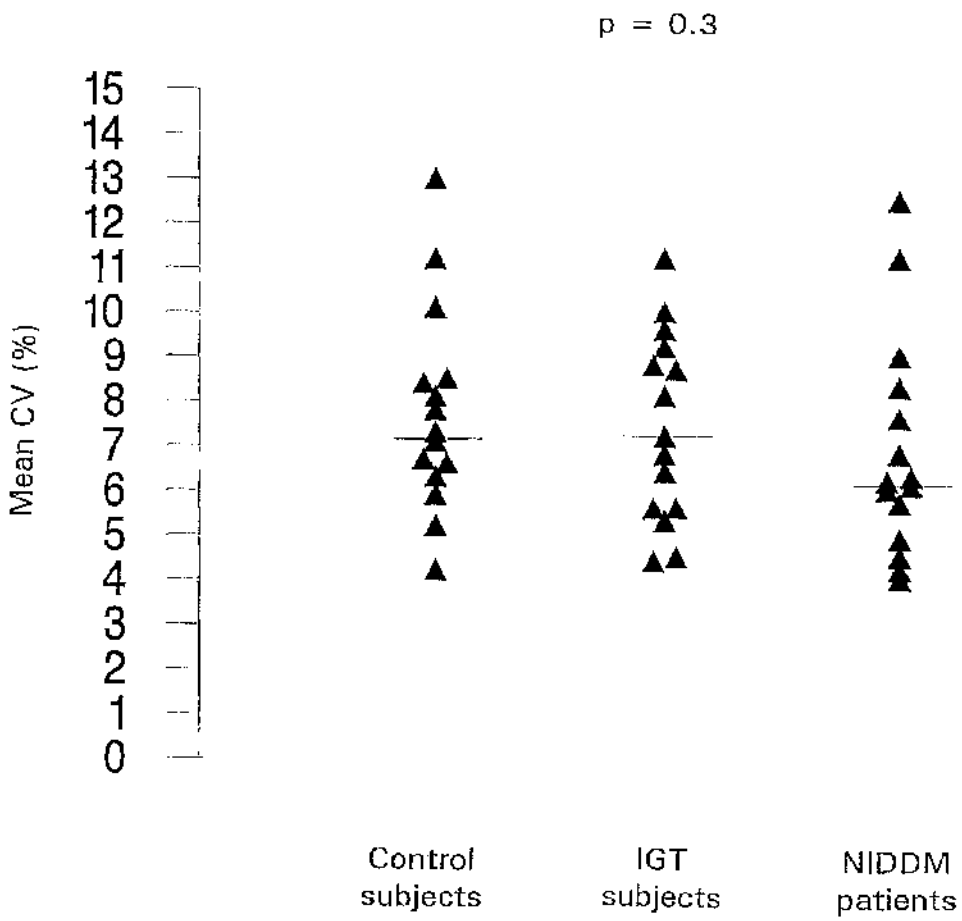


Fig 7.3 *Mean intra-individual variability in basal capillary density in NIDDM patients, subjects with IGT, and control subjects (horizontal bars represent median values)*



density between the 6 sites in the different subject groups (CV = 7.3 (4.2-13.0) % NIDDM patients vs 6.8 (4.4-11.2) % IGT subjects vs 6.2 (4.0-12.5) % control subjects; p = 0.3) (Fig 7.3).

All the groups were well matched for baseline characteristics with no differences in blood pressure (p = 0.1) or BMI (p = 0.4), although plasma glucose concentration was significantly higher in the NIDDM patients (Table 7.1). There was no significant difference in skin temperature (p=0.3) between the three groups.

Discussion:

This study has demonstrated that there are no differences in capillary density between patients with NIDDM, subjects with IGT or control subjects under basal conditions or following venous occlusion. The latter was carried out in an attempt to assess maximum capillary density in case there were differences in basal tissue perfusion between the groups which might have masked a real difference. In addition, there was no difference in the homogeneity of perfusion under basal conditions between the three groups, as the median intra-individual CV for each group was similar.

These results are in agreement with those from other studies, none of which support the hypothesis of impaired early microcirculatory development in skin or other tissues in patients with NIDDM. In one study, derived skin capillary density calculated from combined ^{133}Xenon and ^{111}Indium-Pentetic acid clearance appeared, if anything, to be increased in NIDDM patients[117]. In the kidney, no decrease in glomerular number has been found in both IDDM and NIDDM patients with nephropathy when compared to non-diabetic subjects with normal

renal function, except in IDDM patients with severe nephropathy[224]. In contrast, reduced skeletal muscle capillary density has been found in morphometric studies in obese subjects with normal glucose tolerance[225,226], although there are no data suggesting a similar reduction in non-obese patients with NIDDM or IGT.

This study had a 90% power at the 5% level of detecting a difference in capillary density of 12 caps.mm^{-2}. Although a smaller difference in capillary density may have been missed, this would be unlikely to be of any practical importance as it would represent a difference of less than 15% in capillary number, and, if it is assumed that all capillaries are of similar cross sectional area, a similarly small difference in total cross sectional area, thereby exerting only a negligible influence on blood flow.

The dorsal skin of the middle phalanx of the middle finger was chosen as the site of measurement in the current study as this was an easy area to record. Although the site of maximal hyperaemia response measurement was the dorsum of the foot, there is no reason to expect that comparison of capillary density in that area between the groups would yield different results, as the changes of diabetic microvascular disease are ubiquitous.

It is possible that differences in epidermal or dermal skin thickness could have masked differences in capillary density between the groups of subjects. Although no morphometric measures of skin thickness were made in the subjects in this study, there is little evidence from previous work in patients with diabetes to suggest that differences in skin thickness exist[227].

In summary, these results do not support the concept that there is impaired early development of the skin microcirculation in patients with diabetes or in subjects at risk of developing diabetes in the future, and

suggest that the observed differences in maximum microvascular hyperaemia do not relate to reduced capillary density.

CHAPTER 8

CONCLUSIONS

8.1 Summary of Results.

The work in this thesis has explored the pathophysiology of microvascular disease in NIDDM, by determining the presence of early functional abnormalities in the skin microcirculation. In contrast to the findings in patients with IDDM[77,79], patients with NIDDM do not appear to have elevated skin nailfold capillary pressure[115] or increased forearm microvascular fluid permeability (Chapter 3). The major early microvascular abnormality found in patients with NIDDM is reduced vasodilation in response to a variety of stimuli[121,122], and which is already present at diagnosis[122]. This does not appear to be due to occult large vessel disease (Chapter 4.1) and may improve after a prolonged period of good glycaemic control in recently diagnosed NIDDM patients (Chapter 4.2). The presence of hypertension in addition to NIDDM does not lead to a further reduction in hyperaemic responses, but does result in increased resistance to blood flow (Chapter 4.3).

A similar reduction in hyperaemic responses has been found in subjects at risk of developing NIDDM who have IGT (Chapter 5.1), and also in insulin resistant acromegalic patients (Chapter 5.2). Limited vasodilation does not seem to relate to hyperglycaemia or hyperlipidaemia, but is associated with hyperinsulinaemia and insulin resistance (Chapters 4.1, 5.2, and 5.3). The underlying mechanism of reduced microvascular hyperaemia appears to involve a defect in

endothelium-dependent vasodilation (Chapter 6), in the context of normal skin capillary density (Chapter 7).

8.2 Critical appraisal of the studies

The work of this thesis is based on the skin microcirculation which is ideal for non-invasive in vivo studies. This provides a useful model of what may be happening in other vascular beds, although specialisation of function in individual tissues means that results cannot be directly extrapolated. Studies of the skin microcirculation are of some direct clinical relevance in that foot ulceration, which is the commonest cause of hospital admission in patients with diabetes, ultimately represents local skin microvascular failure, albeit usually secondary to a combination of neuropathy and large vessel disease plus superadded infection. Limitation of protective microvascular hyperaemic responses will affect the inflammatory response to infection and tissue damage, and will also contribute to slow ulcer healing[211].

Most of the techniques used in this thesis provide indirect measures of microvascular function and although they have been thoroughly validated against direct techniques, for some there remains a degree of debate about the interpretation of what is actually being measured. For instance laser Doppler fluximetry is thought to measure total multidirectional red cell flux within a volume of tissue, which is obviously different to unidirectional flow, although in the absence of differences in capillary density between control subjects and those with NIDDM and IGT (Chapter 7), this is unlikely to be of any practical importance. Similarly, assessment of fluid permeability using plethysmography may be critically dependent on forearm tissue

composition and care was therefore taken to ensure that no such differences existed between NIDDM patients and control subjects (Chapter 3). Great care was taken to ensure the intra-individual reproducibility over a period of time of all the techniques used (Chapter 2).

Study design was cross-sectional and results will therefore require confirmation in a prospective study. In view of the subject groups studied and the nature of the research, sample sizes were necessarily small. In Chapters 3 and 7, where no differences were found between patients and control subjects, preceding power calculations ensured that a clinically relevant difference was not missed due to small sample size.

Future studies are likely to be enhanced by the emergence of new and more sophisticated techniques for assessing microvascular function. In the future it is likely that simultaneous assessment of flow, pressure and permeability in single capillaries in vivo will become possible, along with technology allowing in vivo assessment of tissue oncotic pressure. With regard to the techniques used in this thesis, more refined methods for the assessment of iontophoretic responses such as a wide area probe, or a laser Doppler scanner are already available, and preliminary work with the latter holds great promise[228].

8.3 A Unifying hypothesis to explain the pathophysiology and epidemiology of microvascular disease in NIDDM

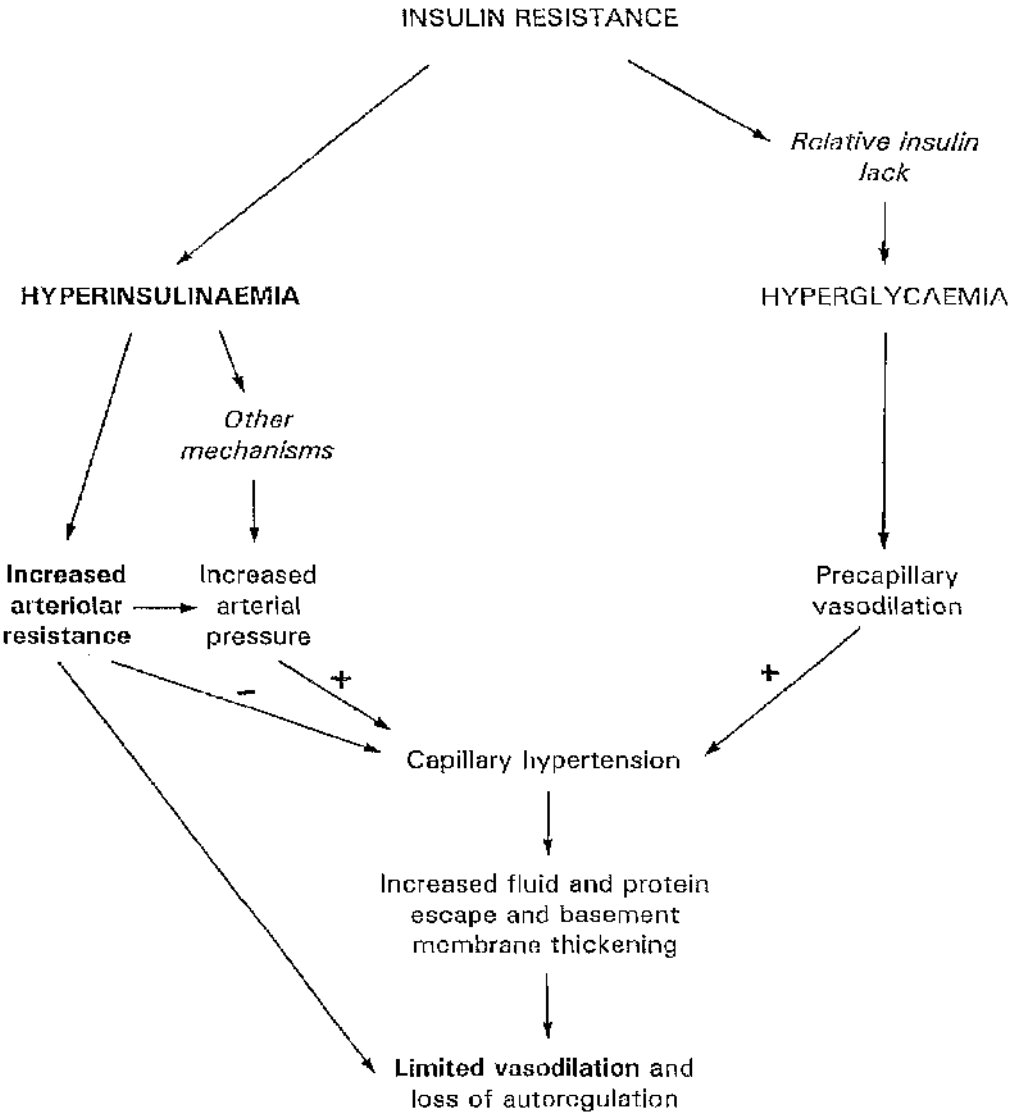
In IDDM poor glycaemic control results in precapillary vasodilation with increased resting microvascular blood flow and pressure. In NIDDM increased microvascular pressure is attenuated whilst at the

same time maximal vasodilatory capacity is severely limited at diagnosis of the disease. This spectrum of microcirculatory abnormalities is consistent with an early rise in arteriolar resistance in NIDDM that is already present at diagnosis and which limits increases in capillary pressure and fluid filtration when hyperglycaemia induces dilation of more distal, immediately pre-capillary, resistance elements (Fig 8.1). At an early stage, in subjects with IGT, hyperinsulinaemia may be the predominant metabolic factor leading to impaired vasodilation due to its effects on microvascular cell proliferation; with the emergence of NIDDM, hyperglycaemia may assume increasing importance leading to further microvascular cellular dysfunction. This sequence of events is in contrast to the situation in IDDM, where hyperglycaemia is the major early metabolic abnormality, although it is noteworthy that after several years of diabetes, insulin resistance emerges in those patients prone to develop nephropathy[229] and a consequent increase in vascular resistance may help to explain why there is no further increase in capillary pressure in IDDM patients with advanced nephropathy[230] compared to those with microalbuminuria[78] despite there being more marked hypertension in the former group.

In NIDDM a rise in arteriolar resistance that antedates the diagnosis of the disease may partly explain the high prevalence of hypertension in this form of diabetes, as well as the modified expression of diabetic microangiopathy which is apparent from epidemiological studies. It is quite plausible that the modifying influence of an early rise in arteriolar resistance may vary in different vascular beds, with the most marked impact being observed in skin and muscle, but less effect in the kidney. This would not be surprising given that the glomerulus is a specialised structure with both an afferent and an efferent arteriole. Although capillary pressure at rest appears to be normal in NIDDM,

Fig 8.1 ***Postulated role of hyperinsulinaemia and hyperglycaemia in the pathogenesis of abnormal microvascular function in NIDDM***

The pathway outlined in bold is the dominant mechanism in the pre-diabetic state, while hyperglycaemia may assume increasing importance with the development of frank diabetes.



such a finding does not negate the haemodynamic hypothesis in this type of diabetes. Despite the presence of structural remodelling at an arteriolar level in response to elevated arterial pressure[169], essential hypertension (in the absence of diabetes) is associated with capillary hypertension[168], i.e. the protection of the capillary bed from raised arterial pressure is incomplete. Arterial hypertension is common in NIDDM and assuming that autoregulation is similarly incomplete in this condition capillary hypertension may prevail in the hypertensive subpopulation who are at highest risk of microangiopathy[231].

Several future lines of research are suggested on the basis of the results of this thesis and the above hypothesis. To further explore the influence of hyperinsulinaemia on microvascular function, studies of insulin resistant subjects with normal glucose tolerance are required In addition, studies of other hyperinsulinaemic groups such as females with polycystic ovarian disease would be interesting. As previously discussed, there is a need for cell culture experiments to look at the interactions of hyperinsulinaemia and endothelial dysfunction in vitro.

The abnormalities observed in the skin microcirculation require confirmation in other microvascular beds in subjects with IGT, and it would be possible to look for abnormalities in retinal blood flow and retinal function (e.g. colour vision); neurophysiology and nerve blood flow; and renal haemodynamics and glomerular charge and size selectivity using available techniques.

The role of raised capillary pressure in hypertensive NIDDM patients deserves further consideration. Finally, it should be possible to investigate the association of reduced hyperaemia with basement membrane or other structural changes in skin using morphometric studies after skin biopsy.

In summary, the pattern of disordered microvascular physiology in NIDDM differs from that found in IDDM, with the predominant abnormality being an early reduction in vasodilation. It is hypothesised that this may relate to the effects of hyperinsulinaemia in the pre-diabetic state and could partly explain the high prevalence of hypertension in NIDDM, and also the modified expression of diabetic microangiopathy which is apparent from epidemiological studies. With the onset of NIDDM, hyperglycaemia may lead to further functional and structural changes in the microcirculation compounded by the effects of arterial hypertension and large vessel disease which are common in this form of diabetes.

REFERENCES

1. Williamson JR, Kilo C. Current status of capillary basement-membrane disease in diabetes mellitus. *Diabetes* 1977; **26**: 65-73.
2. Ajjam ZS, Barton S, Corbett M, Owens D, Marks R. Quantitative evaluation of the dermal vasculature of diabetics. *Q J Med* 1985; **54**: 229-239.
3. Sueki H. Diabetic microangiopathy in subcutaneous fatty tissue. *J Cutan Pathol* 1987; **14**: 217-222.
4. Vracko R. Skeletal muscle capillaries in diabetics: a quantitative analysis. *Circulation* 1970; **41**: 271-283.
5. Ledet T, Neubauer B, Christiansen NJ, Lundbaeck K. Diabetic cardiopathy. *Diabetologia* 1979; **16**: 207-209.
6. Malik RA, Newrick PG, Sharma AK et al. Microangiopathy in human diabetic neuropathy : relationship between capillary abnormalities and the severity of neuropathy. *Diabetologia* 1989; **32**: 92-102.
7. Jaap AJ, Tooke JE. Is microvascular disease important in the diabetic foot? In: Boulton AJM, Connor H, Cavanagh PR eds. *The Foot in Diabetes* 2nd Edn. John Wiley and Sons, Chichester, UK, 1994. 49-56.
8. Ghafour IM, Allan D, Foulds WS. Common causes of blindness and visual handicap in the West of Scotland. *Br J Ophthalmol* 1983; **67**: 209-213.
9. Rettig B, Teutsch SM. The incidence of end-stage renal disease in Type I and Type 2 diabetes mellitus. *Diabetic Nephropathy* 1984; **3**: 26-27.
10. Panzram G. Mortality and survival in Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1987; **30**: 123-131.
11. Greenfield ADM. The circulation through the skin. In: Hamilton WF ed. *Handbook of Physiology - Circulation II.* American Physiological Society, Washington DC, USA, 1963: 1325-1351.

12. Mescon H, Hurley J, Moretti G. The anatomy and histochemistry of the arteriovenous anastamosis in human digital skin. *J Invest Dermatol* 1956; **27**: 133-144.

13. Grant RT, Bland EF. Observations on arteriovenous anastamoses in human skin and in the bird's foot with special reference to reaction to cold. *Heart* 1931; **15**: 385-411.

14. Hales JRS. Skin arteriovenous anastamoses, their control and role in thermoregulation. In: Johansen K, Burggren WW eds. *Cardiovascular Shunts*. Munksgaard, Copenhagen, Denmark, 1985. 433-451.

15. Vascular structure. In: Williams PL, Warwick R, eds. *Gray's Anatomy*, 36th edn. Churchill Livingstone, Edinburgh, London, Melbourne, New York, 1980. 622-630.

16. Levick JR. Solute transport between blood and tissues. In: *An Introduction to Cardiovascular Physiology*. Butterworth's, London, 1991. 117-141.

17. Martinez-Hernandez A, Amenta PS. The basement membrane in pathology. *Lab Invest* 1983; **48**: 656-677.

18. Levick JR. Circulation of fluid between blood, interstitium and lymphatics. In: *An Introduction to Cardiovascular Physiology*. Butterworth's, London, 1991. 142-170.

19. Renkin EM. Relation of capillary morphology to transport of fluid and large molecules: a review. *Acta Physiol Scand* 1979; **463**: 81-91.

20. Curry FE, Michel CC. A fiber matrix model of capillary permeability. *Microvascular Res* 1980; **20**: 96-99.

21. Deckert T, Feldt-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Enevoldsen A. Albuminuria reflects widespread vascular damage. The Steno Hypothesis. *Diabetologia* 1989; **32**: 219-226.

22. Intaglietta M. Vasomation and flowmotion : physiological mechanisms and clinical evidence. *Vasc Med Rev* 1990; **1**: 101-112.

23. Johansson B. Myogenic tone and reactivity : definitions based on muscle physiology. *J Hypertens* **1989; 7** (Suppl 4): 55-58.

24. Harder DR. Pressure-induced myogenic activation of cat cerebral arteries is dependent on intact endothelium. *Circ Res* 1987; **60**: 102-107.

25. Griffith TM, Edwards DH, Davies RLI, Harrison **TJ**, Evans KT. EDRF co-ordinates the behaviour of vascular resistance vessels. *Nature* 1987; **329**: 442-445.

26. Kilbom A, Wennmalm A. Endogenous prostaglandins as regulators of blood flow in man: effect of indomethacin on reactive and functional hyperaemia. *J Physiol* 1976; **247**: 109-121.

27. Yanagisawa M, Kurihara H, Kimura S et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411-415.

28. Porta M, La Selva M, Molinatti P, Molinatti GM. Endothelial cell function in diabetic microangiopathy. *Diabetologia* 1987; **30**: 601-609.

29. Kruithof EKO. Plasminogen activator inhibitors - a review. *Enzyme* 1988; **40**: 113-121.

30. Vanhoutte PM. Serotonin, adrenergic nerves, endothelial cells and vascular smooth muscle. *Prog Appl Microcirc* 1986; **10**: 1-11.

31. Orlidge A, D'Amore PA. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *J Cell Biol* 1987; **105**: 1455-1462.

32. Kuwabara T, Cogan DG. Retinal vascular patterns. VI. Mural cells of the retinal capillaries. *Arch Ophthalmol* 1963; **69**: 492-502.

33. Takahashi K, Brooks R, Kanse S et al. Production of endothelin 1 by cultured bovine retinal endothelial cells and presence of endothelin receptors on associated pericytes. *Diabetes* 1989; **38**: 1200-1202.

34. Gall MA-, Rossing P, Skøtt P et al. Prevalence of micro- and macro-albuminuria, arterial hypertension, retinopathy and large vessel disease in European Type 2 diabetic patients. *Diabetologia* 1991; **34**: 655-661.

35. UK Prospective Diabetes Study 6. Complications in newly diagnosed Type 2 diabetic patients and their associations with different clinical and biochemical risk factors. *Diabetes Res* 1990; **13**: 1-11.

36. United Kingdom Prospective Diabetic Study III. Prevalence of hypertension and hypotensive therapy in patients with newly diagnosed diabetes. *Hypertension* 1985; **7** (Suppl 2): 8-13.

37. Reaven GM. Insulin resistance in human disease. *Diabetes* 1988; **37**: 1595-1607.

38. Cahill GF Jr. Beta-cell deficiency, insulin resistance, or both? *N Engl J Med* 1988; **318**: 1268-1270.

39. Jarrett RJ, Keen H, Fuller JH, McCartney M. Worsening to diabetes in men with impaired glucose tolerance ('borderline diabetes'). *Diabetologia* 1979; **16**: 25-30.

40. Fuller JH, Shipley MJ, Rose G, Jarret RS, Keen H. Coronary-Heart-Disease-Risk and impaired glucose tolerance : The Whitehall Study. *Lancet* 1980; **i**: 1373-1376.

41. Jarrett RJ, McCartney P, Keen H. The Bedford Study : Ten year mortality rates in newly diagnosed diabetics, borderline diabetics, and normoglycaemic controls and risk indices for coronary heart disease in borderline diabetics. *Diabetologia* 1982; **22**: 79-84.

42. Strandness D Jr, Priest RF, Gibbons GE. Combined clinical and pathological study of diabetic and non-diabetic peripheral arterial disease. *Diabetes* 1964; **13**: 366-372.

43. Stout RW. Insulin as a mitogenic factor: Role in the pathogenesis of cardiovascular disease. *Am J Med* 1991; **90** (Suppl 2A): 625-655.

44. Barker DJP, Bull AR, Osmond C, Simmonds SJ. Fetal and placental size and risk of hypertension in adult life. *Br Med J* 1990; **301**: 259-262.

45. Barker DJP, Winter PD, Osmond C, Margetts B, Simmonds SJ. Weight in infancy and death from ischaemic heart disease. *Lancet* 1989; **i**: 577-580.

46. Barker DJP, Hales CN, Fall CHD, Osmond C, Phipps K, Clark PMS. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (Syndrome X) : relation to reduced fetal growth. *Diabetologia* 1993; **36**: 62-67.

47. Klein R, Klein BEK, Moss SE, Davies MD, DeMets DL. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. II. Prevalence and risk of diabetic retinopathy when age of diagnosis is less than 30 years. *Arch Ophthalmol* 1984; **102**: 520-526.

48. Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T. Diabetic nephropathy in Type 1 (insulin-dependent) diabetes: An epidemiological study. *Diabetologia* 1983; **25**: 496-501.

49. Melton LJ, Dyck PJ. Epidemiology. In: Dyck PJ, Thomas PK, Asbury AK, Winegrad AI, Porte Jr D, eds. *Diabetic Neuropathy*. WB Saunders, Philadelphia, 1987. 27-35.

50. Ziegler D, Mühlen H, Gries FA. Neurophysiological tests in Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetic patients with subclinical and symptomatic neuropathy. *Diabetologia* 1992; **35**: 1099-1100.

51. Hendriksen PH, Ooy PL, Wieneke GH, Bravenboer B, Banga JD. Subclinical diabetic neuropathy : similarities between electrophysiological results of patients with Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1992; **35**: 690-695.

52. Sima AAF, Nathaniel V, Bril V, McEwen TAGJ, Greene DA. Histopathological heterogeneity of neuropathy in insulin-dependent and non-insulin-dependent diabetes and demonstration of axo-glial dysjunction in human diabetic neuropathy. *J Clin Invest* 1988; **81**: 349-364.

53. Lehtinen JM, Uusitupa M, Siitonen O, Pyörälä K. Prevalence of neuropathy in newly diagnosed NIDDM and non-diabetic control subjects. *Diabetes* 1989; **38**: 1307-1313.

54. Lehtinen JM, Niskanen H, Hyvönen K, Siitonen O, Uusitupa M. Nerve function and its determinants in patients with newly diagnosed Type 2 (non-insulin-dependent) diabetes mellitus and control subjects. *Diabetologia* 1993; **36**: 68-72.

55. Ziegler D, Gries FA, Spühler M, Lessmann F. Diabetic Cardiovascular Autonomic Neuropathy Multicenter Study Group. The epidemiology of diabetic neuropathy. *J Diab Comp* 1992; **6**: 25-33.

56. Schmitz A, Vaeth M. Microalbuminuria : a major risk factor in non-insulin-dependent diabetes. A 10-year follow-up study of 503 patients. *Diabetic Med* 1988; **5**: 126-134.

57. Tung P, Levin SR. Nephropathy in non-insulin-dependent diabetes mellitus. *Am J Med* 1988; **85** (Suppl 5A): 131-136.

58. Fabré J, Balant LP, Dayer PG, Fox HM, Vernet AT. The kidney in maturity onset diabetes: a clinical study of 570 patients. *Kidney Int* 1982; **21**: 730-738.

59. Friedman R, Gross JL. Evolution of glomerular filtration rate in proteinuric NIDDM patients. *Diabetes Care* 1991; **14**: 355-359.

60. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet* 1982; **i**: 1430-1432.

61. Jarrett RJ, Viberti GC, Argyropoulos A, Hill RD, Mahmud U, Murrells TJ. Microalbuminuria predicts mortality in non-insulin-dependent diabetes. *Diabetic Med* 1984; **1**: 17-19.

62. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. *N Engl J Med* 1984; **310**: 356-360.

63. Knowler WC, Pettitt DJ. Six-year incidence of microvascular complications confirms the need to reassess diagnostic criteria for diabetes. *Exerpta Med Int Congr Ser* 1979; **481**: 122-123.

64. Klein R, Barrett-Connor EL, Blunt BA, Wingard DL. Visual impairment and retinopathy in people with normal glucose tolerance, impaired glucose tolerance and newly diagnosed NIDDM. *Diabetes Care* 1991; **14**: 914-918.

65. Nelson RG, Kunzelman CL, Pettitt DJ, Saad MF, Bennett PH, Knowler WC. Albuminuria in Type 2 (non-insulin-dependent) diabetes mellitus and impaired glucose tolerance in Pima Indians. *Diabetologia* 1989; **32**: 870-876.

66. Kuroda N, Taniguchi H, Baba S, Yamamoto M. The pupillary light reflex in borderline diabetics. *J Int Med Res* 1989; **17**: 205-211.

67. Parving H-H, Viberti GC, Keen H, Christiansen JS, Lassen NA. Haemodynamic factors in the genesis of diabetic microangiopathy. *Metabolism* 1983; **32**: 943-949.

68. Tooke JE. Microvascular haemodynamics in diabetes mellitus. *Clin Sci* 1986; **70**: 119-125.

69. Zatz R, Brenner BM. Pathogenesis of diabetic microangiopathy: the haemodynamic view. *Am J Med* 1986; **80**: 443-453.

70. Gundersen HJG. Peripheral blood flow and metabolic control in juvenile diabetes. *Diabetologia* 1974; **10**: 225-231.

71. Popoff NW. The digital vascular system. *Arch Pathol* 1934; **18**: 295-330.

72. Partsch H. Neuropathies of the ulceromutilating types. Clinical aspects, classification, and circulatory measurements. *Vasa* 1977; Suppl **6**: 1-48.

73. Boulton AJM, Scarpello JHB, Ward JD. Venous oxygenation in the diabetic neuropathic foot : evidence of arteriovenous shunting? *Diabetologia* 1982; **22**: 6-8.

74. Corbin DOC, Young RJ, Morrison DC et al. Blood flow in the foot, polyneuropathy and foot ulceration in diabetes mellitus. *Diabetologia* 1987; **30**: 468-473.

75. Flynn MD, Edmonds ME, Tooke JE, Watkins PJ. Direct measurement of capillary blood flow in the diabetic neuropathic foot. *Diabetologia* 1988; **31**: 652-666.

76. Tymms JA, Tooke JE. The effect of continuous subcutaneous insulin infusion (CSII) on microvascular blood flow in diabetes mellitus. *Int J Microcirc Clin Exp* 1988; **7**: 347-356.

77. Sandeman DD, Shore AC, Tooke JE. Relation of skin capillary pressure in patients with insulin-dependent diabetes mellitus to complications and metabolic control. *N Engl J Med* 1992; **327**: 760-764.

78. Shore AC, Jaap AJ, Tooke JE. Capillary pressure in insulin dependent diabetic patients of long disease duration with and without microangiopathy. *Diabetic Med* 1992; **9** (Suppl 2): S11. (Abstract).

79. Jaap AJ, Shore AC, Gartside IB, Gamble J, Tooke JE. Increased microvascular fluid permeability in young Type 1 (insulin-dependent) diabetic patients. *Diabetologia* 1993; **36**: 648-652.

80. Parving H-H, Noer I, Deckert T et al. The effect of metabolic regulation on microvascular permeability to small and large molecules in short-term juvenile diabetics. *Diabetologia* 1976; **12**: 161-166.

81. Leinonen H, Matikainen E, Juntunen J. Permeability and morphology of skeletal muscle capillaries in Type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1982; **22**: 158-162.

82. Alpert JS, Coffman JD, Balodimos MC, Koncz L, Soeldner JS. Capillary permeability and blood flow in skeletal muscle of patients with diabetes mellitus and genetic prediabetes. *N Engl J Med* 1972; **286**: 454-460.

83. Parving H-H, Munkgaard Rasmussen S. Transcapillary escape rate of albumin and plasma volume in short- and long-term juvenile diabetics. *Scand J Clin Lab Invest* 1973; **32**: 81-87.

84. Parving H-H, Rossing N. Simultaneous determination of transcapillary escape rate of albumin and IgG in normal and long-term juvenile diabetic subjects. *Scand J Clin Lab Invest* 1973; **32**: 239-244.

85. Rayman G, Hassan A, Tooke JE. Blood flow in the skin of the foot related to posture in diabetes mellitus. *Br Med J* 1986; **292**: 87-90.

86. Hassan AAK, Tooke JE. Mechanism of the postural vasoconstrictor response in the human foot. *Clin Sci* 1988; **75**: 379-387.

87. Shore AC, Price KJ, Sandeman DD, Tripp JH, Tooke JE. Abnormalities of the postural vasoconstriction response in diabetic children and adolescents. *Diabetic Med* 1990; **7** (Suppl 2): 34A. (Abstract).

88. Rayman G, Williams SA, Spencer PD, Smaje LH, Wise PH, Tooke JE. Impaired microvascular hyperaemic response to minor skin trauma in Type 1 diabetes. *Br Med J* 1986; **292**: 1295-1298.

89. Walmsley D, Wales JK, Wiles PG. Reduced hyperaemia following skin trauma : evidence for an impaired microvascular response to injury in the diabetic foot. *Diabetologia* 1989; 32: 736-739.

90. Tooke JE, Östergren J, Lins P-E, Fagrell B. Skin microvascular blood flow control in long duration diabetics with and without complications. *Diabetes Res* 1987; **5**: 189-192.

91. Walmsley D, Wiles PG. Myogenic microvascular responses are impaired in long-duration Type 1 diabetes. *Diabetic Med* 1990; **7**: 222-227.

92. Shore AC, Price KJ, Sandeman DD, Green EM, Tripp JH, Tooke JE. Impaired microvascular hyperaemic response in children with diabetes mellitus. *Diabetic Med* 1991; **8**: 619-623.

93. Lembeck F. Sir Thomas Lewis' nocifensor system, histamine and substance P-containing primary afferent nerves. *Trends Neurosci* 1983; 6: 106-108.

94. Parkhouse N, Le Quesne PM. Impaired neurogenic vascular response in patients with diabetes and neuropathic foot lesions. *N Engl J Med* 1988; **318**: 1306-1309.

95. Walmsley D, Wiles PG. Early loss of neurogenic inflammation in the human diabetic foot. *Clin Sci* **1991**; **80**: 605-610.

96. Tooke JE, Lins P-E, Östergren J, Fagrell B. Skin microvascular autoregulatory responses in Type 1 diabetes: the influence of duration and control. *Int J Microcirc Clin Exp* 1985; 4: 249-256.

97. Kastrup J, Norgaard T, Parving H-H, Lassen NA. Decreased distensibility of resistance vessels of the skin in Type 1 (insulin-dependent) diabetic patients with microangiopathy. *Clin Sci* 1987; **72**: 123-130.

98. Christiansen JS, Gammelgaard J, Frandsen M, Parving H-H. Increased kidney size, glomerular filtration rate, and renal plasma flow in short-term insulin-dependent diabetics. *Diabetologia* 1981; **20**: 451-456.

99. Zatz R, Dunn R, Meyer TW, Anderson S, Rennke HG, Brenner BM. Prevention of diabetic glomerulopathy by pharmacological amelioration of capillary hypertension. *J Clin Invest* 1986; **77**: 1925-1930.

100. Parving H-H, Kastrup H, Smidt UM, Andersen AR, Feldt-Rasmussen BF, Sandahl Christiansen J. Impaired autoregulation of glomerular filtration rate in Type 1 (insulin-dependent) diabetic patients with nephropathy. *Diabetologia* 1984; **27**: 247-252.

101. Kohner EM, Hamilton AM, Saunders SJ, Sutcliffe BA, Bulpitt CJ. The retinal blood flow in diabetes. *Diabetologia* 1975; **11**: 27-33.

102. Chahal P, Falon TJ, Jennings SJ, Chowienczyk PJ, Kohner EM. Vitreous fluorophotometry in patients with no or minimal diabetic retinopathy. *Diabetes Care* 1986; **9**: 134-139.

103. Patel V, Rassam S, Newsom S, Wiek J, Kohner E. Retinal blood flow in diabetic nephropathy. *Br Med J* 1992; **305**: 678-683.

104. Grunwald JE, Riva CE, Sinclair SH, Brucker AV. Altered retinal vascular response to 100% O_2 breathing in diabetes mellitus. *Ophthalmol* 1984; **91**: 1447-1452.

105. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerve of rats with experimental diabetic neuropathy. *Brain* 1984; **107**: 935-950.

106. Low PA, Tuck RR, Dyck PJ, Schmelzer JD, Yao JK. Prevention of some electrophysiologic and biochemical abnormalities with oxygen supplementation in experimental diabetic neuropathy. *Proc Natl Acad Sci USA* 1984; **81**: 6894-6898.

107. Tesfaye S, Harris N, Jakubowski J, Mody C, Ward JD. Impaired blood flow and A-V shunting in human diabetic sural nerve. *Diabetic Med* 1990; **7** (Suppl 2): 29A (Abstract).

108. Newrick PG, Wilson AJ, Jakubowski J, Boulton AJM, Ward JD. Sural nerve oxygen tension in diabetes. *Br Med J* 1986; **293**: 1053-1054.

109. Dyck PJ, Karnes JL, O'Brien P, Okazaki H, Lais A, Engelstad J. The spatial distribution of fiber loss in diabetic polyneuropathy suggests ischaemia. *Ann Neurol* 1986; **19**: 440-449.

110. Korthals JK, Gieron MA, Dyck PJ. Intima of epineurial arterioles is increased in diabetic polyneuropathy. *Neurology* 1988; **38**: 1582-1586.

111. Dyck PJ, Hansen S, Karnes J et al. Capillary number and percentage closed in human diabetic sural nerve. *Proc Natl Acad Sci (USA)* 1985; **52**: 2513-2517.

112. Britland ST, Young RJ, Sharma AK, Clarke BF. Relationship of endoneurial capillary abnormalities to type and severity of diabetic polyneuropathy. *Diabetes* 1990; **39**: 909-913.

113. Tooke JE. Capillary pressure in non-insulin-dependent diabetes. *Inter Angiol* 1983; **2**: 167-171.

114. Flynn MD, Boolell M, Tooke JE, Watkins PJ. The effect of insulin infusion on capillary blood flow in the diabetic neuropathic foot. *Diabetic Med* 1992; **9**: 630-634.

115. Shore AC, Jaap AJ, Tooke JE. Capillary pressure in patients with NIDDM. *Diabetes* 1994; **43**: 1198-1202.

117. Katz MA, McCuskey P, Beggs JL, Johnson PC, Gaines JA. Relationship between microvascular function and capillary structure in diabetic and non-diabetic human skin. *Diabetes* 1989; **38**: 1245-1250.

118. Valensi P, Attali JR, Behar A, Sebaoun J. Isotopic test of capillary permeability to albumin in diabetic patients: effects of hypertension, microangiopathy, and duration of diabetes. *Metabolism* 1987; **36**: 834-839.

119. Valensi P, Attali JR, Behar A, Attalah M. Increased capillary permeability to albumin and diabetic neuropathy. *Diabetes Res Clin Prac* 1991; **11**: 41-46.

120. O'Hare JA, Ferriss JB, Twomey B, O'Sullivan DJ. Poor metabolic control, hypertension and microangiopathy independently increase the transcapillary escape rate of albumin in diabetes. *Diabetologia* 1983; **25**: 260-263.

121. Tur E, Yosipovitch G, Bar-On Y. Skin reactive hyperaemia in diabetic patients. A study by laser Doppler flowmetry. *Diabetes Care* 1991; **14**: 958-962.

122. Sandeman DD, Pym C, Green EM, Seamark C, Shore AC, Tooke JE. Microvascular vasodilation in feet of newly diagnosed non-insulin dependent diabetic patients. *Br Med J* 1991; **302**: 1122-1123.

123. Katz MA, McNeill G. Defective vasodilation response to exercise in cutaneous procapillary vessels in diabetic humans. *Diabetes* 1987; **36**: 1386-1396.

124. Menon RK, Grace AA, Burgoyne W, Fonseca VA, James IM, Dandona P. Muscle blood flow in diabetes mellitus. Evidence of abnormality after exercise. *Diabetes Care* 1992; **15**: 693-695.

125. Laakso M, Edelman SV, Brechtel G, Baron AD. Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes* 1992; **41**: 1076-1083.

126. McVeigh GE, Brennan GM, Johnston GD et al. Impaired endothelium-dependent and independent vasodilation in patients with Type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 1992; **35**: 771-776.

127. Ishida K, Ishibashi F, Takashina S. Comparison of renal haemodynamics in early non-insulin-dependent and insulin-dependent diabetes mellitus. *J Diab Comp* 1991; **5**: 143-145.

128. Vora JP, Dolben J, Dean JD et al. Renal haemodynamics in newly presenting non-insulin dependent diabetes mellitus. *Kidney Int* 1992; **41**: 829-835.

129. Grunwald JE, Riva CE, Martin DB, Quint AR, Epstein PA. Effect of an insulin-induced decrease in blood glucose on the human diabetic retinal circulation. *Ophthalmol* 1987; **94**: 1614-1620.

130. Tesfaye S, Harris N, Jakubowski J, Mody C, Ward JD. Impaired nerve blood flow and arterio-venous shunting in human diabetic sural nerve. *Diabetic Med* 1990; **7** (Suppl 2): 29A. (Abstract).

131. Yoshida A, Kojima M, Ogasawara H, Ishiko S. Oscillatory potentials and permeability of the blood-retinal barrier in non-insulin-dependent diabetes mellitus without retinopathy. *Ophthalmol* 1991; **98**: 1266-1271.

132. Myers BD, Nelson RG, Williams GW et al. Glomerular function in Pima Indians with non-insulin-dependent diabetes of recent onset. *J Clin Invest* 1991; **88**: 524-530.

133. Schmitz A, Christensen T, Taagehoej Jensen F. Glomerular filtration rate and kidney volume in normoalbuminuric non-insulin-dependent diabetics. Lack of glomerular hyperfiltration and renal hypertrophy in uncomplicated NIDDM. *Scand J Clin Lab Invest* 1989; **49**: 103-108.

134. Nelson RG, Loon N, Bennett PH, Berg RL, Mitch WE, Myers BD. Glomerular filtration and barrier function in non-insulin-dependent diabetes mellitus. *Diabetes* 1990; **39**: 287A. (Abstract).

135. Ishibashi F, Ishida K, Takashina S. The aetiology of microalbuminuria in early non-insulin-dependent diabetes mellitus. *J Diab Comp* 1991; **5**: 107-109.

136. Yamazaki M, Tani N, Igarashi K et al. Changes in the glomerular pore size selectivity in patients with Type 2 diabetes mellitus. *J Diab Comp* 1991; **5**: 138-139.

137. Ikenaga H, Suzuki H, Ishii N, Itoh H, Saruta T. Enzymuria in non-insulin-dependent diabetic patients: signs of tubular cell dysfunction. *Clin Sci* 1993; **84**: 469-475.

138. Chiba Y, Tani N, Yamazaki M, Nakamura H, Ito S, Shibata A. Glomerular charge selectivity in non-insulin-dependent diabetes mellitus. *J Diab Comp* 1991; **5**: 135-137.

139. Lorenzi M, Cagliero E, Toledo S. Glucose toxicicty for human endothelial cells in culture. Delayed replication, disturbed cell cycle, and accelerated death. *Diabetes* 1985; **34**: 621-627.

140. Lee T-S, Saltsman A, Ohashi H, King GL. Activation of protein kinase C by elevation of glucose concentration: proposal for a mechanism in the development of diabetic vascular complications. *Proc Natl Acad Sci USA* 1989; **86**: 5141-5145.

141. Greene DA, Lattimer SA, Sima AAF. Sorbitol, phosphoinositides and sodium-potassium ATPase in the pathogenesis of diabetic complications. *N Engl J Med* 1987; **316**: 599-606.

142. Brownlee M, Vlassara H, Cerami A. Non-enzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Int Med* 1984; **101**: 527-537.

143. Pfeifle B, Ditschuneit H. Effect of insulin on growth of cultured human arterial smooth muscle cells. *Diabetologia* 1981; **20**: 155-158.

144. King GL, Goodman AD, Buzney S, Moses A, Kahn CR et al. Receptors and growth-promoting effects of insulin and insulinlike growth factors on cells

from bovine retinal capillaries and aorta. *J Clin Invest* 1985; **75**: 1028-1036.

145. Taggart H, Stout RW. Control of DNA synthesis in cultured vascular endothelial and smooth muscle cells. *Atherosclerosis* 1980; **37**: 549-557.

146. Ledbetter SR, Wagner CW, Martin GR, Rohrbach DH, Hassel JR. Response of diabetic basement-membrane producing cells to glucose and insulin. *Diabetes* 1987; **36**: 1029-1034.

147. Ginsberg HN. Lipoprotein physiology in nondiabetic and diabetic states : relationship to atherogenesis. *Diabetes Care* 1991; **14**: 839-855.

148. Bucala R, Tracey KJ, Cerami A. Advanced glycation products quench nitric oxide and mediate defective endothelium-dependent vasodilation in experimental diabetes. *J Clin Invest* 1991; **87**: 432-438.

149. Christlieb AR, Janka H-V, Kraus B et al. Vascular reactivity to angiotensin II and norepinephrine in diabetic subjects. *Diabetes* 1976; **25**: 268-274.

150. Viberti GC, Benigni A, Bognetti E, Remuzzi G, Wiseman MJ. Glomerular hyperfiltration and urinary prostaglandins in Type 1 diabetes mellitus. *Diabetic Med* 1989; **6**: 219-222.

151. Baynes JW. Role of oxidative stress in development of complications in diabetes. *Diabetes* 1991; **40**: 405-412.

152. Williamson JR, Chang K, Frangos M et al. Hyperglycaemic pseudohypoxia and diabetic complications. *Diabetes* 1993; **42**: 801-813.

153. Collier A, Rumley A, Rumley AG et al. Free radical activity and haemostatic factors in NIDDM patients with and without microalbuminuria. *Diabetes* 1992; **41**: 909-913.

154. Ford I, Singh TP, Kitchen S, Makris M, Ward JD, Preston FE. Activation of coagulation in diabetes mellitus in relation to the presence of vascular complications. Diabetic Med 1991; 8: 322-329.

155. Walmsley D, Hampton KK, Grant PJ. Contrasting fibrinolytic responses in Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes. *Diabetic Med* 1991; **8**: 954-959.

156. Ernst E, Matrai A. Alterations in red and white blood cell rheology in Type 2 diabetes. *Diabetes* 1986; **35**; 1412-1415.

157. Frier BM, Hilsted J. Does hypoglycaemia aggravate the complications of diabetes? *Lancet* 1985; **ii**: 1175-1176.

158. Viberti GC, Keen H, Wiseman M. Raised arterial pressure in parents of proteinuric insulin-dependent diabetic patients. *Br Med J* 1987; **295**: 515-517.

159. Mangili R, Bending JJ, Scott GS, Li LK, Gupta A, Viberti GC. Increased sodium-lithium countertransport activity in red cells of patients with insulin-dependent diabetes and nephropathy. *N Engl J Med* 1988; **318**: 146-149.

160. Ubbink DT, Jacobs MJ, Tangelder GJ, Slaaf DW, Reneman RS. Posturally induced microvascular constriction in patients with different stages of leg ischaemia: effect of local skin heating. *Clin Sci* 1991; **81**: 43-49.

161. Bongard O, Fagrell B. Discrepancies between total and nutritional skin microcirculation in patients with peripheral arterial occlusive disease. *Vasa* 1990; **19**: 105-111.

162. Junger M, Frey-Schnewlin G, Bollinger A. Microvascular flow distribution and transcapillary diffusion at the forefoot in patients with peripheral ischaemia. *Int J Microcirc Clin Exp* 1989; **8**: 3-24.

163. McEwan AJ, Ledingham IM. Blood flow characteristics and tissue nutrition in apparently ischaemic feet. *Br Med J* 1971; **III**: 220-224.

164. Seifert H, Jager K, Bollinger A. Analysis of flow motion by the laser Doppler technique in patients with peripheral arterial occlusive disease. *Int J Microcirc Clin Exp* 1988; **7**: 223-236.

165. Ubbink DT, Jacobs MJ, Slaaf DW, Tangelder GJ, Reneman RS. Microvascular reactivity differences between the two legs of patients with unilateral lower limb ischaemia. *Eur J Vasc Surg* 1992; **6**: 269-275.

166. Ubbink DT, Jacobs MJ, Slaaf DW, Tangelder GJ, Reneman RS. Capillary recruitment and pain relief on leg dependency in patients with severe lower limb ischaemia. *Circulation* 1992; **85**: 223-229.

167. Jacobs MJ, Beches RC, Jorning PJ, Slaaf DW, Reneman RS. Microvascular haemodynamics before and after vascular surgery in severe limb ischaemia. *Eur J Vasc Surg* 1990; **4**: 525-529.

168. Williams SA, Boolell M, MacGregor GA, Smaje LH, Wassermann S, Tooke JE. Capillary hypertension and abnormal pressure dynamics in patients with essential hypertension. *Clin Sci* 1990; **79**: 5-8.

169. Williams SA, Tooke JE. Noninvasive estimation of increased structurally-based resistance to blood flow in the skin of subjects with essential hypertension. *Int J Microcirc Clin Exp* 1992; **11**: 109-116.

170. Orlandi C, Rossi M, Finardi G. Evaluation of the dilator capacity of skin blood vessels of hypertensive patients by laser Doppler flowmetry. *Microvasc Res* 1988; **35**: 21-26.

171. Carberry PA, Shepherd AMM, Johnson JM. Resting and maximal forearm skin blood flows are reduced in hypertension. *Hypertension* 1992; **20**: 349-355.

172. Parving H-H, Gyntelberg F. Transcapillary escape rate of albumin and plasma volume in essential hypertension. *Circ Res* 1973; **32**: 643-651.

173. Whitney RJ. The measurement of volume changes in human limbs. *J Physiol* 1953; **121**: 1-27.

174. Gamble J, Garside IB. A four-channel recording system using an IBM PC/XT compatible computer for low frequency data sampling during mercury-in-rubber strain gauge plethysmography in humans. *J Physiol* 1989; **413**: 7p (abstract).

175. Gamble J, Gartside IB, Christ F. A Reassessment of mercury in silastic strain gauge plethysmography for microvascular permeability assessment in man. *J Physiol* 1993; **464**: 407-422.

176. Poulson HL, Nielsen SL. Water filtration in the forearm in short- and long-term diabetes mellitus. *Diabetologia* 1976; **12**: 437-440.

177. Henriksen O. Local nervous mechanism in regulation of blood flow in human subcutaneous tissue. *Acta Physiol Scand* 1977; **97**: 385-391.

178. Fauchald P, Norseth J, Jervell J. Transcapillary colloid osmotic gradient, plasma volume and interstitial fluid volume in long-term Type 1 (insulin-dependent) diabetes. *Diabetologia* 1985: **28**: 269-273.

179. Gartside IB, Gamble J. Apparatus for the production of step changes in cuff pressure for venous occlusion plethysmography in man. *J Physiol* 1991; **438**: 10p. (Abstract).

180. Nilsson GE, Öberg PÅ, Tenland T. A new instrument for continuous measurement of tissue blood flow by light beating spectroscopy. *IEEE Trans Biomed Eng* 1980; **27**: 12-19.

181. Kiel JW, Riedel GR, Di Resta GR, Shepherd AP. Gastric mucosal blood flow measured by laser Doppler velocimetry. *Am J Physiol* 1985; **249**: G539-G545.

182. Stern MD, Lappe DL, Bowen PD et al. Continuous measurement of tissue blood flow by laser Doppler spectroscopy. *Am J Physiol* 1977; **232**: H441-H448.

183. Saumet JL, Dittmar A, Leftheriotis G. Non-invasive measurement of skin blood flow: comparison between plethysmography, laser-Doppler flowmeter and heat thermal clearance method. *Int J Microcirc Clin Exp* 1986; **5**: 73-83.

184. Tooke JE, Östergren J, Fagrell B. Synchronous assessment of human skin microcirculation by laser Doppler flowmetry and dynamic capillaroscopy. *Int J Microcirc Clin Exp* 1983; **2**: 277-284.

185. Nilsson GE, Tenland T, Öberg PÅ. Evaluation of a laser Doppler flowmeter for measurement of tissue blood flow. *IEEE Trans Biomed Eng* 1980; **27**: 597-604.

186. Fagrell B. Laser Doppler flowmetry for evaluating skin microcirculation and its relation to nutritional capillary flow. *Int J Microcirc Clin Exp* 1984; **3**: 434.

187. Saumet JL, Kellogg DL Jr, Taylor WF, Johnson JM. Cutaneous laser-Doppler flowmetry: influence of underlying muscle blood flow. *J Appl Physiol 1988*; **65**: 478-481.

188. Johnson JM, O'Leary DS, Taylor WF, Kosiba W. Effect of local heating and cooling on forearm reactive hyperaemia. *Clin Physiol* 1986; **6**: 337-346.

189. Westerman RA, Widdop RE, Hannaford J et al. Laser Doppler velocimetry in the measurement of neurovascular function. *Aust Phys Eng Sci Med* 1988; 11: 53-66.

190. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; **288**: 373-376.

191. Westerman RA, Widdop RE, Low A, Hannaford J, Kozak W, Zimmet P. Non-invasive tests of neurovascular function: reduced axon reflex responses in diabetes mellitus of man and streptozotocin-induced diabetes of the rat. *Diabetes Res Clin Prac* 1988; **5**: 49-54.

192. Walmsley D, Wiles PG. Assessment of the neurogenic flare response as a measure of nociceptor C fibre function. *J Med Eng Technol* 1990; **14**: 194-196.

193. Parkhouse N, Le Quesne PM. Quantitative objective assessment of peripheral nociceptive C fibre function. *J Neurol Neurosurg Psych* 1988; **51**: 28-34.

194. Hornyak ME, Naver HK, Rydenhag B, Wallin BG. Sympathetic activity influences the vascular axon reflex in the skin. *Acta Physiol Scand* 1990; **139**: 77-84.

195. Flynn MD, Williams SA, Tooke JE. Clinical television microscopy. *J Med Eng Technol* 1989; **13**: 278-284.

196. Anderson RR, Parrish JA. The optics of the human skin. *J Investigative Dermatol* 1981; **77**: 13-19.

197. Fowler CJ, Carroll MB, Burns D, Howe N, Robinson K. A portable system for measuring cutaneous thresholds for warming and cooling. *J Neurol Neurosurg Psychiatry* 1987; **50**: 1211-1215.

198. Wiles PJ, Pearce SM, Rice PJS, Mitchell JMO. Vibration perception threshold: influence of age, height, sex and smoking, and calculation of accurate centile values. *Diabetic Med* 1991; **8**: 157-161.

199. Michel CC. The investigation of capillary permeability in single vessels. *Acta Physiol Scand* 1979; **463** (Suppl): 67-74.

200. Shami SK, Chittenden SJ, Sarin S. Microvascular vasodilation in non-insulin dependent diabetes. *Br Med J* 1991; **302**: 1465.

201. Scarpello JHB, Martin TRP, Ward J. Ultrasound measurement of pulse-wave velocity in the peripheral arteries of diabetic subjects. *Clin Sci* 1980; **58**: 53-57.

202. Ferrier TM. Radiologically demonstrable arterial calcification in diabetes mellitus. *Austral Ann Med* 1964; **13**: 222-228.

203. Tovey FI, Karanjia N, Plant GR, Rees M. Oscillotonometry : a simple non-invasive method of differentiating proximal arterial obstruction from distal microangiopathy in the vascular assessment of diabetics. *Practical Diabetes* 1990; **7**: 203-205.

204. Andersen L, Dinesen B, Jørgensen PN, Poulsen F, Røder ME. Enzyme immunoassay for intact human insulin in serum or plasma. *Clin Chem* 1992; **39**: 578-582.

205. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993; **329**: 977-986.

206. UK Prospective Diabetes Study Group. UK Prospective Diabetes Study (UKPDS) VIII. Study design, progress and performance. *Diabetologia* 1991; **34**: 877-890.

207. Ewing DJ, Clarke BF. Diagnosis and management of diabetic autonomic neuropathy. *Br Med J* 1982; *285*: 916-918.

208. Camerini-Davalos RA, Velasco C, Glasser M, Bloodworth JMB Jr. Drug-induced reversal of early diabetic microangiopathy. *N Engl J Med* 1983; **309**: 1551-1556.

209. Raskin P, Pietri AO, Unger R, Shannon WA Jr. The effect of diabetic control on the width of skeletal muscle capillary basement membrane in patients with type 1 diabetes mellitus. *N Engl J Med* 1983; **309**: 1546-1550.

210. Ulrych M. Pathogenesis of essential hypertension : Role of the postcapillary segment of the circulation. *Angiology* 1979; **30**: 104-116.

211. Pecoraro RE, Ahroni JH, Boyko EJ, Stensel VL. Chronology and determinants of tissue repair in diabetic lower extremity ulcers. *Diabetes* 1991; **40**: 1305-1313.

212. WHO Study Group. Report on diabetes mellitus. *World Health Organisation, Technical Report Series 727,* 1990, Geneva.

213. Forrest RD, Jackson CA, Yudkin JS. The abbreviated glucose tolerance test in screening for diabetes: the Islington diabetes survey. *Diabetic Med* 1988; **5**: 557-561.

214. Finch CF, Zimmet PZ, Alberti KGMM. Determining diabetes prevalence : a rational basis for the use of fasting plasma glucose concentrations? *Diabetic Med* 1990; **7**: 603-610.

215. Melmed S. Acromegaly. *N Engl J Med* 1990; **322**: 966-75.

216. Wass JAH, Cudworth AG, Bottazzo G, Woodrow JC, Besser GM. An assessment of glucose tolerance in acromegaly and its response to medical treatment. *Clin Endocrinol* 1980; **80**: 53-59.

217. Wright AD, Hill DM, Lowy C, Russel Fraser T. Mortality in acromegaly. *Q J Med* 1970; *39*: 1-13.

218. Teale JD, Marks V. Inappropriately elevated plasma insulin-like growth factor II in relation to insulin-like growth factor I in the diagnosis of non-islet cell tumour hypoglycaemia. Clin Endocrinol 1990; 33: 87-98.

219. Clemmons DR, Van Wyk JJ, Ridgway EC, Klinman B, Kjellberg RN, Underwood LE. Evaluation of acromegaly by radioimmunoassay of somatomedin-C. *N Engl J Med* 1979; **301**: 1138-1142.

220. Hosker JP, Matthews DR, Rudenski AS et al. Continuous infusion of glucose with model assessment : measurement of insulin resistance and ß-cell function in man. *Diabetologia* 1985; **28**: 401-411.

221. Nakano S, Uchida K, Kigoshi T et al. Circadian rhythm of blood pressure in normotensive NIDDM subjects. Its relationship to microvascular complications. *Diabetes Care* 1991; **14**: 707-711.

222. UK Prospective Diabetes Study Group. UK Prospective Diabetes Study (UKPDS) XI: Biochemical risk factors in Type 2 diabetic patients at diagnosis compared with age- matched normal subjects. *Diabetic Med* 1994; **11**: 534-544.

223. White WB. Assessment of ambulatory blood pressure recorders : accuracy and clinical performance. *Clin Invest Med* 1991; 14: 202-211.

224. Bendtsen TF, Nyengaard JR. The number of glomeruli in Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 1992; **35**: 844-850.

225. Lithell H, Lindgarde F, Hellsing K et al. Body weight, skeletal muscle morphology and enzyme activities in relation to fasting serum insulin concentration and glucose tolerance in 48-year old men. *Diabetes* 1981; **30**: 19-25.

226. Lillioja S, Young AA, Culter CL et al. Skeletal muscle capillary density and fibre type are possible determinants of in vivo insulin resistance in man. *J Clin Invest* 1987; **80**: 415-424.

227. Malik RA, Metcalfe J, Sharma AK, Day JL, Rayman G. Skin epidermal thickness and vascular density in Type 1 diabetes. *Diabetic Med* 1992; **9**: 263-267.

228. Wårdell K, Naver HK, Nilsson GE, Wallin BG. The cutaneous vascular axon reflex in humans characterised by laser Doppler perfusion imaging. *J Physiol* 1993; **460**: 185-199.

229. Yip J, Mattock MB, Morocutti A, Sethi M, Trevisan R, Viberti G. Insulin resistance in insulin-dependent diabetic patients with microalbuminuria. *Lancet* 1993; **ii**: 883-887.

230. Sandeman DD, Shore AC, Tooke JE. Captopril reduces nailfold capillary pressure in Type 1 diabetic patients with established nephropathy. *Diabetic Med* 1991; **8** (Suppl 1): 37A.

231. Knowler WC, Bennett PH, Ballintine EJ. Increased incidence of retinopathy in diabetics with elevated blood pressure. A six-year follow-up study in Pima Indians. *N Engl J Med* 1980; **302**: 645-650.