CUTANEOUS MICROVASCULAR HAEMODYNAMICS
IN DIABETES MELLITUS

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PREFACE

ABSTRACT

In this thesis the laser Doppler flowmeter and other microvascular methods were used to investigate the skin microcirculation in non-diabetic and diabetic subjects in order to gain a greater understanding of the normal microcirculation and to define abnormalities relevant to the diabetic state. The principle findings were-

1. The normal skin microvascular response to thermal and mechanical injury is a substantial increase in blood flow. In diabetic subjects with and without complications this hyperaemic response was reduced and degree of impairment was found to be greatest in those with the severest complications.

2. In diabetic patients, the diameter of foot skin capillaries was reduced and the basement membrane width was found to increase progressively with increasing severity of complications. These structural changes may partly explain the reduced hyperaemic responses and their relationship with severity of complications. These structural and functional abnormalities may be implicated in the pathogenesis and impaired healing of diabetic foot lesions.

3. In normal subjects, blood flow in the toe pulp fell by 80% when the foot was lowered 50 cm below the heart. Toe blood flow in neuropathic diabetic subjects was three fold higher than in normal subjects, and on lowering the foot this difference was even greater; dependent flow was seven fold higher and the fall in blood flow was only 50%. These findings are compatible with reduced central sympathetic tone and/or peripheral sympathetic nerve failure.

4. In young non-neuropathic diabetic subjects, the more severe stress of sitting still for 50 minutes with the foot 1 meter below heart level, also revealed an increase in toe pulp blood flow. This was associated with elevated capillary pressure, failure in the expected rise in plasma osmotic pressure, and increased foot swelling. These results provide evidence of capillary hypertension and impairment of oedema preventing mechanisms in the dependent foot of diabetic subjects. These abnormalities may be important in initiating structural and functional damage to the skin microcirculation.

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CHAPTER I

INTRODUCTION AND REVIEW

1.1 INTRODUCTION

Diabetic foot complications are a major cause of morbidity and mortality and are amongst the most frequent reasons for admission of a diabetic person to hospital. In the United Kingdom, Connor (1987) estimated that in Hereford in 1979-1980, diabetic foot problems accounted for approximately 47% of all diabetes bed related days. The relative risk of lower limb amputation in people with diabetes is approximately 15 times that of people without diabetes, and diabetic patients account for approximately 50% of all non-traumatic amputations (Bild 1989). In 1987 in the United States of America 56,000 lower limb amputations were performed in people with diabetes, which represents a estimated direct cost of about 500 million dollars (Division of Diabetes Translation, 1990).

The pathological mechanisms involved in diabetic foot ulceration include large vessel disease, peripheral neuropathy and increased susceptibility to infection. Although large vessel disease is the major reason for amputation, in as many as 50 % of diabetic patients with foot ulceration foot pulses are present (Edmonds 1986). In such patients neuropathy and foot deformities with the resulting high foot pressures and callous formation have been shown to be important risk factors (Boulton 1985). The patient with an insensitive neuropathic foot is at risk of painless mechanical, thermal, or chemical injury. Once initiated, what would often be considered to be relatively minor and inconsequential injury in a non-diabetic subject, in the diabetic patient frequently results in delayed healing and occasionally extensive tissue breakdown. The role of microvascular disease in such patients is unclear, but perhaps in an effort to counter the therapeutic

nihilism and inappropriate care associated with the belief that irremediable small vessel disease is the cause of foot lesions, several authors have suggested that microvascular disease is of little or no importance (LoGerfo 1984, Faris 1984)

The aim of the studies described in this thesis was to investigate microvascular haemodynamics in the skin of diabetic patients free from macrovascular disease in an attempt to define functional microvascular abnormalities which may be of importance in the pathogenesis of diabetic foot ulceration.

1.2 THE NORMAL FOOT SKIN MICROCIRCULATION

The arterial supply to the foot comprises the posterior tibial, the dorsalis pedis and lateral peroneal arteries. The posterior tibial divides to form the medial and lateral plantar arteries which anastamose to form the plantar arch, which receives a contribution from the dorsalis pedis artery. The digital arteries arise from the plantar arch. Small arteries pierce the dermis and divide into the perpendicularly or diagonally distributed arterioles of the subpapillary arteriolar plexus. The capillary loops in the epidermis arise in a candelabra like pattern from this subpapillary arteriolar plexus. The papillary system of the upper dermis comprises the indentations of the epidermal rete pegs into the dermis and the extensions of the dermis with its nerves and blood vessels into the epidermis. Usually there is only one, seldomly as many as three capillaries supplying each papilla. Capillary density varies considerably from one area to another. On the dorsum of the digits it is about 50-70 per mm^2 but decreases to between 20-40 per mm^2 on the lower leg (Wetzel 1926).

The capillary is composed of the ascending or arteriolar limb, the apex or capillary loop which has a hairpin turn, and the descending or venular limb. The latter drains into a venule of the subpapillary venous

plexus. The diameter of the capillary lumen varies from 5.0 to 7.5 μm in the ascending limb to 6-10 μm in the descending limb (Higgins 1981), and the length varies from 150 to 500 μm depending on the site of the skin examined (Braverman 1977). The capillary wall in normal skin consists of a single layer of endothelial cells with usually two and no more than three endothelial cells in a transverse section. The abluminal aspect of the capillary wall is surrounded by the basement membrane in which lies the pericyte.

The basement membrane is composed of several different proteins and glycoproteins which include Type IV and V collagen, laminin, heparan sulphate proteoglycans and fibronectin. The microvascular basement membrane is believed to serve at least three functions. It provides structural rigidity to the capillary wall and is thought to be responsible for the resistance of these vessels to overdistension and rupture when exposed to high capillary pressure and trauma (Murphy 1975). Type IV collagen, the most abundant basement membrane protein, provides most of the structural rigidity. The basement membrane is also believed to provide a framework on which regenerating endothelial cells migrate and spread following injury (Vracko 1970). By virtue of its porosity and its specific highly negatively charged heparan sulphate proteoglycans the basement membrane is believed to contribute to the selective permeability of the capillary (Williamson 1988).

The vascular endothelium envelopes the circulating blood in a continous monolayer. The view of the endothelium as simply a passive semi-permeable membrane through which nutrients are exchanged has in recent years been markedly altered with the ability to culture endothelial cells. The endothelium is now recognised as a complex tissue with multiple functions which include the maintainance of the fluidity of the blood through anticoagulant properties, and the regulation of blood flow through the synthesis of a number of vasoactive substances. These include angiotensin-

converting enzyme, prostacyclin, endothelium-derived releasing factor (EDRF), endothelin-1, various growth promoting factors, tissue-type plasminogen activators and inhibitors, and von Willebrand factor (Vane 1990).

A specific feature of the skin microcirculation of the hands and feet is the presence of arteriovenous anastamoses. These are channels connecting arterial and venular sides of the microcirculation through which nutrients are not normally exchanged. Such communications were first described by Sucquet (1862) and later confirmed by others including Hoyer (1887) who was the first to suggest that they may be involved in temperature regulation. They are most numerous in the nail beds, pulps of the digits, palms of the hands and soles of the feet but are absent on the dorsum of the hands and feet (Grant 1931). The vessels are absent at birth and develop in humans from about the age of 4 months from simple direct connections (Hale 1960). The anastamoses are coiled channels with thick muscular walls and an average luminal diameter of 35 μm, but may vary in size from 10 to 100 μm depending on their state of contraction or dilatation (Sherman 1963). By connecting arterioles and venules in the dermis they bypass the capillary network and are thus also referred to as shunt vessels. These vessels are primarily under control of the sympathetic nervous system. As originally suggested by Grant (1931) the anastomoses are believed to serve two functions, local regulation of skin temperature (for example, protecting the extremity from extreme cold by 'cold vasodilation') and general regulation of body temperature- by allowing an enormous increase in skin blood flow thereby aiding the dispersal of heat.

1.3 THE CONTROL OF SKIN BLOOD FLOW

From the above description it can be seen that (total) blood flow to the skin of the hands and feet may be considered as having two distinct components.

The nutritive component which comprises flow through the capillary network, and the thermoregulatory or shunt component, comprising flow through arteriovenous anastamotic channels. In a comfortable environment skin blood flow is normally subjected to a high degree of sympathetic vasoconstrictor tone. This was first demonstrated by Claude Bernard (1852) who found that cervical sympathetic transection in the rabbit caused the ear on the same side to become flushed and warm. In humans, Adson (1925) found warming and increased circulation through the toes following lumbar sympathectomy, and Walker (1950) demonstrated that after sympathectomy hand blood flow increased from around 5 ml/100 gm tissue per minute to between 25 and 60 ml/100 gm tissue per minute. Thermoregulation is achieved primarily by changes in vasoconstrictor activity in sympathetic nerves. This was demonstrated in microneurographic recordings from human skin suppling the hands and feet (Normell 1974, Bini 1980). In these studies, sympathetic activity in post-ganglionic C fibres was shown to increase with body cooling and decrease with moderate warming. Increased and decreased sympathetic activity were accompanied by decreased and increased finger blood flow respectively, the latter determined by measuring pulse amplitude using a plethysmograph. In addition transient bursts of increased sympathetic neural activity were noted to be followed by transient reductions in pulse amplitude.

The magnitude of the changes in blood flow that can be achieved by alterations in sympathetic vasoconstrictor control of arteriovenous anastamoses can appreciated by assuming blood to be a Newtonian fluid and applying Poiseuille's equation, where flow is related to the fourth power of the radius. A shunt vessel dilating from a diameter of 10 μm to 100 μm would increase flow by 10,000 fold, moreover flow through such an open shunt would be 40,000 fold greater than through a capillary with an internal diameter of 7 μm. Thus the opening of shunt vessels floods the venous

plexus of the cooler dermis with warm blood and by this means heat is dissipated.

In addition to the central thermoregulatory component, blood flow to the extremities is also influenced by alerting stimuli and local physical stimuli such as local skin temperature, tissue injury, ischaemia and changes in transmural pressure. Each of these will be briefly considered.

Alerting stimuli, for example sudden noises and deep inspiration (Bolton 1936) produce rapid and transient vasoconstriction in the hands and feet. These changes are caused by a burst of sympathetic activity and as such have been used as tests of sympathetic function (Aminoff 1980, Fagius 1982).

Post-occlusive reactive hyperaemia is the increase in blood flow which follows arterial obstruction of blood flow. It is a local phenomenon independent of vasomotor nerves as demonstrated by Lewis (1926) in chronically denervated limbs. The duration of the hyperaemia and the magnitude of the peak blood flow is related to the duration of the ischaemia and the local skin temperature (Fagrell 1981). The mechanism underlying reactive hyperaemia is not well understood. It is believed to be related to the accumulation of vasodilator metabolites, and to myogenic relaxation of the vascular smooth muscle as transmural pressure falls distal to the occlusion (Carlsson 1987). This reaction has been extensively investigated in diabetic skin and muscle.

In addition to central body temperature, changes in local skin temperature can have profound effects on skin blood flow. At temperatures of between 0-10 °C, blood flow in the extremities falls to zero, but with the onset of pain, flow is re-established and the pain disappears. This is followed repeat cessation of flow and recurrence of pain. The cycle continues as long as the exposure is maintained. This so called 'cold vasodilatation' is brought about by opening of arteriovenous anastamoses (Grant 1930) the object of which is to protect the tissue from cold injury. Through the temperature

ranges of 10°C to 42°C there is increasing vasodilatation. Temperatures above 43 °C and freezing result in tissue injury. In the skin, injury whether thermal, mechanical, chemical or exposure to ultra-violet light results in similar changes in skin blood flow and involves the so called 'triple response' described by Lewis (1924). For example, the injury induced by a firm stroke from a sharp instrument, results in a sharply defined red line confined to the line of the stroke which develops within 20 seconds. This is followed within another 10 seconds by a red flush with irregular margins extending about 2 cm on either side of the line, and after a further 30-50 seconds, a wheal. Lewis attributed the flare to arteriolar vasodilatation and the wheal to fluid exudation. Since the flare was present immediately after nerve section but disappeared after the distal parts had degenerated, Lewis concluded that this aspect of the response was mediated by an axon reflex. It has been established that C fibres with primary afferent units through polymodal nociceptors are involved and substance P, VIP and CGRP have all been proposed as the neurotransmitter substance (Foreman 1987). In contrast, the red line still occurs in chronically denervated skin and is believed to results from direct tissue injury. The precise mechanism for this local hyperaemia is not known.

Changes in vascular transmural pressure also alter skin blood flow. Two principle mechanisms are involved, autoregulation and the veno-arteriolar reflex. Autoregulation is the tendency to maintain constant blood flow during changes in arteriolar perfusion pressure (Johnson 1964). It is present in various tissues including the brain, skeletal muscle, and adipose tissue. The mechanism is unknown. Neurogenic mechanisms do not appear to be involved since autoregulation is still present in chronically denervated skin (Folkow 1949). As originally proposed by Bayliss (1902), intrinsic myogenic smooth muscle reactions to stretch have been suggested (Folkow 1964, Johnson 1989), as have vascular resistance adjustments to accumulated local metabolites (Berne 1964).

In the limbs autoregulation has been demonstrated in muscle, cutaneous and subcutaneous tissues (Henriksen 1973). Thus, when transmural pressure is increased by placing the limb below heart level, blood flow in all these tissues remains relatively constant. However, Henriksen (1977) showed that once the limb is lowered beyond approximately 40 cm, autoregulation is over-ridden and pronounced vasoconstriction occurs with flow measured by the Xenon clearance technique falling to approximately 50% of the value at heart level. He suggested that this is due to a local sympathetic axon reflex (veno-arteriolar reflex) involving impulse transmission from small veins to arterioles, as the response could also be elicited by venous distension (elevating of venous pressure to 25 cm Hg), and was abolished by local anaesthesia and chronic sympathectomy, but not by acute spinal blockade.

Hassan (1988a) using the laser Doppler flowmeter has recently re-examined the veno-arteriolar reflex to determine the nature of local mechanism and the relative contributions of central and local mechanisms. By studying blood flow in both legs simultaneously he has demonstrated that in addition to the fall in blood flow in the dependent foot there is a small fall in flow in the other foot kept at heart level. Acute spinal blockade abolished the latter and resulted in a small attenuation of the veno-arteriolar reflex in the dependent foot. This suggests that there is a small central contribution to the postural vasoconstrictor response, mediated via sympathetic efferent fibres. Like Henriksen, Hassan found that local anaesthesia completely blocked the veno-arteriolar response suggesting a local neurogenic response. However at very low concentrations of lignocaine (3×10^{-4} mol/l), said to interfere with nervous impulse transmission and not myogenic activity, lowering of the limb still caused a small reduction in flow (19% compared with 83% before infiltration). If nerve impulses were indeed blocked this would suggest a small myogenic component to the reflex. This suggestion is supported by the finding that venous pressure elevation by venous occlusion

to the same level as that produced by dependancy, produced a slightly less marked fall in flow (Hassan 1988a).

The postural changes in blood flow are believed to be important in preventing dependent oedema. The increase in precapillary resistance, limits the expected rise in capillary pressure (Levick 1978), thereby reducing filtration. In the presence of considerably reduced blood flow continued filtration results in an increase in plasma osmotic pressure (Noddeland 1981) which acts as a further brake to fluid filtration.

In recent years a number of locally active substances produced by the endothelium have been discovered which are believed to have important physiological roles in local blood flow regulation. EDRF (nitric oxide) is a powerful vasodilator which is believed to be continuously released in healthy humans thereby keeping the vasculature in a dilated state. The strongest evidence to support this lies in the results of intravenous injection of N^G-monomethyl-L-arginine, the inhibitor of nitric oxide formation from L-arginine. In animals this causes an immediate and substantial rise in blood pressure (Rees 1989) which can be reversed by L-arginine. In humans there is also evidence that vessels are continuously vasodilated by EDRF released from endothelial cells, since intra-arterial injection of N^G-monomethyl-L-arginine into the forearm causes substantial vasoconstriction which is reversed by L-arginine (Vallance 1989). Endothelin-1 is a linear 21-amino acid peptide which is released by the endothelial cell. The most striking property of this slowly released molecule is its long lasting hypertensive action. Intravenous infusion in humans causes intense vasoconstriction and reduction of blood flow. The function of this peptide is not well understood; however it has been suggested that together with EDRF it is important in maintaining basal vascular tone.

1.4 MEASUREMENT OF SKIN BLOOD FLOW

There have been a large number of studies, using a variety of different methods to investigate blood flow in the skin of patients with diabetes. The results have often been conflicting. This relates to the great difficulty in measuring skin blood flow, which is in part related to the complex nature of its control and distribution within the skin. Any discussion of the results of previous studies in diabetics must therefore include consideration of the methods used. This section deals with the commonly used methods, their advantages and disadvantages.

Venous occlusion plethysmography measures the volume change of a digit or limb in which venous outflow has been obstructed for a short period. Provided arterial inflow is not restricted, the rate of volume change is equal to the rate of arterial inflow. The volume changes may be measured by air or water displacement with the limb enclosed in a rigid sealed container or by the use of a mercury-in-rubber strain gauge. The latter technique, first described by Whitney (1953) is the most commonly used plethysmographic method since it is simple to use, portable, and does not require the immobilization of the limb in a sealed water- or air-filled container.

Although venous occlusive plethysmography has been extensively used and has provided considerable information on peripheral blood flow, it has several disadvantages. It measures total blood flow to the area under investigation, which will often include muscle and subcutaneous tissue. Shunt and nutritive blood flow cannot be separated. The technique cannot be used to measure blood flow in the dependent limb because in this position the veins are already filled (Greenfield 1954). At high flow rates particularly when measurement are made in the digits, the part fills within a few beats making accurate flow measurements difficult (Sumner 1982). Blood flow cannot be continuously measured as the method involves intermittent venous

obstruction. Furthermore venous obstruction elicits the veno-arteriolar reflex which will reduce blood flow.

The radioactive isotope clearance technique introduced by Kety (1949) relies on the measurement of the rate of disappearance from the skin of an injected freely diffusible isotope such and Xenon. Although relatively simple, there are a number of problems associated with this technique. It is invasive and requires the exposure of the subject to radiation. It relies on very high blood-tissue permeability of the tracer, for only then will the clearance be flow limited. Whereas at normal skin flow rates permeability for most tracers is not a problem, at high flow rates such as during hyperaemic conditions, flow will be underestimated by a tracer with restricted diffusion (Spence 1985). The most widely used isotope, Xenon, has the disadvantage of having a high affinity for fat so that the blood-tissue partition coefficient will vary with the composition of the tissue. This makes the differences in fat content of the subcutaneous tissue between individuals an significant variable. Perhaps the most important drawback of this technique is the effect that the trauma of injecting the isotope has on blood flow. Exponents of the technique suggest that injection trauma is minimal but allow 30 minutes to elapse after injection before measurements are made so as to allow the injection hyperaemia to subside. There is no doubt that the the hyperaemia persists well beyond this period and evidence for this is presented in Chapter 3. This raises considerable doubts as to the ability of this technique to measure resting blood flow and vasoconstrictor reflexes. Injection trauma can be avoided by exposure of the skin to Xenon gas in sealed chamber placed on the skin's surface, the so called epicutaneous labelling technique (Sejrsen 1969). This method is technically difficult, and in practice Hutchinson found (1983) high rate constants during arterial occlusion suggesting that there is significant back diffusion from the skin, a potential source of error. The technique cannot be used in areas of skin heavily endowed with sweat glands as approximately 60% of the tracer is

lost through sweating and the method cannot be used when skin temperature exceeds 39°C (Al-Siaidy).

The thermal clearance probe is a non-invasive method which assesses the rate of removal of heat from a heated area in the centre of the probe by the skin blood flow. The conventional probe consists of a central copper disc surrounded but separated from a concentric segmented copper annulus (Holti 1974). The central disc is heated usually to about 2-3°C above skin temperature and the differential between this and the outer annulus is measured using thermocouples; alternately the power needed to maintain a given temperature difference is determined. By varying the distance between the disc and annulus its is possible to vary the depth sensitivity of the instrument (Delpy 1983). It is claimed that the instrument can be used to measure nutrient flow (Holti 1978). Brown (1980) has argued that thermal clearance due to the thermal conductivity of the skin and underlying tissue is greater than the thermal clearance of the nutritive capillary supply, furthermore the presence of large blood vessels within a few millimetres may remove heat more rapidly than the perfusing capillary network. In this respects it is of interest that the recently described 18 mm probe with an apparent depth sensitivity limited to less than 1 mm was found to reflect anastamotic flow rather than nutritive flow (Corcoran 1987).

The measurement of transcutaneous oxygen tension using a modified Clark-type polarographic electrode (Clark 1956) in heated skin was introduced by Hutch (1972). Initially intended for monitoring arterial oxygen tension in neonates it has been suggested as a useful technique for measuring tissue perfusion pressure in the ischaemic limb (Lancet editorial 1984). The method depends on maximally vasodilating the microvasculature by heating the skin to 44°C. Although abnormalities can be detected at critical levels of perfusion, the method cannot be considered a reliable measure of blood flow and there must be some doubt when it is used to compare groups such as diabetic and non-diabetic subjects since epidermal

thickness, capillary density, the oxygen diffusion characteristics of the microvasculature and dermal tissue, oxygen affinity, metabolic rate of the underlying tissue and the ability of the local microcirculation to maximally dilate will all influence the measurement (Lubbers 1979).

The measurement of capillary blood flow using television microscopy is at present the only direct method of measuring nutritive blood flow. It was first introduced by Zimmer (1964) and further refined by Bollinger (1974). The technique is greatly facilitated by the arrangement of the capillaries in the fingernail fold which lie parallel to the skin surface. These are observed through an ordinary microscope to which a television camera is mounted. The images are displayed on a television monitor and can be stored on videorecorder. The nailfold capillaries are illuminated by a tungsten lamp with a blue filter or mercury vapour lamp to obtain maximum contrast between the red cells and the surrounding tissue. Measurement of red cell velocity can be made either manually or with the use of a cross-correlator. The former method involves frame to frame measurement of the distance moved by a column of red cells in a capillary. Knowing the final magnification and the frame speed, the velocity can be calculated. The method is clearly time consuming. The cross-correlator technique was described by Intaglietta (1975). This involves the use of two videophotometric windows displayed on the TV screen which are placed upstream and downstream of the capillary under investigation. The outputs from these windows are proportional to the brightness of the enclosed area, thus plasma gaps which appear brighter than red cells give a greater signal. The outputs from the two windows are fed into the cross-correlator which produces a signal related to the red cell velocity.

Whilst capillary velocity measurement using televison microscopy has the great advantage of being the only technique for measuring nutritive blood flow it is technically difficult, limited to the assessment of a small number of capillaries which may not be representative of the average

nutritional flow, and at present cannot be used at high flow rates precluding any assessment of maximum nutritive blood flow. Furthermore, because of the relative fixed apparatus the method does not easily permit continuous measurement of blood flow with change in posture.

In the studies described in this thesis, laser Doppler flowmetry, a relatively recent technique, was used to measure skin blood flow. The methodology and validation are described in detail in Chapter 2, and hence to avoid repetition only a brief description is given here. The technique has been used for many years in industrial research to measure the movement of macromolecules in fluids (Yeh 1964), but it was Riva (1972) who first used the technique to measure blood flow in the retinal vessels of rabbits. The use of the instrument to measure skin microvascular blood flow was first suggested by Stern (1975) when he demonstrated that coherent light backscattered from skin showed spectral Doppler broadening which was shown to be the result of blood flow in the microcirculation. Since then several different prototype and commercial instruments have been developed. These have been used to examine blood flow in a variety of tissues including skin, bone, muscle, retinal tissue, liver and kidneys. The principle underlying the laser Doppler method is the same as that used for ultra-sound Doppler in the assessment of blood flow in larger vessels. Laser light in this case, on encountering a moving object undergoes as frequency shift related to the velocity of the object. In the skin the measurement is derived from blood flowing in the most superficial blood vessels, the backscattered light being derived from a depth of 1-1.5 mm (Stern 1977). Both the Doppler shifts (related to the velocity of red cell movement) and the proportion of the backscattered light which has undergone a Doppler shift (related to the number of moving particles) are measured. The final output of the instrument is related to the product of these, in other words to the quantity and velocity of blood flow.

This method has many advantages over existing methods for measuring blood flow. It is relatively simple to use and quick to set up, it does not disturb the underlying tissue, and can be used in small areas of skin. Furthermore it can be used to the study dynamic changes such as the effect of change in position or posture on blood flow. Validation and disadvantages of the method are discussed in Chapter 2.

1.5 THE DIABETIC SKIN MICROVASCULATURE

1.5.1 Resting skin blood flow, environmental temperature, metabolic status and distal autonomic function in diabetes.

There are now a number of studies which show that in the absence of macrovascular disease, in the resting state, total limb blood flow is elevated in diabetic patients with peripheral neuropathy. The simple measure of skin temperature, though only a rough index because of its non linear relationship with blood flow (Cooper 1955) suggests that blood flow is increased in the neuropathic foot. Martin (1953) found that despite being exposed for more than an hour to a room temperature of between 18 and 20°C, 9 of 20 patients with peripheral neuropathy had toe temperatures which remained above 30°C. Sundkvist (1986) found that after 20 minutes in a room with a constant temperature of 19°C, mean toe skin temperature in diabetics with autonomic neuropathy was approximately 29°C compared with 24°C in those without neuropathy, a highly significant difference. At a room temperature of 20-22°C, Archer (1984) found a mean toe temperature of 33.5°C in diabetics with peripheral neuropathy, significantly higher than that of the control group (25.8°C), and similarly, at a room temperature of 22 ± 0.5 °C, Flynn (1989a) also found a significantly higher mean toe temperature in neuropathic (32.6°C) compared with control subjects (27.1°C).

Doppler ultrasound studies also suggest that resting blood flow is increased in the neuropathic foot (Scarpello 1980, Edmonds 1982b). Patients with neuropathy were found to have markedly abnormal blood velocity profiles (sonograms), consisting of increased forward flow in systole and decreased reverse flow in diastole. These findings are consistent with reduced peripheral vascular resistance as found in arteriovenous shunting (Rutherford 1978). Since the abnormalities were almost exclusive to patients with peripheral neuropathy, and previous studies have shown that sympathetic denervation increases shunt blood flow (Cronenwett 1977) it was suggested that the findings indicated peripheral sympathetic denervation.

Direct evidence of increased flow has been provided by Archer (1984) using venous occlusion plethysmography. Toe and mid-foot blood flow in patients with painful and sensory neuropathy were elevated approximately 5 fold, to values consistent with release of sympathetic tone and increased shunt flow. In addition a number of diabetic patients in the non-neuropathic group studied also had elevated foot blood flow. Archer suggested that this might indicate early peripheral sympathetic neuropathy before the development of clinical neuropathy.

It is important note that all the above studies were performed at environmental temperatures below 23°C when resting blood flow is heavily influenced by sympathetic tone. Had higher room temperatures been employed differences may not have been detected. In this respect it is of interest that Christensen (1969) did not find a difference in resting blood flow between non-diabetic and diabetic subjects with neuropathy when using a water filled venous occlusion plethysmograph heated to 32°C. Similarly, Scott (1988) found no significant difference in resting foot blood flow in diabetics with autonomic neuropathy compared with normal subjects when the foot was maintained at 34°C and subjects were exposed to environmental temperature of 25-26°C. However, when exposed to a temperature of 16°C

in a water-perfused suit, foot blood flow was significantly higher in patients with autonomic neuropathy.

Indirect evidence exists to support the suggestion that the increased flow in the neuropathic limb is due to arteriovenous shunting. The simple observation by Ward (1983) that the veins are distended in the neuropathic foot, led to the demonstration that venous pressure is elevated in. this condition. Boulton (1982) measured the oxygen tension of blood sampled from the veins on the dorsum of the feet and reported a mean PO_2 of 60.3 mmHg in diabetic patients with neuropathy and foot ulcers, and 53.8 mmHg in those with neuropathy but without foot ulcers; both significantly raised compared to a value of 45.5 mmHg in control subjects. Although these findings support the concept of shunting, increased capillary blood flow (reduced capillary transit time) might also give similar results. More conclusive evidence of shunting is provided by Partsch (1977) who injected 20-30 μm diameter radio-labelled human albumin microspheres intra-arterially and measured the radioactivity over the lungs. Microspheres are trapped in the capillary bed, but in the presence of shunting reach the lungs. Shunt volume in the neuropathic diabetic subjects was 8.45% compared with 5% in the normal subjects.

Further evidence supporting the suggestion that peripheral sympathetic denervation results in abnormalities of blood flow regulation is the demonstration by Christensen (1969) that patients with diabetic neuropathy have reduced spontaneous variation in resting blood flow and that the degree of abnormality correlates with the degree of neuropathy. Burton (1939) had previously shown that spontaneous variations in blood flow reflects changes in sympathetic nervous activity and that such variations are absent in sympathectomised limbs.

Other tests of sympathetic vasomotor function reveal evidence of peripheral sympathetic denervation in diabetic patients with peripheral neuropathy. Low (1983) examined the effect of inspiratory gasp, valsalva

manoeuvre, and the immersion of the face in cold water (cold pressor test) on peripheral blood flow. Each of these results in abrupt reductions in peripheral blood flow dependent on an intact sympathetic vasoconstrictor fibres. Although differences were detected in patients with neuropathy, numbers were not specified and the responses in the control subjects were very variable. This variability may have arisen because central vasoconstrictor reflexes were attenuated by directly warming the limb to between 34 and 35 °C. Archer (1984) using Doppler ultrasound also examined sympathetic vasoconstrictor responses by measuring the changes in the pulsatility index (a measure of peripheral resistance) induced by coughing. Diabetic patients with sensory neuropathy in comparison with control subjects had a significantly lower pulsatility index which did not increase on coughing. Fagius (1982) has directly measured sympathetic vasomotor activity by simultaneously recording microneurographic sympathetic nerve activity and digital pulse plethysmography in diabetic patients with peripheral neuropathy. In the majority of patients with neuropathy, no recording could be made, and in two third of such patients this was accompanied by absent vasoconstricton in response to a startle reaction.

Thus total blood flow is increased in the neuropathic limb but also in a small number of diabetic subjects without neuropathy. Corbin and Young (1987) have questioned the assumption that increased limb blood flow is the result of sympathetic denervation. Using Doppler waveform analysis they confirmed the presence of abnormal blood flow in patients with painful neuropathy, painless neuropathy associated with foot ulceration, and some patients free from symptomatic neuropathy but "with only minor autonomic dysfunction". Since the blood flow abnormalities did not appear to parallel the changes in cardiac autonomic function they suggested that factors other than peripheral sympathetic neuropathy should be sought to explain the elevated blood flow. An alternative explanation may be that central cardiac

autonomic function tests do not necessarily reflect peripheral sympathetic denervation. This view is supported by Ryder (1990) who using the acetylcoline sweat test (Ryder 1988), demonstrated that peripheral sympathetic denervation was invariably present in diabetic patients with neuropathic foot ulceration, whereas Young (1986) had previously questioned the importance of autonomic dysfunction in diabetic foot ulceration as he had found that cardiac tests of autonomic denervation did distinguish patients with neuropathic ulceration from those with other neuropathic syndromes.

Although Young may not be correct in dismissing peripheral sympathetic neuropathy as the cause of elevated flow in the neuropathic foot, he is correct in suggesting that other factors may increase peripheral blood flow. Using venous occlusion plethysmography, Christensen (1970) demonstrated increased resting forearm blood flow in untreated young insulin dependent diabetic subjects and in diabetic subjects after insulin withdrawal. This abnormality was reversed with correction of the hyperglycaemia and ketosis. Similar findings were reported by Gundersen (1976) who suggested the abnormality may to be related to the increased metabolic rate which is associated with poor control (Leslie 1986). In patients with autonomic neuropathy, however, strict metabolic control with continuous insulin infusion does not correct the blood flow abnormality (Scott 1988) .

In view of the vulnerability of the neuropathic diabetic foot to ulceration it may seem paradoxical to find an increased blood flow. To explain this finding it has been suggested that the increased flow is limited to shunt vessels, opening of the arteriovenous anastamoses resulting in a 'capillary steal' phenomenon (Ward 1983). Evidence from animal studies exists to support this proposal. Grant (1931) noted that when arteriovenous anastamoses opened there was occasionally reflux of blood (reverse flow) into the venous limb of the capillary, and Luckner (1955) showed that

opening of shunts results in a rise in local venous pressure which spreads to the venous limb of the capillary. The effect would be a reduction in the effective capillary perfusion pressure and a reduction in blood flow. Furthermore an increase in capillary pressure would increase fluid filtration. This has been suggested to be the mechanism of neuropathic oedema (Edmonds 1982).

In humans there have been few studies of nutritive blood flow in the feet because of the limitations of the techniques. Studies employing television capillary microscopy have been mainly limited to the fingers. Fagrell (1984) found no difference in resting red cell velocity in finger nail fold capillaries in diabetics with retinopathy, nor did Tooke (1985a) in uncomplicated Type I diabetics of various durations. In a recent study resting capillary blood velocity in the finger nailfold was found to be normal in Type II diabetic subjects (Pazos-Moura 1990). In contrast, in a limited study, Tooke (1982) found reduced red cell velocity in finger nailfold capillaries of Type II diabetics during poor diabetic control associated with increased shunt flow (measured by venous occlusion plethysmography). These changes were reversed by improvement in diabetic control. In the only study of nailfold capillary blood flow in the diabetic foot, Flynn (1988) found that rather than a reduction, there was an increase in capillary flow in diabetics with neuropathy in whom shunt blood flow was increased, thus refuting the 'capillary steal' hypothesis. It was however suggested that although capillary flow was increased this may be have been insufficient to meet the increased oxygen consumption of the warmer neuropathic feet.

In summary there is good evidence to support the concept of arteriovenous shunting of blood flow in the neuropathic foot and recent evidence suggests that this is associated with increased rather than reduced capillary flow. This loss of blood flow regulation appears to be related to peripheral sympathetic denervation, however, similar blood flow

abnormalities in some diabetics apparently free from neuropathy suggests that either peripheral sympathetic neuropathy is one of the earliest forms of neuropathy or that in these subjects the blood flow abnormalities are related to some other disturbance.

1.5.2 Autoregulation and postural control of blood flow.

Autoregulation depends on normal vascular smooth muscle function. Impaired autoregulation has been demonstrated in the brain (Kastrup 1986), and kidney (Parving 1984). Faris (1983) using the Xenon clearance technique found impaired autoregulation in the foot in response to elevation, and Kastrup (1985) using the same technique found evidence for the loss of autoregulation on dependency in the subcutaneous tissue of the feet of diabetic subjects with arteriolar hyalinosis. Kastrup (1987a) has also shown that this abnormality does not reverse with improvement in diabetic control suggesting that it is related to a structural rather than metabolic defect. In contrast, Hilsted (1979) also using Xenon clearance found normal autoregulation in subcutaneous tissue of the ankle in diabetic subjects with severe complications. The reasons for these conflicting results is not obvious, except that slightly different methods were used. Whereas Hilsted did not disturb the local circulation, Kastrup abolished the veno-arteriolar reflex using lignocaine at a dose which was claimed not inhibit the myogenic autoregulatory response in normal smooth muscle. An increase in the sensitivity of diabetic smooth muscle to lignocaine rather than loss of autoregulation may explain why Kastrup's findings differed from those of Hilsted.

Hilsted (1979) has examined the veno-arteriolar reflex in subcutaneous tissue in response to lowering the foot 50 cm in diabetics with and without orthostatic hypotension using the Xenon clearance technique. Whereas blood flow reduced by 50% in normal subjects and diabetics

without complications, those with autonomic neuropathy showed complete absence of the veno-arteriolar reflex. In contrast Kastrup (1987a) found normal postural reflexes in diabetics with complications which included subjects with evidence of autonomic neuropathy. Possible explanations for these very different results are discussed in Chapter 5 where the findings are considered in relation with the changes in blood flow detected with the laser Doppler technique.

1.5.3 Hyperaemic skin blood flow.

A considerable number of studies in which a variety of different techniques have been used, demonstrate impairment of hyperaemic blood flow in the skin of patients with diabetes. In early studies, abnormal vasodilation could only be inferred, because the techniques used to measure blood flow were not sufficiently accurate and the methods used to elicit vasodilatation were dependent on an intact autonomic nervous system making separation of vascular from neural abnormalities impossible.

Using the histamine flare response described by Lewis (1926), Starr (1930) was the first to examine microvascular vasodilatation in diabetic skin. The method involves pricking the skin seven times through a drop of histamine to form a 5 mm diameter circle. The normal reaction consists firstly of an immediate red spot followed and obliterated by a wheal, and surrounded by a flare several centimetres in diameter. Responses were graded into, normal (Grade I); delayed- wheal and flare after 5 minutes (Grade II); and absent (Grade III). Of 100 diabetic patients studied, 32 had normal responses, 34 Grade II and 34 Grade III responses. Only 7 of the 50 subjects with atherosclerosis had normal responses. In the remaining 50 subjects, 25 had either Grade II (17 cases) or Grade III (8 cases) responses. Starr concluded that the absence of a response in patients free from large vessel disease indicated the presence of small vessel disease. Hutchinson

(1974, 1983) has also found impaired histamine-induced flare responses in diabetic subjects, but unlike Starr suggested that this represented a defect in the sensory nerves mediating the axon reflex rather than a defect in the small vessels. Similar conclusions have been made by Parkhouse (1988) and Westerman (1987) using iontophoresis of acetylcholine in diabetic subjects with neuropathy.

As an index of blood flow, Handelsman (1952) used the change in foot skin temperature to examine the vascular response to the vasodilator Priscoline (2-benzyl imidazoline hydrochloride) in 16 diabetics and 14 normal subjects. Seven of the diabetics had no response, as did 4 non-diabetic subjects (2 of whom had heart failure and one tabes with trophic leg changes). The authors concluded that a large percentage of diabetics were unable to maximally vasodilate the skin vessels. The imprecise methods used, and the failure to include an adequate control group, the mean age of which was substantially different to that of the diabetics who failed to vasodilate, render this conclusion doubtful.

Megibow (1953) studied digital circulation using plethysmography in 47 diabetic subjects free from peripheral vascular disease and under the age of 55 years. After the administration of nitroglycerine, vasodilatation in the two big toes were compared. Differences between the two sides was taken to indicate digital vascular disease. Interpretation of these studies is difficult since no control group was included and as pointed out by Christensen (1972) differences between the sides may be related to differences in starting sympathetic tone.

Mendlowitz (1953) measured heat dissipation by calorimetry to determine blood flow in the big toes of 38 diabetic patients free of complications and with diabetes less than 10 years duration, and 30 control subjects, all under the age of 50 years. After indirect heating and sympathetic blockade with tetraethyl-ammonium chloride, 9 of the diabetic subjects had a vascular response below the lowest of the normal subject.

This was taken as evidence of "obstruction of the smallest channels in the foot". Similarly, Bárány (1955) found reduced heat dissipation measured by calorimetry, following indirect heating and after nerve blockade, in 20 of 120 young diabetic subjects. He concluded that capillary circulation was subnormal. However, the calorimetric method does not be accurately reflect blood flow particularly at hyperaemic flow rates (Christensen 1972).

Faris (1982) has determined skin vascular resistance in diabetic patients with peripheral vascular disease using external counter pressure over the site of intradermal injection of radio-isotope in the skin of the calf maximally vasodilated by histamine injection. Since vascular resistance was increased in the diabetic group compared to a non-diabetic group with a similar degree of peripheral vascular disease, it was suggested that the findings related to microvascular disease in the skin. The test relies on the production of maximum vasodilation. Holloway (1980) has shown that histamine does not produce maximum vasodilatation; the response is considerably less than that produced by heating, furthermore the response is dose dependent. The differences observed could thus be explained by differences in vascular response to histamine or differences in histamine diffusion in diabetic tissue. Moreover, if as suggested by Fauchald (1985) interstitial fluid volume is increased in diabetic tissue, dilution of the histamine may explain the reduced vasodilatation in the diabetic subjects.

Kastrup (1987c) investigated the distensibility of skin blood vessels in the dorsum of the foot using the Xenon clearance technique. The method consists of injecting Xenon and histamine together; the latter to relax the vascular bed. Blood flow is then measured before and after lowering the foot 50 cm. Since the vascular bed is paralysed, the veno-arteriolar reflex is abolished. Lowering the leg increases transmural pressure and distends the vessels. The relative increase in blood flow was significantly less in diabetic subjects with (24%) and without (48%) complications, compared with non-diabetic subjects (79%). Although these findings suggest that the vessels are

less distensible, the question previously raised regarding the use of histamine also applies to this study.

Several studies have claimed that the abnormalities in transcutaneous oxygen measurements found in the skin of patients with diabetes indicate impairment of microvascular blood supply. Using the oxygen electrode at 37 °C, Ewald (1981) found a reduction in peak $TcPO_2$ following vascular occlusion in children with diabetes without signs of microvascular disease. Similar findings were reported in adults by Railton (1983) and Haitas (1984). Although as claimed this may represent a reduction in microvascular post-occlusive reactive hyperaemia, the differences could be related to skin thickness, capillary density, oxygen diffusion, oxygen affinity and the metabolic rate of the underlying tissue. Using the probe at 44 °C, Railton (1983), Gaylarde (1988), and Breuer (1988) have found reduced resting $TcPO_2$ measurements in the feet of diabetic, although Gilbey (1989) has failed to confirm this in diabetic patients with autonomic neuropathy and nephropathy. The former authors have suggested that the abnormality indicates reduction in the ability of the microvascular bed to vasodilate in response to heating. As previously discussed other abnormalities may explain for these findings.

Blood flow post occlusive reactive hyperaemia has also been examined in diabetic subjects using capillary microscopy and laser Doppler techniques. Following 1 minute of arterial occlusion, time to peak capillary blood velocity in finger nail fold capillaries has been demonstrated to be significantly prolonged in both insulin (Tooke 1985) and non-insulin dependent diabetic subjects (Pazos-Moura 1990) and in the latter study peak response was reduced. Walmsley (1990) using laser Doppler flowmetry has also demonstrated reduced peak flow blood flow responses post-ischaemia in diabetic subjects. Similarly, Newrick (1988) found that the peak hyperaemic response in the soles of the feet after the ischaemia of standing was significantly reduced and prolonged in diabetic patients with neuropathy.

Prolonged time to peak and reduced peak responses may represent impaired myogenic responses due to reduced clearance of metabolites, rheological abnormalities or increased vascular resistance.

Further evidence supporting reduction in precapillary vasodilatation comes from the studies of finger nailfold capillary pressure by Tooke (1980). In young insulin dependent diabetics, resting capillary pressure was not significantly different from that in normal subjects but post occlusion, capillary pressure was significantly less.

1.5.4 Structural microvascular abnormalities and structure/ function relationships in diabetic skin.

Although it has been assumed that structural abnormalities may be implicated in the pathogenesis of the previously described blood flow abnormalities and in foot ulceration, histological studies of the foot skin microvessels, as distinct from studies in other areas of skin, are relatively few, furthermore the relationship between structure and function has seldom been explored.

Goldenberg (1959) was the first to identify pathological changes in the diabetic foot microvessels considered to be specific for diabetes. He examined by light microscopy, 92 diabetic and 30 non-diabetic amputation specimens. Whether the studies were of muscle or skin vessels was not stated. The major findings were of endothelial proliferation, endothelial thickening, and the deposition of PAS positive material in the vessel walls. These abnormalities were considered to be causally related to diabetic foot ulceration as they were present in all the diabetic specimens but in only 3 of the non-diabetic specimens. This study has received much critical attention (LoGrefo 1984) particularly as subsequent prospective studies with clear documentation of diabetic status and 'blinded' histological assessment have failed to identify endothelial proliferation.

Handelsman (1962) suggested that the changes described by Goldenberg may have been the result of ischaemia, and therefore examined the forearm skin from 19 diabetic subjects (both insulin treated and non-insulin dependent) and 13 normal subjects, using light microscopy. He confirmed Goldenberg's findings, but as with that study abnormalities were based purely on subjective assessment. There was marked proliferation of endothelial cells and an increase in PAS positive basement membrane-like material in the vessels of diabetic subjects. Furthermore, all 6 subjects with diabetes of long duration and complications fell into the severest grade of PAS positive vessel wall thickening in comparison with only 2 of the 13 of short duration. This suggested the involvement of these changes in the development of complications.

Aagenaes (1961) also using using light microscopy and a subjective scoring system reported increased PAS positive material in the small vessels from the pulps of the fingers and toes of 22 diabetic subjects compared with 9 normal subjects. The abnormality was greatest in those with diabetic complications and in whom diabetes had developed before the age of 40 yrs. Unlike Goldenberg (1959) and Handelsman (1962) no endothelial cell abnormalities were found. In the same study, a limited electron microscopic examination of the skin from the finger pulps of 2 normal subjects and 4 diabetic patients was attempted. It was stated that "most of the capillaries from the diabetic patients were found to have far thicker walls". No quantitative analysis was performed.

Säve-Söderbergh (1966) examined by light microscopy the small blood vessels of the feet in 38 insulin treated diabetic subjects and 17 normal subjects. Vessel wall thickness, assessed in a semi-quantitive manner, showed a positive correlation with age, duration of diabetes and retinopathy status. By subjective assessment, endothelial proliferation was not a feature of the vessels from diabetic subjects. There was relationship between vessel wall

thickness and the increase in toe temperature following indirect heating, a crude method of assessing vasodilator capacity.

The light microscopic study by McMillan (1966) of forearm skin is purely qualitative, the vessels being considered as abnormal, intermediate, or normal. As with most other studies there was an association between the presence of diabetic complications and the degree of PAS positive staining.

Banson (1964) examined the skin capillaries in the toes of amputation and post mortem specimens from 18 diabetic and 17 non-diabetic subjects by electron microscopy. Endothelial cell proliferation was not evident but capillary basement membrane thickness in the diabetic group (1.33 ± 0.12 μm) was significantly increased compared with that in the control subjects (0.59 ± 0.12 μm; p<0.001). This study has been criticised by Friederici (1966) on the basis that the subjects had peripheral circulatory disturbances. He was unable to find any significant difference in basement membrane thickness between diabetic and non-diabetic subjects when capillaries from the dorsum of the fingers were examined.

Ajjam (1985) has undertaken a quantitative study of dermal microvessels from the forearm. The study included both non-insulin dependent and insulin treated diabetics as well as patients with and without peripheral vascular disease. Since the study was limited to light microscopy, differentiation between capillaries, arterioles and venules would have not been possible; furthermore the precision and accuracy of the measurements will have been limited. Nevertheless, a significant increase in vessel wall thickness was observed in diabetic patients (Type I and II) with small and with large vessel disease. A significant reduction in vascular luminal size was observed in the same groups. Vascular responses were assessed by measuring the temperature change following vascular occlusion , the extent of the flare response to intradermal histamine, and the area of hyperaemia following topically applied Transvasin (ethyl nicotinate). Although these methods are crude, vascular responses were found to be reduced in all

diabetic groups. The authors do not discuss the relationship between these parameters and the structural abnormalities. Presumably none was found, which may not be surprising considering the methods used.

As previously described Kastrup (1978) using Xenon clearance demonstrated reduced distensibility of microvessels in the foot. Skin biopsies were taken from the same individuals. A significant inverse relationship was found between vessel distensibility and the degree of arteriolar hyalinosis based on a crude semi-quantitative scoring of the amount of PAS positive staining in the vessel wall.

In contrast, Katz (1989) using electron microscopy was unable to demonstrate structural abnormalities in forearm skin capillaries in Type II diabetic subjects. This may be related to the selection of patients free from complications and, as later discussed, the choice of forearm skin.

A number of comments can be made regarding the aforementioned studies. The great majority have employed light microscopy which provides insufficient assessment of detailed pathological changes and cannot reliably distinguish capillaries from arterioles and venules. In the majority, the methods used to assess microvascular abnormalities have been semi-quantitative or purely qualitative. Some studies have not been blinded with respect to which specimens were from diabetic and non-diabetic subjects. In many studies, crude subjective scoring systems had been used to determine the degree of capillary wall and basement membrane thickness. Finally, in many studies the biopsies have been taken from sites other than the foot and may therefore have no direct relevance to foot complications. Thus, the subjective endothelial proliferation described by Goldenberg and Handelsman has not been confirmed by electron microscopy. Increased capillary wall and basement membrane thickness has been found in some studies and not in others. It is noteworthy that whereas thickening of the basement membrane has been demonstrated in all studies of foot skin, those studies that have failed to confirm this (Katz 1989, Friederici 1966) have

been performed on skin from the forearm or finger. This difference may be explained by Vracko's (1970) demonstration that the relationship of increasing skeletal muscle capillary basement membrane thickness with increasing vertical distance from the heart is exaggerated in subjects with diabetes. Thus, there may be no significant difference in basement membrane thickness in diabetic and non-diabetic subjects when upper limb skin capillaries are compared, whereas in the same subjects, the diabetic group may demonstrate significantly increased basement membrane thickness when the foot skin capillaries are examined.

A number of studies have attempted to measure the capillary luminal diameter by in vivo assessment of erythrocyte column width in the nailfold using capillary microscopy. Landau (1960) found a greater percentage of capillaries showing 'venous congestion' (diameter >12 μm) and severe narrowing of the arteriolar limb (diameter <3.5 μm) in diabetic subjects. Karlander (1985) using a purely subjective means found a higher frequency (38%) of dilated capillaries in the nailfold of the toes of diabetic subjects compared with non-diabetic subjects (18%). Pazos-Moura (1990) found no difference in finger nailfold capillary width. Flynn (1988) found an increase in the erythrocyte column width (10.6 μm) in diabetic subjects with neuropathy compared with non-diabetic subjects (8.1 μm). Although these studies appear to show that capillary diameter is increased in diabetic subjects, as pointed out by Flynn (1988), differences may simply be related to reduction in the marginal layer of plasma sheathing the erythrocytes. Furthermore, Fagrell (1977) has shown that erythrocyte column width increases with skin temperature and blood flow. Thus, the wider capillary diameters in the diabetic subjects may simply reflect increased capillary flow and greater skin temperature. In this respect it is interesting to note that in Flynn's study the diabetic subjects with peripheral neuropathy had significantly higher skin temperatures and capillary blood flows than the control subjects.

1.6 THE HAEMODYNAMIC HYPOTHESIS IN THE PATHOGENESIS OF DIABETIC MICROVASCULAR COMPLICATIONS.

Parving (1983) was the first to put forward the haemodynamic hypothesis for the development of microangiopathy. According to the hypothesis, early in the disease, loss of blood flow regulation results in raised microvascular flow and increased capillary pressure which damages the microvascular bed and eventually leads to microvascular sclerosis and limitation of capillary perfusion. There is considerable evidence in the retinal and renal circulations to support this hypothesis (Parving 1983, Zatz 1986, Tooke 1989). Is there any evidence in the skin microcirculation?

As previously described many studies have demonstrated increased blood flow in subjects with peripheral neuropathy. However, according to the hypothesis the increased perfusion should precede the development of complications. Gunderson (1976) and Christensen (1970) found increased forearm blood flow soon after diagnosis and during periods of poor metabolic control in subjects without complications. Moreover, although resting blood flow is not elevated in the majority of diabetic subjects without clinical neuropathy, several studies (Corbin and Young 1987, Archer 1984, Flynn 1989) have shown that some diabetics in this group despite satisfactory diabetic control have abnormally elevated foot blood flow. Thus, perhaps early on in the disease, interspersed between periods of normal blood flow control are periods when regulation is lost, either as the result of sub-optimal control, elevated metabolic rate or perhaps transient peripheral sympathetic dysfunction. This would explain why at any one time only a proportion of patients without complications are found to have elevated blood flow. Repeated episodes of high flow and elevated capillary pressure may then result in minor but progressive vascular damage, this may further impair vascular responsiveness, with the process becoming self-escalating. According to the haemodynamic hypothesis this would lead to

progressive vascular sclerosis and limitation of microvascular perfusion. The previously described histological and functional studies provide some evidence in support of this.

In attempting to attribute the microvascular changes in the foot to the haemodynamic hypothesis, a number of special features of the foot skin circulation need to be considered. It could be argued that if as suggested, increased shunt flow results in 'capillary steal', the capillary being protected from high blood flow would not be expected to not develop structural changes. However, the study by Flynn (1989) suggests that 'capillary steal' does not occur and that flow is in fact increased. Even if there were 'capillary steal', open arteriovenous shunts would result in elevated venous limb capillary pressures. The structural hallmark of diabetic microangiopathy is basement membrane thickening. Williamson and Kilo (1977) suggested that elevated capillary pressure provides the stimulus to basement membrane formation. In support of this hypothesis is the demonstration by Williamson and Kilo (1971) that in normal subjects and the giraffe, basement membrane thickness increases linearly with the vertical distance from the heart and hence with intracapillary hydraulic pressure, and that in infants where postural hydrostatic gradients have not developed this variation does not exist; furthermore Longhurst (1975) showed that cardiac failure in non-diabetic patients was associated with an increase in basement membrane material. Recent studies confirm increased finger nailfold capillary pressure at heart level, even in short duration diabetic subjects free from complications (Sandeman 1990). In the dependent foot the capillaries are subjected to the highest capillary pressures in the body. They are partially protected by postural pre-capillary vasoconstriction which limits the rise in capillary pressure. Were this reflex to be lost in patients with diabetes, the capillary pressures in the dependent limb will be even more elevated with respect to those at heart level which may explain Vracko's (1970) demonstration that the postural variation in

basement membrane thickness is exaggerated in diabetic subjects. An increased capillary pressure would be expected to be associated with increased filtration, increased extravasation of macro-molecules and plasma proteins, and with time increased basement membrane thickening. It is possible to envisage that such changes would eventually impair capillary function, result in further microvascular sclerosis and further alter the intricate regulation of microvascular blood flow.

This aim of the work presented in this thesis was to investigate the microcirculation of the foot skin in patients with diabetes to determine whether there exists any evidence of abnormal blood flow regulation which would support the haemodynamic hypothesis, and to determine whether microvascular abnormalities exist which may contribute to the impaired healing of diabetic foot lesions. The first two experimental chapters examine the skin microvascular vasodilator response to injury and its relationship to microvascular structure in particular the basement membrane thickness. The last two experimental chapters examine the regulation of blood flow during change in posture and the effect of such changes on fluid filtration in the skin of the diabetic foot.

CHAPTER 2

METHODOLOGY

2.1 STUDY CONDITIONS

Environmental temperature is a major determinant of skin blood flow particularly in areas of the hands and feet supplied by arteriovenous anastamoses (Wilson 1937). It was thus necessary to conduct all the vascular studies under temperature controlled conditions. At Charing Cross Hospital, studies were performed in the Microvascular Laboratory, a temperature controlled room, maintained at 22.0 ± 1 °C by the central hospital heating system. When necessary an independent air conditioning and heating unit mounted on an exterior wall would automatically over ride this system. Room temperature was regularly checked (at approximately thirty minute intervals), and was never allowed to vary by more than 1 °C during a study. All studies were conducted within the above temperature range with the exception of those in which the effect of posture on fluid filtration in the foot was being studied (Chapter 6). Here, tighter control was considered important (22.0 ± 0.5 °C). In studies performed at the Ipswich hospital (Chapter 4) the room temperature was maintained by the central hospital heating system at a slightly higher temperature (23.0 ± 1.0 °C).

Subjects were were acclimatised, resting on the measurement couch for at least 30 minutes prior to any blood flow measurements. The subjects were lightly clad and the limb or area of skin being studied was exposed to the ambient temperature.

During the early studies radiant heat from sunshine through the windows was noticed to promote vasodilatation. Sunshine was therefore excluded by heavy curtains and the room was restfully lit by fluorescent

room lights. Ambient noise was kept to a minimum as sudden noise is known to provoke cutaneous vasoconstriction (Abramson 1940).

2.2 SKIN MICROVASCULAR BLOOD FLOW MEASUREMENT

Skin microvascular blood flow was measured using a laser Doppler flowmeter. This method was chosen as it allows measurements to be made in minute areas of tissue, does not disturb the local vasculature, can be used in a variety of postures and unlike other methods allows continuous measurements to be made. For a comparison with other methods the reader is referred to Chapter 1.

In the present studies the Periflux laser Doppler flowmeter (Perimed Ltd, Stockholm, Sweden) was used. This is shown in Figure 2.1 and simplified block diagram of the instrumentation is shown in Figure 2.2. The instrument contains a low power of 2 mW Helium-Neon laser. This emits continuous monochromatic radiation within the visible wavelength range at 632.8 nm. Light from the instrument is conducted by an optical fibre to the skin surface. Within the skin, the photons are scattered by stationary structures such as connective tissue and by moving objects, principally erythrocytes. The frequency of a photon scattered by a stationary object remains unchanged. However, a photon which collides with a moving blood cell undergoes a frequency shift which according to the Doppler effect is related to the velocity of the cell's movement. In practice each photon undergoes multiple frequency shifts depending on the number of cells encountered. In addition, because red blood cells move at different velocities in the microvascular network, and because the light is scattered at many different angles, the Doppler shifted light is not of a single frequency but a spectrum of frequencies. A small proportion of the light (0.1%) is backscattered to two efferent optical fibres contained within the same measuring probe as the afferent fibre. These efferent optical fibres transmit
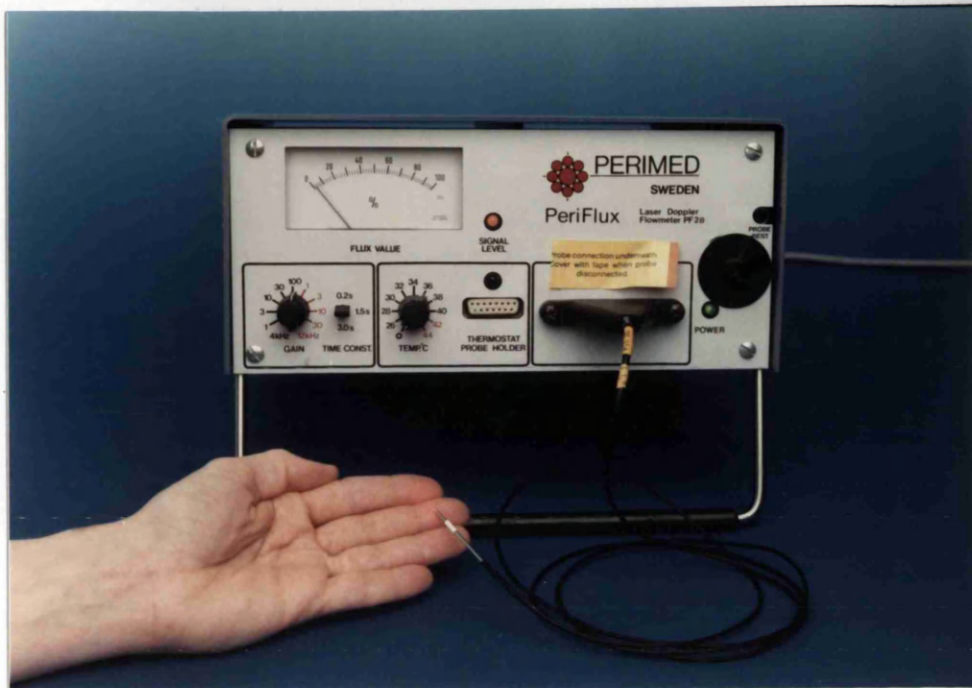
Figure 2.1: Photograph showing the Periflux PF 2b.
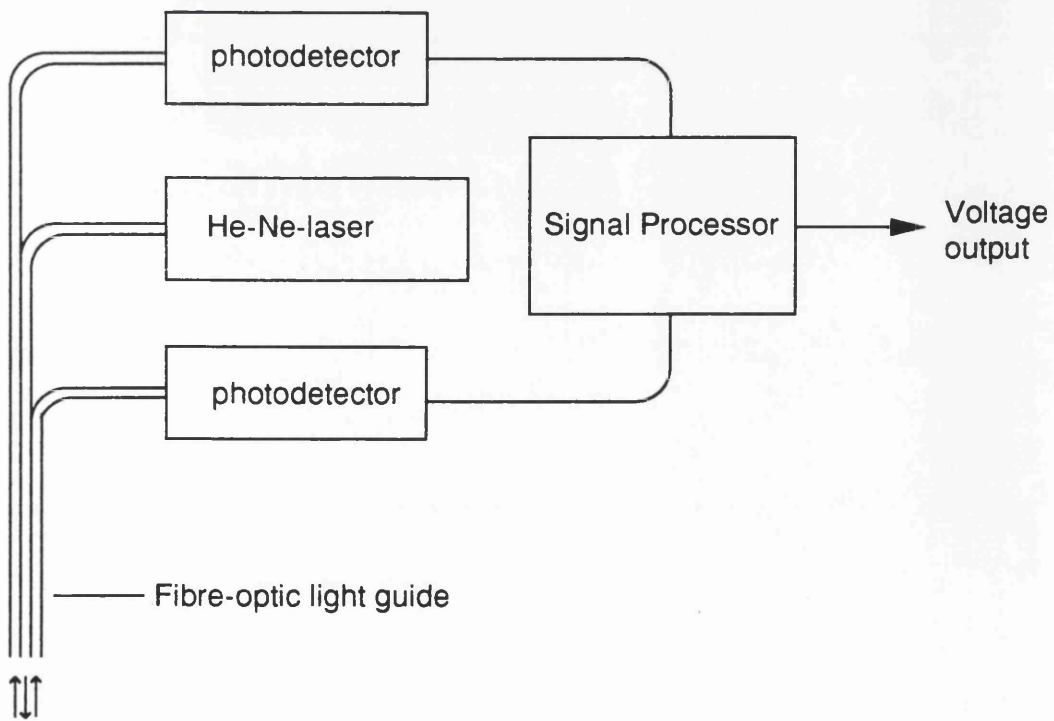
Figure 2.2: Block diagram of the laser Doppler flowmeter

the light to the instrument's two photodetectors. In each detector Doppler shifted and unshifted light is mixed resulting in the creation of beat frequencies equal to the Doppler shifts (Bonner 1981).

From the above description, it is clear that light of a single frequency is fundamental to the instrument. If a light source emitting multiple frequencies were used, distinguishing between shifted and unshifted light backscattered from the tissues would be impossible. The output from the laser contains a few "rogue" frequencies which if unsuppressed would interfere with the bandwith of interest, making continuous measurement impossible. To overcome this problem, a dual channel arrangement of efferent fibres and photodetectors is employed. Thus, interference as well as some external source noise will be in phase in the two channels and can be suppressed. However, Doppler shifted photons in the two channels will not be in phase because they originate from different, though adjacent parts of the microcirculation. This method allows for amplification of the signal to noise ratio. The signal is subjected to an electronic transformation in which the tissue perfusion is mathematically related to the unprocessed Doppler signal using an algorithm (Nilsson 1980a). The final voltage output of the instrument is related to both the velocity and the number of moving red cells, irrespective of their direction of movement, within a hemisphere of tissue 1-1.5 mm in diameter (the depth from which the backscattered light originates- Stern 1977). Because the measurement is independent of the direction of red cell movement it is sometimes termed red cell flux. In the studies later described, for ease of use this measurement is referred to as laser Doppler blood flow, although the more correct term would be laser Doppler flux.

The studies performed at the Charing Cross Hospital (all Chapters with the exception of Chapter 4) employed the Periflux, Model PF1 Mark VII. At Ipswich Hospital a more recent model, the Periflux PF2b was used (Figure 2.1). The instruments are essentially similar with the exception that

the Periflux PF2b has finer fibre-optic fibres which help to reduce artefacts produced by movement of the fibre-optic cable.

Since the completion of the studies described in this thesis, two relatively new laser Doppler flowmeters have been introduced; the TSI Laserflo (TSI Incorporated, St. Paul, MN, USA) and the Moor MBF3 laser Doppler flow monitor (Moor Instruments, Devon, UK.). These permit the signals related to red cell velocity and the proportion of shifted backscattered light (equivalent to the volume of vascular tissue) to be analysed separately. In comparison with the Periflux laser Doppler flowmeters these instruments use a laser diode which emits light at a frequency of 780 nm and detects flow from a slightly deeper depth of 2-3 mm.

2.2.1 Use of the Periflux laser Doppler flowmeter

Before being used the instrument was allowed to warm up for a period of at least 60 minutes. It was then zeroed against a white background as recommended by the manufacturers. A calibration standard supplied by the manufacturers consisting of a colloidal suspension of latex particles was used to check the performance of the PF2b at weekly intervals. Adjustments were never found to been necessary. This calibration standard was not available for the studies in which the PF1 was used. After instrument warm up, the probe was placed in the probe holder on the skin surface. Apart from the studies which involved thermal injury, the standard black plastic probe holder was used to support and maintain the probe's contact with the skin surface. This was affixed to the skin with double sided sticky discs (Double-Stick Discs- 3M, St Paul, Minnesota, USA). Probe holders used on the pulp of the big toe were heat moulded so as to conform more closely to the curvature of the toe. The 1.5 meter fibre-optic line from the instrument was secured where appropriate by adhesive tape to fixed structures in order to minimise artefacts resulting from sudden movements of the line.

Recordings of the voltage output from the instrument were made on a Vitatron (model 2001) two-channel pen recorder. This instrument allows a variety of paper speeds to be selected. Generally a speed of 20 or 50 mm/minute was used. Prior to each experiment the chart recorder was calibrated by operating the full scale deflection switch on the Periflux. The PF1 produced a full scale deflection of 4.77 volts. The more recent models such as the PF2b have maximum outputs of 10.0 volts. With the former instrument the pen recorder was adjusted so that the full scale deflection on the Periflux was equivalent to 0.477 times the full width of the scale on the recording paper, giving a theoretical 10 volts full span of the paper. This was done to facilitate easier reading of the results from the traces. With the PF2b the recorder was adjusted so that the full scale deflection of 10 volts gave a full scale deflection on the paper.

The Periflux PF1 has 3 different gain settings- x1, x10, and x100. Blood flow in unstimulated skin on the dorsum of the foot is low and was thus measured at the x100 gain setting. When made hyperaemic, for example when heated, the x10 gain was used. Resting blood flow in the pulp of the toe is generally high because of the large numbers of arteriovenous anastamoses and was usually measured using the x10 setting. Skin blood flow was seldom high enough for the x1 gain to be used. With the PF1, blood flow never exceeded the full voltage output of 4.77 volts, similarly the PF2b never exceeded 10 volts. The PF2b has the additional settings of x3 and x30 which were used when appropriate.

On both the Periflux PF1 and PF2b instruments a switch allows the operator to select one of two bandwidth filters, a wide waveband (12 kHz) or a narrow waveband (4 kHz). At low blood flow rates, as recommended by the manufacturers, the 4 kHz filter was used, since the Doppler shifts are usually less than 4 kHz. In these circumstances, use of the 12 kHz filter would allow unwanted 'noise' to be represented in the instrument's output. At high blood flow rates the 12 kHz filter was selected since Doppler shifts

then exceed 4 kHz. Use of the 4 kHz filter in these circumstances would cut part of the signal and underestimate of the blood flow. When recording a large change in blood flow, such as that which occurs when the skin is heated, a change from 4 kHz to 12 kHz was necessary.

Time constants of 0.2, 1.5 or 3 seconds can be selected by a switch located on the front of the flowmeter. Artefacts due to sudden movement of the fibre-optic line could be easily identified on the recording trace as sudden upward deflections ('spikes') when either the 0.2 or 1.5 seconds time constants are used. A longer time constant averages the output signal making movement artefacts difficult to identify in the 'smoothed out' trace. In all the experiments a time constant of 1.5 seconds was found to be appropriate.

When used in tissues in which blood flow has been arrested, the laser Doppler flowmeter produces a small signal believed to be due to small to- and fro- movements of red cells as fluid is exchanged across vessels and to the movement of macromolecules (Nilsson 1990). Thus, after each experiment an arterial occlusion was performed to determine this 'biological zero' which was taken into account when calculating the laser Doppler blood flow.

Blood flow recordings frequently showed marked spontaneous variations in frequency and amplitude making estimations of average blood flow by inspection alone unreliable. Therefore a Hipad digitizing pad (Houston Instruments, Austin, Texas, USA) linked to a Research Machines 380Z computer (Research Machines Ltd., Oxford, UK.) was used to measure the average flow. In practice the recording trace was placed on the digitizing pad and by means of a cursor the area between the 'biological zero line' on the paper and the ink trace was entered into the computer. From the calculated area and the recording time interval the average flow was ascertained.

2.2.2 Calibration and Validation of the Laser Doppler Flowmeter

The laser Doppler technique has been validated against mechanical models, isolated tissue and in vivo. In various mechanical models laser Doppler flowmeters perform well (Nilsson 1980, Smits 1986), however, calibration in living tissue is more difficult since often there is no good reference standard, other methods do not measure blood flow in precisely the same volume of tissue, and the optical properties of different tissues vary, making calibration in absolute units valid only for the conditions under which a particular study has been performed.

Shepherd (1982) found a close correlation (0.97) between intestinal mucosal blood flow measured using the laser Doppler flowmeter and total intestinal blood flow measured using an electromagnetic flowmeter. Similarly, good correlations have been obtained in renal tissue when compared with blood flow measured by microspheres (Smits 1986) and the accumulation of ^{51}Cr labelled red cells (Roman 1985). However, in all these studies although the correlations remained good, the slopes of the correlations were different at different tissue sites in the same preparation.

Tyml (1985) has compared the laser Doppler method with the measurement of red cell flow by video microscopy in the sartorious muscle preparation of unanaesthetised frogs. When the two methods were used at the same site, excellent correlations were found with altering perfusion rates (0.83-0.99). Furthermore, over several different sites, good correlations in resting blood flow were obtained (0.76-0.95) provided that the penetration of the laser light was limited to 0.3-0.4 mm. However, if the laser light was allowed to penetrate to a greater depth correlation was poor. This suggests that important spatial variations in flow exist in areas separated by more than 0.4 mm. This suggestion is supported by the finding of marked spatial variations in resting blood flow measured by the videomicroscope in areas on the surface of the muscle separated by less than a millimetre.

In the skin, the laser Doppler flowmeter has been compared with venous occlusion plethysmography, Xenon clearance, thermal clearance and capillary microscopy. Tooke (1983) compared the laser Doppler method with the actual measurement of red cell velocity using dynamic capillary microscopy in the nailfold of the finger. When resting flow was measured there was poor correlation between the methods, but this as suggested by Tooke probably related to the separation of the measurement areas by 2.5 mm, the relative limited number of subjects studied, the narrow range of skin temperatures, and the possibility that at this site the laser Doppler flowmeter may be measuring blood flow in anastamotic vessels as well as capillaries. In contrast, when the blood flow responses to venous occlusion and release of arterial occlusion were examined there was broadly comparability between the techniques. Hassan (1988) has also compared capillary microscopy and laser Doppler flowmetry when examining the effect of changes in posture on nailfold blood flow. Good comparability between the two methods was found, blood flow falling by $91.0 \pm 2.0\%$ and $85.0 \pm 3.0\%$ when measured by capillary microscopy and laser Doppler flowmetry, respectively. Hassan found the correlation between the methods to be between 0.98 and 0.82 in four different studies.

Engelhart (1983) measured blood flow using the Xenon clearance and laser Doppler techniques in the finger tip and the skin fold between the first and second fingers. Whereas the two methods recorded similar changes in skin fold blood flow during reactive hyperaemia, venous pressure elevation, and orthostatic manoeuvres, there was no agreement at the finger tip. Based on the assumption that the Xenon clearance technique measures only nutritive flow, Engelhart suggested that the lack of correlation at the finger tip was due to the inclusion of shunt flow by the laser Doppler, whereas in the skin fold there was better agreement because at this site there is little shunt flow and thus both methods were measuring predominantly nutritive blood flow. In support of the suggestion was the finding that the laser

Doppler flowmeter recorded considerably higher flow in the finger compared with the skin fold, whereas the Xenon clearance method, which does not measure shunt flow, demonstrated little difference.

In comparison to Engelhart's findings, Kastrup (1987) found a far greater fall in laser Doppler measured skin blood flow during orthostatic change than he had found with the Xenon clearance technique. Assuming that the latter technique to be the "gold standard", he concluded that the laser Doppler was not suitable for the measurement of postural changes. These findings contrast with those of Engelhart's in which the same techniques were compared. Furthermore as previously mentioned, Hassan found very good agreement between capillary microscopy and laser Doppler techniques when used to determine the change in blood flow in the nailfold of the toe with the foot lowered 50 cm compared with that at heart level (a fall of 91% and 85%, respectively); far greater than the changes reported with the Xenon clearance technique (50%). This would suggest that contrary to Kastrup's opinion the Xenon clearance technique does not fully reflect posturally induced changes in blood flow, whereas the laser Doppler technique does. The hyperaemia following the trauma induced by the injection may explain why the Xenon clearance technique fails to mirror the large fall in blood flow seen in undisturbed skin.

Johnson (1984) has compared the laser Doppler technique with venous occlusion plethysmography and found excellent correlation coefficients, ranging from 0.90 to 0.98, when forearm skin blood flow responses to whole body heating were measured. Saumet (1986) in similar studies obtained correlations averaging 0.90. Although both studies found good linearity between the methods, the slope of the relationship varied from individual to individual and from site to site in the same individual. Similarly, when the laser Doppler was compared with thermal clearance methods, both Saumat (1986) and Nitzan (1988) found good correlations between the methods (0.86-0.92 and 0.90 respectively), however as with

plethysmography the slopes varied between different areas of skin and from individual to individual.

In summary, comparative studies between the laser Doppler flowmeter and a variety of other methods used in a variety of different tissues yield good correlations and provide strong evidence that the laser Doppler signal is a linear measure of blood flow. However site-to-site and between subject variations exist. These variations probably arise because the laser Doppler samples a very small amount of tissue and is therefore be affected by microregional variations in vascular density, skin thickness, orientation of blood vessels, and ratios of the different types of microvessels in the tissue. For these reasons and because no other method measures blood flow in precisely the same volume of tissue it is not possible to calibrate the instrument in order to produce an absolute measure of volumetric flow. The measurement is thus expressed in arbitrary units of volts. Although site-to-site variations exist, the instrument may be used to compare groups, provided that blood flow is sampled from multiple sites so as to reduce this variation or provided that sufficiently large numbers of subjects are studied and the differences in blood flow are large.

2.2.3 Preliminary Observations with the Laser Doppler Flowmeter

In agreement with Engelhart (1983), in preliminary studies, marked differences in blood flow were recorded when the laser Doppler probe was used in areas heavily supplied with arteriovenous anastomoses such as the pulps of the toes and fingers compared with areas devoid of arteriovenous anastamoses, such as the dorsum of the foot or forearm. An example illustrating such differences is provided in Figure 2.3 where recordings were obtained from the pulp of the big toe and the dorsum of the foot. It was also found that changes in laser Doppler blood flow in the pulp of the
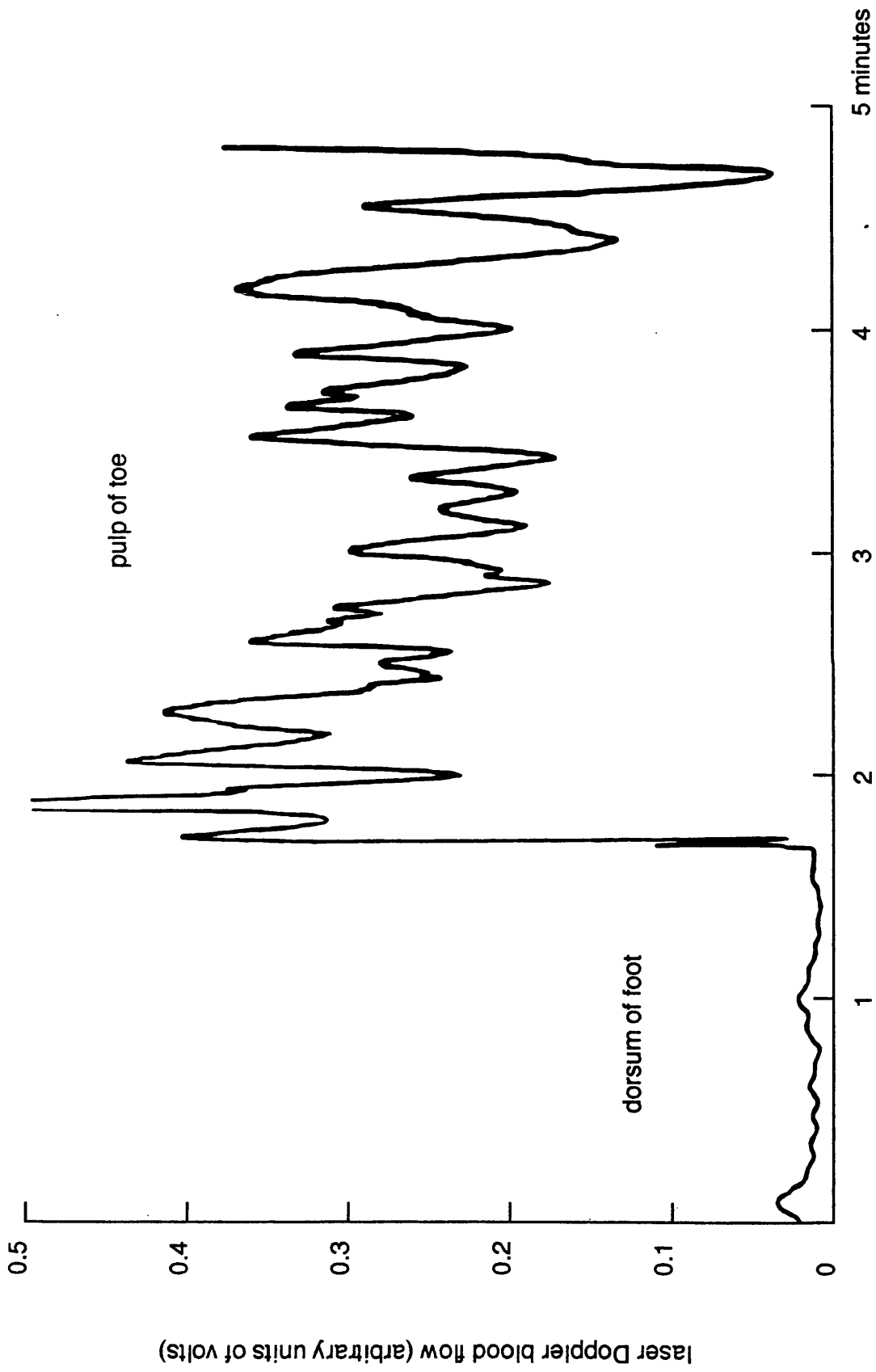
Figure 2.3- Laser Doppler flow recordings from skin in a non-shunt flow area (dorsum of foot) compared with a shunt flow area (pulp of big toe).

toe closely paralleled changes in toe volume, measured at the base of the toe using mercury-in-silastic strain gauge, Figure 2.4. Both these observations strongly suggest that when used in areas endowed with arteriovenous anastamoses the measurement relates to both capillary and shunt flow. Similar conclusions have been reached by other workers (Tooke 1983, Engelhart 1983)

Apart from the differences in measurements made in areas of skin subserving different functions, another problem encountered with the laser Doppler technique was the spatial variation in measurements recorded from areas of skin separated by no more than a few millimetres. This is observation, first made by Tenland (1982) has as previously mentioned been ascribed to microregional differences in vascular supply, but may also relate to differences in epidermal thickness. Bonnor and Nossal (1989) have suggested that epithelial pigmentation does not affect the laser Doppler signal, however because doubt exists over this issue, subjects with pigmented skin were excluded from all studies and the probe was never used over freckled areas. The influence of capillary density and epidermal thickness on the laser Doppler signal were determined in the following studies.

2.2.3.i The effect of capillary density on laser Doppler measurements

Since the instrument's output is related to the number of moving particles within a given volume the result should be dependent on the volume of vascular tissue enclosed within the area from which the light is backscattered. To determine whether this is the case, laser Doppler measurements were made in areas of skin shown to differ in capillary density. The latter was determined in the skin on the dorsum of the middle finger and the dorsum of the foot, using a videomicroscope system, as described later in this chapter. The studies were conducted on 5 normal subjects. Mean capillary density on the dorsum of the middle finger was 100.0 ± 10.6 capillaries/mm^2 compared with 50.7 ± 5.7 capillaries/mm^2 on

the dorsum of the foot. Hyperaemic blood flow response to thermal injury, a reproducible measurement of maximum blood flow (see below) was also significantly greater in the finger compared with the foot (finger- 1.18 ± 0.04 Volts; foot- 0.71 ± 0.11 Volts) suggesting that capillary density does indeed influence the measurement.

2.2.3.ii The effect of epidermal thickness on laser Doppler measurements

Epidermal thickness can vary from around 40 μm on the trunk to as much as 400 μm at the fingertips (Whitton 1973). To determine the importance of epidermal thickness on the laser Doppler measurement, hyperaemic responses (see below) were measured at several different skin sites found to have differing maximum responses. When blood flow was constant a section of skin 120 μm thick, previously removed from the heel pad and kept moist in liquid paraffin was placed between the probe and the skin surface. As Figure 2.5 shows, the signal was reduced but only by approximately 10 % despite skin thickness being increased 2 and 3 fold. Thus, provided similar areas of skin are being examined, minor differences in skin thickness are unlikely to be important when comparing groups.

2.2.3.iii Thermal injury and the use of the heating probe

Heating the skin to 43°C induces mild thermal injury (Storm 1979), abolishes microvascular reflexes and results in maximal vasodilation (Johnson 1986). This was found to produce relatively constant laser Doppler blood flow measurements, uninfluenced by environmental conditions, state of relaxation of the subject and reflex stimuli. For these reasons the measurement of maximum blood flow was found to be ideal for investigating the influence of capillary density and epidermal thickness, as described above.

Both the Perimed PF1 and PF2b are supplied with heater probes which permit the user to examine changes in microvascular perfusion at
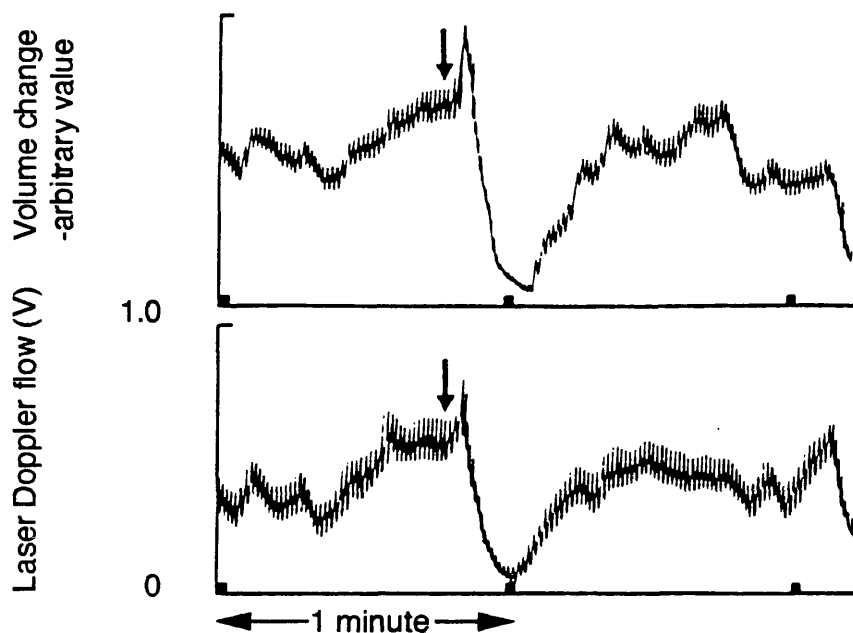
Figure 2.4: Volume changes in the big toe (upper trace) measured using strain gauge plethysmography compared with simultaneously measured laser Doppler flow from the pulp of the same toe. The arrow marks the start of a maximal deep inspiration. Redrawn from original traces.

Laser Doppler signal from hyperaemic skin (V)
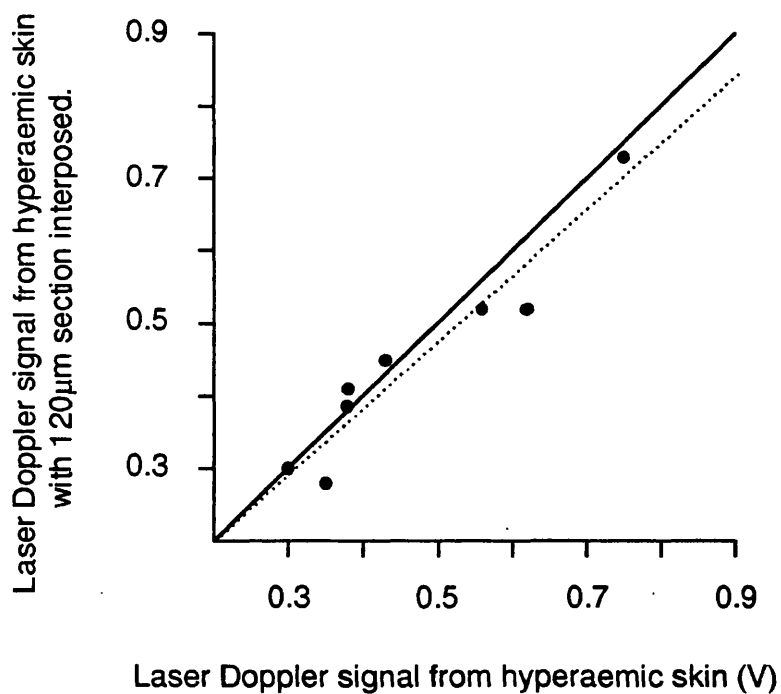
Figure 2.5: The effect of epidermal thickness on the laser Doppler signal. 120μm of skin has been interposed between the probe and the hyperaemic skin surface. Results are in arbitrary units of volts.

different skin temperatures. The heater probe is a 4 cm heated disc with a central hole through which the laser probe is inserted. In practice this heating probe was found to be unsatisfactory. The central area over which blood flow is being measured is not directly heated. The heat distribution over the rest of the face of the probe was found to be uneven, and more importantly the skin did not attain the temperature set on the flowmeter, the final temperature varying with the blood flow. The latter problem arises because the instrument does not contain a feed-back thermostat. According to the manufacturers, the heating element is calibrated at the time of manufacture by placing a small thermistor between its face and a block of styrofoam. The power required to attain various desired temperatures is then determined. In use, the same fixed power is delivered for that particular temperature setting. Naturally the skin will dissipate heat by conduction and by increasing its blood flow. This explains why in practice skin temperature seldom exceeds 40˚C when the probe is set to 44˚C. Others have made the same observation (Cochrane 1985).

To overcome the heater problem, a heating probe based on the type used for transcutaneous oxygen tension measurements was specifically designed by the author. This was kindly manufactured by Dr David Delpy at the Medical Physics Department, University of London. It consists of a cylindrical brass core heated by a small heating element, Figure 2.6 (a). Into the brass core, close to the face which is to be in contact with the skin are two thermistors which feed back to a thermostat control unit. The thermostat regulates the output to the element so as to maintain the surface temperature set by the operator. The circular end face (0.9 cm diameter) of the brass core is held in direct contact with the skin to be heated by a plastic collar, previously affixed to the skin with a double-sided adhesive ring (Double-Stick Discs- 3M, St Paul, Minnesota, USA).

The probe was also designed to deal with the problem of poor within-subject reproducibility due to site-to-site variations in blood flow, by

constructing it so as to permit multiple sampling. This was achieved by eccentrically locating the channel for the laser Doppler probe, Figure 2.6 (b). Rotation of the heater probe within the plastic collar allows blood flow to be recorded from different sites each of which will have been directly heated immediately prior to rotation. In comparison, the manufacturer's heater probe does not directly heat the area from which flow is to be measured. In practice the probe was rotated through 40° for each measurement, thus blood flow was recorded from a total of nine sites.

Figure 2.7 illustrates the response to thermal injury in skin on the dorsum of the foot. Blood flow increases to reach a peak after about 15 minutes, however in the studies later described, to ensure that full vasodilatation was achieved the skin was heated for 30 minutes before recordings were made. The tracing also shows the effect of placing the leg 50 cm below heart level. Dependency normally elicits the veno-arteriolar reflex (Henriksen 1977), however heating inhibits vascular reflexes so that the increase in transmural pressure produced by lowering the foot distends the vessels and further increases blood flow.

To determine the reproducibility of the maximal thermal injury response based on a single site measurement and to compare it with that based on the multiple sampling technique, nine normal subjects were studied on 2 separate occasions separated by 24 hours. Thermal injury was provoked as described above by heating the foot skin to 44 °C for 30 minutes and lowering it 50 cm. When the Periflux PF2b was used the coefficients of variations based on single measurements varied from 15.9 to 40.1%, Table A 2.1. The coefficient of variation was considerably improved when the thermal injury response was based on the average of the nine sites. The coefficient of variation calculated using the method for paired samples (Raggatt 1989) was 8.2%. The coefficient of variation when the the Periflux PF1 was used was 7.6%, Table A 2.2.
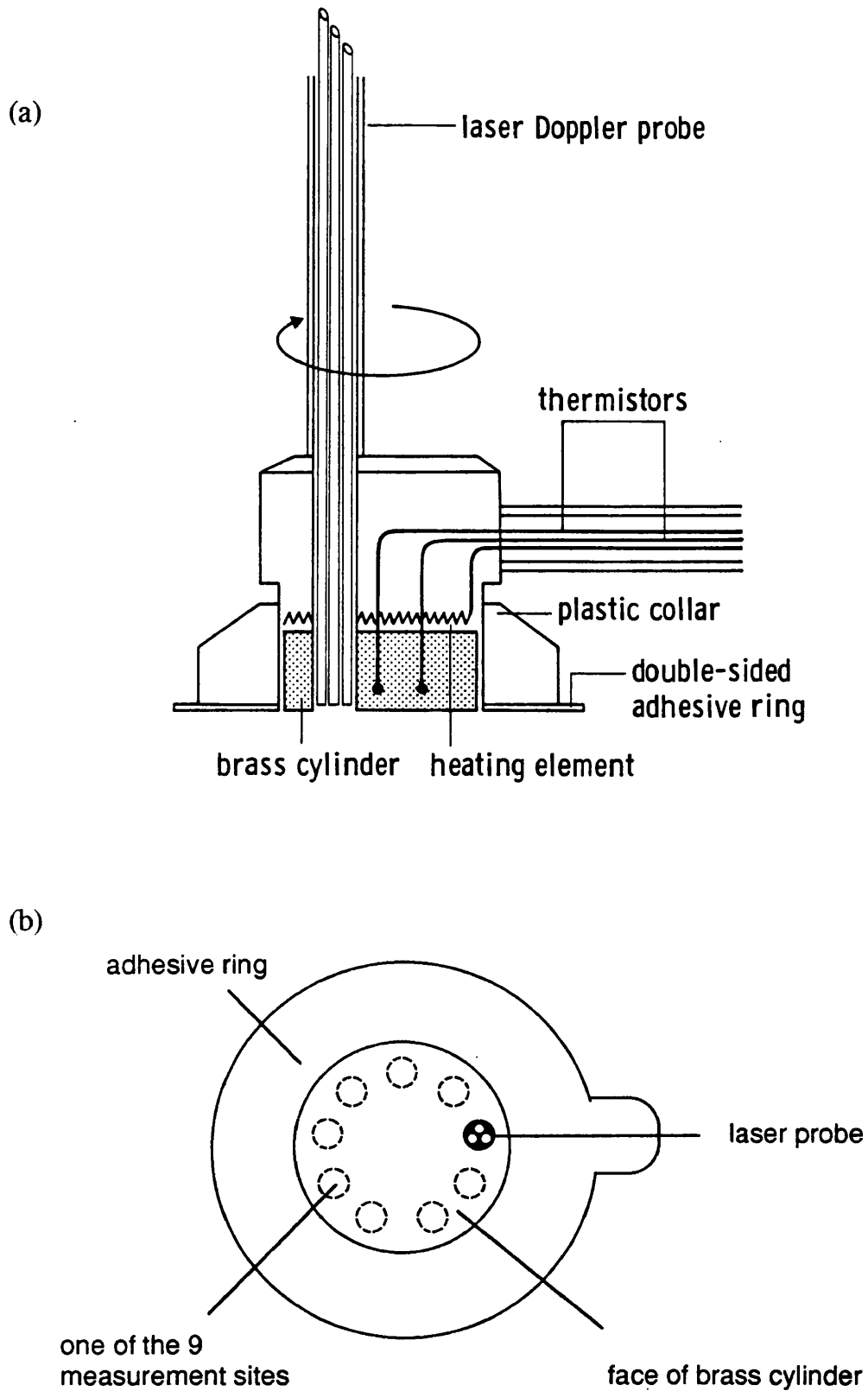
Figure 2.6- (a) Cross sectional diagram of heating probe with modified laser Doppler probe in place. Arrow indicates rotation of the probe within the plastic collar. (b) Skin surface view of probe showing possible points of rotation.
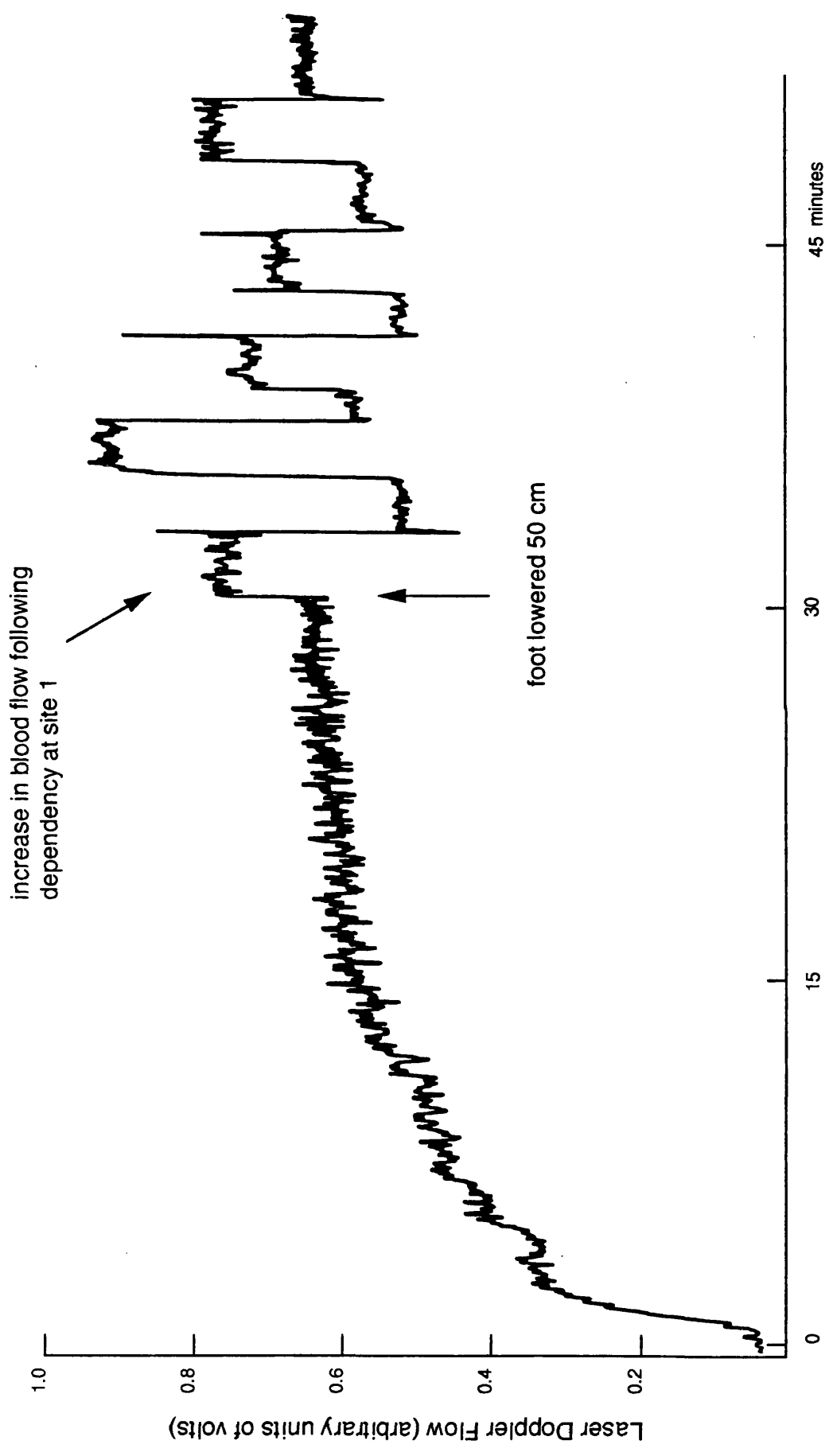
Figure 2.7: This trace shows the changes in skin blood flow recorded from the start of heating. The increase in blood flow following dependency is indicated, and flow from each of the 9 sites appears on the trace.

2.3 TELEVISION MICROSCOPY

A television microscope system was used to determine capillary density. The system consisted of a standard black and white television camera (Hitachi HV720K) mounted directly over the lens using a standard phototube and mount (Leitz 543345 and 512737), Figure 2.8. These were attached to a focusing block which was supported on a stout pillar. Unlike most microscope systems, focusing was achieved by moving the camera and lens rather than the tissue under study. The latter was viewed through a low power objective lens (Leitz UM 10/0.22) which encompassed a field of 1.0 square millimetres. The tissue was illuminated by light from a 50 Watt mercury vapour lamp (Wotan HB050) conducted via a fibre optic cable (Leitz KL155). The image was viewed on a high resolution television monitor (Hitachi VM-AE906/K). The images were stored on magnetic videotape using a high quality VHS format recorder (Panasonic AG6800)

The skin on the dorsum of the foot between the first and second metatarsal heads, was painted with clear nail varnish to render the capillaries visible. The subject sat with the foot under the camera as shown in Figure 2.9. The magnification was measured with a graticule slide in horizontal and vertical planes. As the monitor was found to distort the images at the corners of the monitor, a central circle was drawn which covered an area equivalent to 0.5 square millimetres of skin and avoided the areas of distortion. Images were recorded from two separate areas and later analysed by two independent and 'blinded' observers to derive the mean number of capillaries per mm^2 of skin surface. The correlation between observers performed on 22 recordings was good ($r=0.93$). The coefficient of variation on repeat measurements on nine subjects on two separate days was 10.8%.
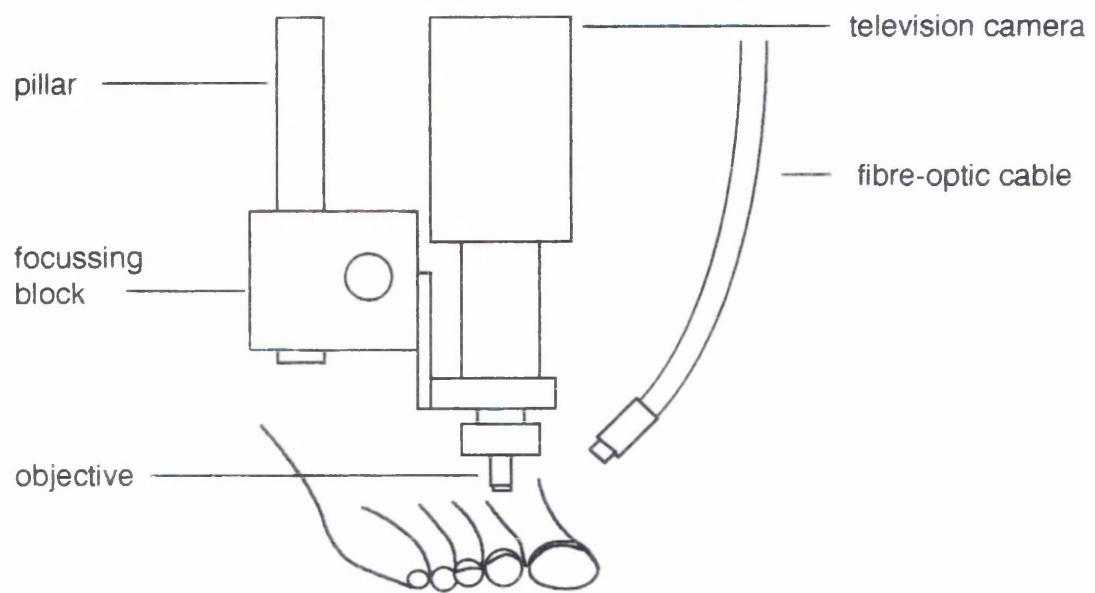
Figure 2.8: Diagram illustrating the television microscope system
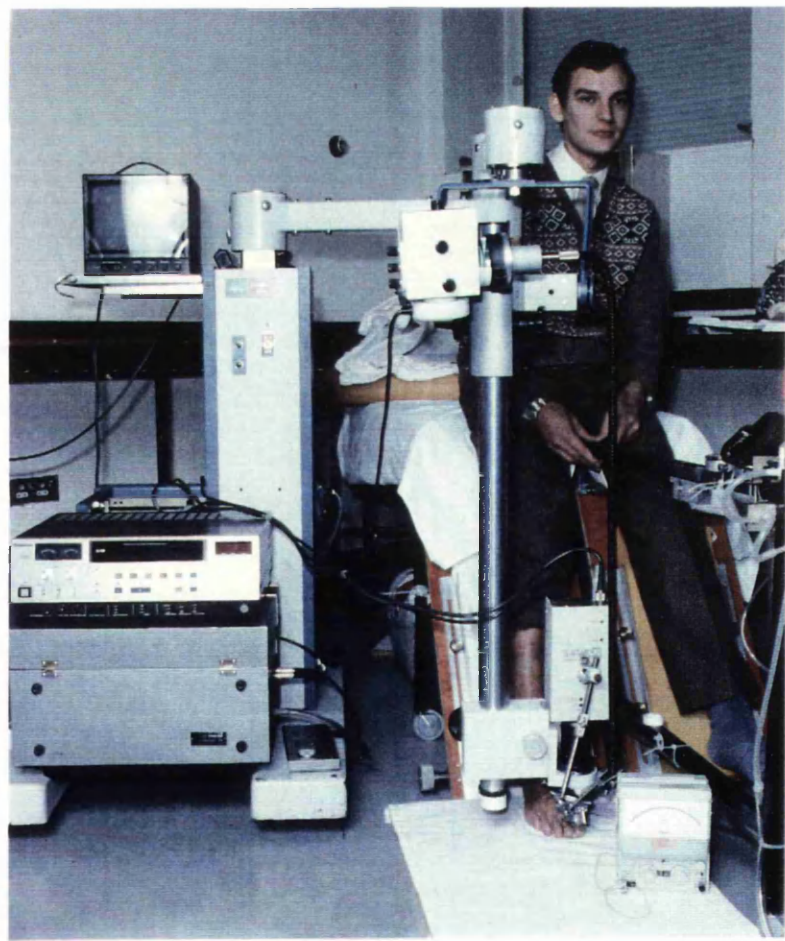
Figure 2.9: Photograph of subject undergoing capillary microscopy

2.4 CAPILLARY PRESSURE MEASUREMENT

I am grateful to Dr JE Tooke who performed all the capillary pressure measurements. A modification of the Landis (1930) microinjection technique described by Levick and Michel (1978) was employed (Tooke 1981). This technique involves cannulation of individual capillaries in the nailfold of the toe by a glass micropipette connected in series to a water filled manometer. The apparatus is illustrated in Figure 2.10.

The micropipettes used for cannulation were made from glass Palladium tubes and prepared using a micropipette puller. The blind end of the pulled pipette was converted into a cannula by carefully breaking and then grinding the tip viewed under a stereomicroscope. The pipette was filled with sterile heparin solution (5000 units/ml) which had been lightly coloured with sterile Patent Blue V dye (30μl/ml heparin solution) to aid visualization of the pipette. The pipette was held in a perspex pipette holder modified from a design of Michel (1969). The large end of the micropipette was connected in series through the pipette holder to a water manometer. A large bore syringe connected via a T junction to the manometer line was used to raise and lower the manometer pressure as required.

To permit ease of cannulation the subject sat on a seat on a stage raised above the investigator; thus placing the foot at eye level with the investigator seated, Figure 2.11. The height of the subject's seat was adjusted to bring the distance between the sternal notch and the toe to 93 cm. This distance was maintained during capillary cannulation.

The capillaries were visualized using an Olympus zoom Stereo microscope, model SZ-Tr, a drop of glycerol having been placed on the skin beforehand to render the skin transparent. Lighting was provided by a Schott cold light source KL150 B.

Cannulation was aided by the use of a micromanipulator. The cannula was positioned above and slightly distal to the capillary, and the tip then

brought down vertically onto the epidermis and advanced. A sudden rush of red cells into the cannula indicated successful cannulation. Capillary pressure was then determined by raising the manometer pressure to expel the majority of the red cells, and until the remaining red cells are seen to oscillate back and forth in the pipette with each heart beat, there being no net movement in or out. The manometer level was considered to be equal to true capillary pressure provided that capillary flow was not interrupted and an increase or decrease in manometer pressure of 0.5 cm H_2O caused efflux and influx of red cells respectively.

2.5 SKIN TEMPERATURE MEASUREMENT

Skin temperature was measured using a Comark Electronic Thermometer Type 1625 (Comark Electronics Ltd., Sussex, UK). The instrument uses extremely lightweight and minute copper/constantan thermocouples which are ideal for use close to the site of microvascular measurement. The thermocouples were affixed to the skin between the adhesive disc and the laser Doppler probe holder and within a few millimetres of the area from which flow measurements were being made.

The instrument is specified to be accurate to within ± 0.5 °C at 23 °C. Regular calibration checks were performed against a standardised mercury thermometer using melting ice and a stirred water bath of varying temperature range (12-50 °C).
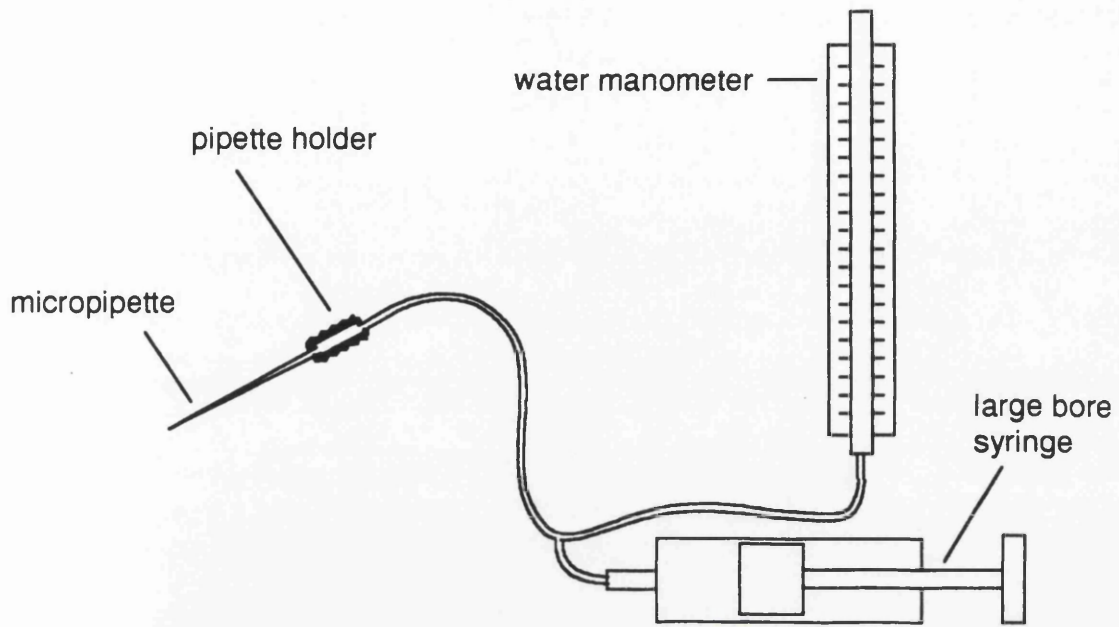
Figure 2.10: Micropipette holder and water manometer system
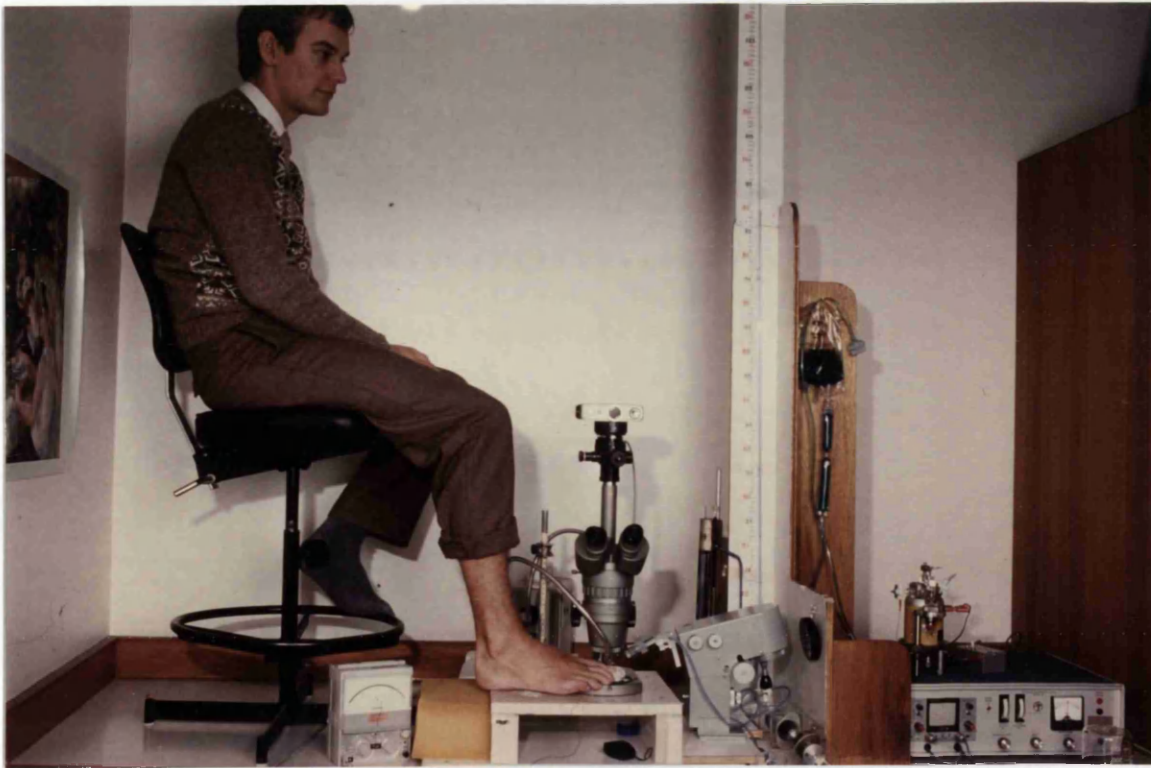
Figure 2.11: Capillary pressure measurement in a seated subject

2.6 VIBRATION SENSORY THRESHOLD

Vibration sensory threshold was measured at the tip of first toe using a hand held Bio-thesiometer (Bio-medical Instrument Company, Newbury, Ohio, USA.). The instrument has a vibrator button which vibrates at 100 Hz. The amplitude of vibration can be increased or decreased by altering the applied voltage.

In practice the vibrator button was placed on the tip of the hallux. Since it has been shown that the application pressure can influence the measured threshold, the button was rested so that majority of the the weight of the instrument was transmitted to the hallux and thus provided a relatively constant application pressure. The voltage was increased to above the threshold of sensation so that the patient could clearly recognise the stimulus. It was then reduced to below the threshold and again slowly increased until first perceived by the patient. This was repeated on two further occasions. From the three readings, the mean voltage calculated to the nearest whole number represented the vibration threshold.

There is an element of subjectivity and the test is open to a degree of operator bias. Thus, in all subjects the test was performed prior to the vascular studies. Despite the subjectivity, the method has been shown to have satisfactory reproducibility (Guy 1985). A centile chart based on the study of 519 non-diabetic subjects showing an age related increase in vibration threshold has been published by Bloom (1984). In the present studies results were compared with this chart and those above the 90th centile for age were considered abnormal.

2.7 AUTONOMIC FUNCTION TESTS

Autonomic function was assessed using a series of tests described below.

(a) Blood pressure response to standing.

A drop in brachial systolic blood pressure of greater than 30 mmHg measured using a standard mercury sphygmomanometer was considered abnormal.

b) Heart rate response to Valsalva manoeuvre.

Heart rate was determined by measuring the R to R' interval recorded on a standard electrocardiogram. The method of Ewing (1973) was followed. The subject was instructed to blow into a mouth-piece connected to a manometer and maintain the mercury column at 40 mmHg for 15 seconds. The Valsalva ratio was calculated by dividing the longest R-R interval after the manoeuvre by the shortest during the manoeuvre. A value of >1.21 was considered normal.

c) Heart rate response to standing.

The immediate heart rate response to standing was assessed using the method described by Ewing (1978). After three minutes of rest, the subject was asked to rise rapidly and then stand motionless. The ratio of the 30th and 15th R-R intervals was calculated from the ECG recording. A value of <1.00 was considered abnormal.

d) Heart rate variation during deep breathing.

After resting supine the heart rate was recorded during six maximal deep-breathing manoeuvres performed over one minute. The Expiration/Inspiration ratio (E:I ratio) was calculated by dividing the mean of the longest R-R interval during inspiration by the shortest during expiration for each of the six breaths. A value of > 1.10 was considered normal (Sundkvist 1979).

2.8 GLUCOSE MEASUREMENT

Capillary blood for glucose measurement was obtained from the pulps of the finger tips using an Autolet finger pricking device (Owen Mumford Ltd, Woodstock, UK.). Glucose was measured using the Reflocheck blood glucose monitor (Boehringer Mannhein Corporation, Mannheim, W. Germany). The system was found to be accurate and precise for glucose values between the range of 3-22 mmol/l (Rayman 1984). The method correlated well with the routine hospital laboratory assay (r= 0.99; y = 0.37 + 0.99 x; n=101). Because of the detection limits and the unreliability of the instrument at glucose levels below 3.0 mmol/l, results outside the range of 4-22.0 mmol/l were verified by laboratory measurement. Regular checks on the performance of the instrument were performed using standard glucose solutions and coloured test strips supplied by the manufacturer.

2.9 GLYCOSYLATED HAEMOGLOBIN MEASUREMENT

Total glycosylated haemoglobin (HbA1) was measured using the column method with a Bio-Rad kit (Bio-Rad Laboratories Ltd, UK. To remove the unstable fraction, prior to measurement the blood samples were incubated for 5 hours at 37 °C and then kept overnight at 2-8 °C. The normal range was 4-7% and the interassay coefficient of variation was 6%.

2.10 HAEMATOCRIT MEASUREMENT

Blood haematocrit was measured using the micro-haematocrit method. Blood was drawn-up by capillary action into heparinised capillary tubes and the end sealed with plasticine. The tubes were spun at 5,000 rpm for 10 minutes in a Hawksley Microhaematocrit centrifuge and the haematocrit then read using a microhaematocrit reader (Medical and Scientific

Equipment, UK). For each sample two measurements were made and the mean used in data analysis. Paired results never differed by more than 1%.

2.11 PLASMA COLLOID OSMOTIC PRESSURE MEASUREMENT

I am grateful to Dr J Gamble for the measurement of plasma colloid osmotic pressure. The measurements were performed using a modified Hansen osmometer coupled to a Bell and Howell Physiological Transducer (type 4-422-0002) and fitted with "backed" Amicon PM 30 membranes. The osmometer was assembled under degassed saline and kept airtight when not in use, allowing each membrane to be used for several weeks without apparent deterioration. The signal from the pressure transducer was amplified by a Devices Transducer Pre-amplifier (type 3552) on a 0-100 mmHg range and displayed on a standard chart recorder.

2.12 MEASUREMENT OF FOOT SWELLING RATE

The rate of volume increase in the foot during dependency was measured using the mercury strain gauge technique first described by Whitney in 1953. A mercury filled rubber or silastic tube which makes contact with electrodes at either end is made to encircle the limb. As the limb expands or contracts the length of the tubing changes by a corresponding amount. Since the electrical resistance of the mercury varies with its the length, variations in the voltage drop across the gauge will reflect changes in circumference and is therefore related to the change in limb volume as described below.

Assuming that the part is cylindrical, the volume (V) bears the following relationship with the radius (r) and length (L)

$$V = \pi r^2 L$$

Since L remains constant the volume (V) is related to

$$\pi r^2$$

As the limb swells, the radius increases by Δr, so that the new volume becomes

$$V+\Delta V=\pi(r+\Delta r)^2 = \pi(r^2+2r\Delta r+\Delta r^2)$$

Subtracting the original volume (πr^2), the volume increase is therefore

$$\Delta V=2\pi r\Delta r+\pi\Delta r^2$$

The ratio of the volume change $\Delta V/V$ is

$$2\Delta r/r+\Delta r^2/r^2$$

The circumference (C) of the limb $= 2\pi r$; hence

$$r=C/2\pi$$

$$\Delta r=\Delta C/2\pi$$

Substituting the last two equations in the equation for the ratio of the volume change

$$\Delta V/V=2\Delta C/C+\Delta C^2/C^2$$

Since the change in circumference following dependency is minute compared with the original circumference, $\Delta C^2/C^2$ can be neglected. Therefore the relative change in volume is equivalent to twice the relative change in circumference

$$\Delta V/V=2\Delta C/C$$

In the studies described in Chapter 6 the volume changes occurring in the foot during dependency were measured using mercury-in-silastic gauges supplied by Jansen Scientific Instruments (Beerse, Belgium). The gauges were chosen for their elasticity and lightness. The silastic tubing is considerably thinner than that of other manufacturers gauges (external diameter 0.6mm, internal diameter 0.3mm). Very little force is needed to stretch these gauges (200mg weight for a 1% extension) reducing the risk of underestimating volume increases because of compression of the underlying tissue.

The gauges are double stranded and were placed so as to encircle the foot approximately mid-way between the metatarsal heads and the ankle. A selection of gauge sizes were available, the choice depending on the size of the foot. Generally the 45cm length was appropriate. Artefacts due to the gauge slipping or rolling as the circumference increased were avoided by taping the silastic tubing to the skin at intervals of approximately 2cm using thin strips (0.5cm) of micropore tape. This does not interfere with circumferential expansion. The change in electrical resistance was measured using a Jansen Scientific Instrument Periflow Mercury-Gauge Amplifier (Beerse, Belgium). Recordings of the instrument's voltage output were made on a Vitatron 2001 Dual channel chart recorder at a paper speed of 50 mm/min. The Periflow contains an inbuilt electronic calibration system which produces a series of voltage changes equivalent to a volume change of 0.1, 0.5, and 1.0%. The chart recorder was set so that these recorded deflections on the paper of 4, 20, and 40 mm respectively.

Figure 2.10 shows a typical recording obtained from a normal subject sitting with the foot dependent 90 cm below heart level. The initial rapid increase over the first 30 seconds results from rapid filling and distension of the veins (venous compliance). Volume increase between 5 and 10 minutes was considered to be a combination of both fluid filtration into the foot and continued distension. Foot swelling rate due to filtration alone was considered to occur between 10-20 minutes of dependency and was obtained by calculating the slope of the line drawn through this part of the trace. The coefficient of variation for this method based on two measurements in six individuals was 5.3%, Table A 2.3.
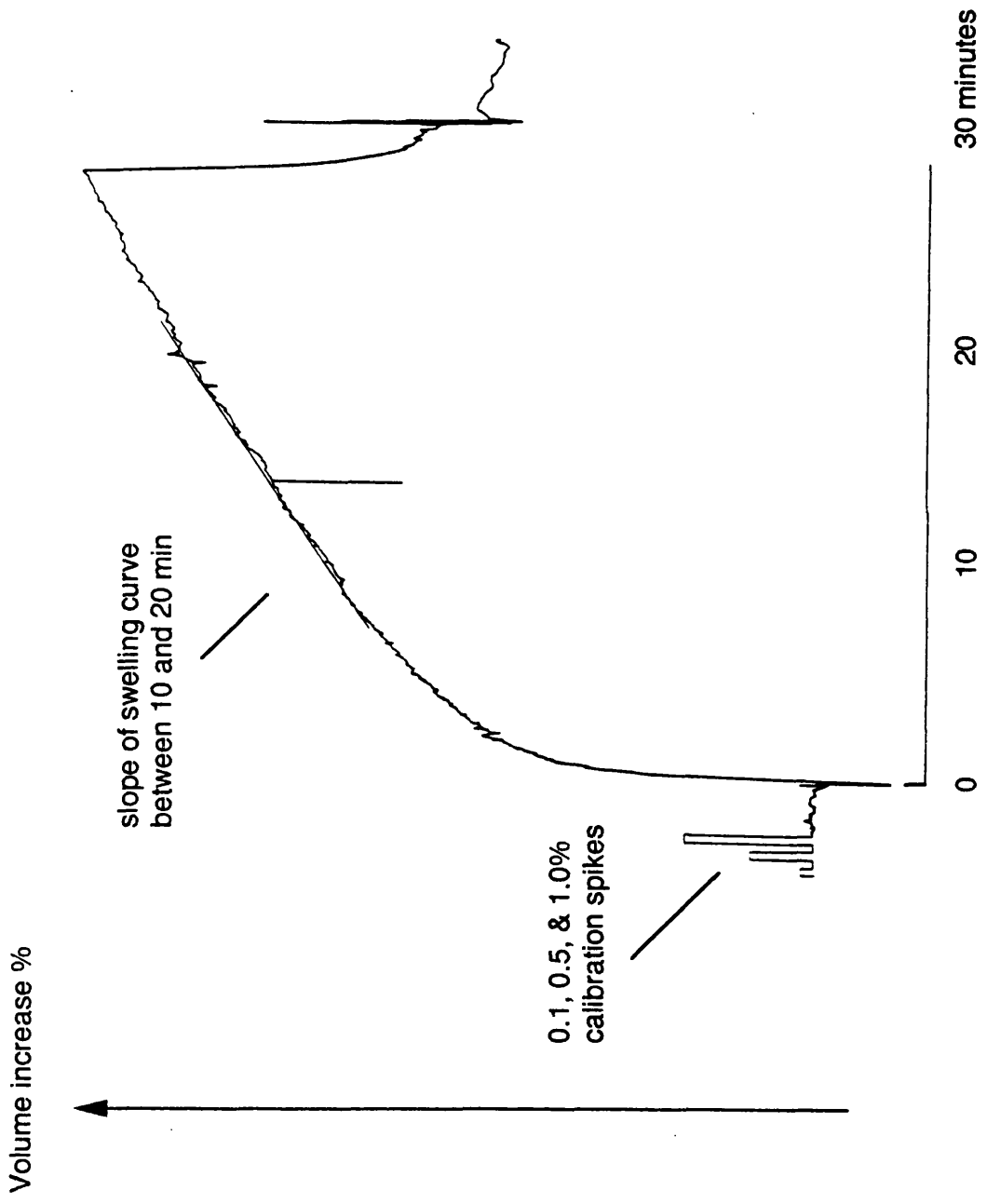
Figure 2.12- Typical changes in the strain gauge output during dependency. At time 0 the foot was placed 1 meter below heart level. Swelling rate was calculated from the slope at 10 to 20 minutes.

2.13 STATISTICAL ANALYSIS

Data was analysed on a Macintosh SE (Apple Computer, Inc., Cupertino, Calif.) using the StatView 512+ program (BrainPower, Inc., Calabasas, Calif.). Results are presented as means ± SD except for skewed data when the median and range are shown. Comparisons were made using the Mann Whitney-U test. When several groups were compared, one-way analysis of variance was employed. When the analysis of variance indicated a F statistic significant at p<0.05, pairwise comparisons between the means of the separate groups were made using the Mann-Whitney test. Spearman rank correlation coefficient and linear regression analysis were used when appropriate to assess the relationship between sets of data.

When determining reproducibility, studies were repeated on two separate occasions in a number of subjects and the mean within subject coefficient of variation calculated using the method described for paired samples (Raggatt 1989). In this, the SD for each pair is the difference between tests divided by the square root of 2. The combination of the estimates of the SD for all the subjects studied gives an estimate of the underlying imprecision. Thus:

$$\text{mean SD} = \sqrt{\frac{\sum^n SD_1^2 + SD_2^2 + ...SD_n^2}{n-1}}$$

Where n is the number of pairs. The mean within subject CV is the mean SD divided by the mean of all the results.

CHAPTER 3

THE SKIN MICROVASCULAR RESPONSE TO INJURY

3.1 INTRODUCTION

In diabetic patients, trauma, often relatively minor, is recognised to be an important factor in the development of foot ulcers, irrespective of the presence or absence of large vessel disease. Subjects with peripheral neuropathy are particularly venerable as the normal protection afforded by sensory nerves is absent, and the skin is subjected to excessive trauma particularly in the presence of deformities and callosities where high pressure loading occurs (Boulton 1983). An important component of the normal vascular reaction to such injury is local hyperaemia which serves to remove injurious substances and deliver substances involved in the repair of tissue and defence from infection.

The experiments described in this chapter were performed to determine 1) the microvascular blood flow response to injury in normal skin, and 2) to determine whether the response is any different in diabetic skin. Two forms of injury were studied- thermal injury and mechanical injury. Prior the introduction of the laser Doppler flowmeter such studies would not have been possible as the then available methods for measuring blood flow required relatively large volumes of tissue necessitating unacceptably large areas of skin trauma. In contrast the laser Doppler technique measures blood flow in minute areas of skin making it uniquely suited to assessment of the injury response where tissue trauma must be kept to a minimum.

3.2 THE MICROVASCULAR RESPONSE TO THERMAL INJURY

3.2.1 Introduction

Heating the skin to above 42°C results in maximal microvascular vasodilation (Johnson 1986). At this temperature mild thermal injury consisting of transient first-degree burns (erythema) is seen (Storm 1979). The vascular response to such injury was investigated in the skin on the dorsum of the foot in normal and diabetic subjects.

3.2.2 Subjects

Young insulin-dependent diabetics were chosen for these studies on the pretext that their findings were less likely to be complicated by large vessel disease and the duration of diabetes could be more definitely established. Significant large vessel disease was considered to be present and such patients excluded from further study if the ankle pressure index (posterior tibial systolic pressure/brachial systolic pressure) was less than 1.0 (Yao 1969). All the patients underwent independent ophthalmic fundus assessment through dilated pupils. Only those with normal fundi or minimal background retinopathy were selected for study. All subjects had intact ankle reflexes, and vibration sensory thresholds were within normal centiles for age assessed using published charts (Bloom, 1984). Case records were reviewed for evidence of albuminuria. Subjects found to be albustix positive on one or more occasions in the three most recent clinic visits were excluded from study. Patients admitted with hyperglycaemic ketoacidosis within the previous three months were not selected for study. Smokers and hypertensive subjects were excluded from both the diabetic and the control groups and none of the subjects had a history of foot complications.

Details of the subjects with diabetes are summarised in Tables 3.1 and 3.2. Twenty-three diabetics (thirteen male and ten female) were studied.

Their mean age was 30.9 ± 6.4 years (range 18 to 45 years) and mean duration of diabetes was 13.3 ± 8.8 years (range 1 to 29 years). Eight of these subjects were found to have minimal background retinopathy. Capillary blood glucose at the time of the study was generally good (mean 9.1 ± 3.9 mmol/l); only five of the diabetic subjects had values above 12 mmol/l. No subject became hypoglycaemic during study. Diabetic control over the preceding months was also judged to be satisfactory, the mean glycosylated haemoglobin for the group being 9.2 ± 1.3% (range 7.1 to 11.8%; normal range 4.0 to 7.0%).

Details of the control subjects are summarised in Table 3.3. Twenty-one subjects were studied. They comprised healthy volunteers from the academic and technical staff of the Charing Cross Medical School. There were eleven men and ten women. The mean age of the group was 29.7 ± 5.2 years (range 20 to 40 years).

3.2.3 Methods

3.2.3.i Acclimatisation and study conditions

All studies were conducted after a minimum acclimatisation period of thirty minutes. Environmental temperature was controlled to between 21°C and 23°C. The subjects were rested on a couch, and during acclimatisation the legs were kept horizontal with the feet exposed to the room temperature. During this rest period subject details, blood glucose, blood pressure and vibration sensory thresholds were recorded.

3.2.3.ii Blood flow measurements

Towards the end of the rest period the specially constructed heater probe with the laser Doppler probe inserted (described in Chapter 2), was affixedto the skin on the dorsum of the right foot using double sided adhesive disks (Double-Stick Discs- 3M, St Paul, Minnesota, USA). To

TABLE 3.1

Clinical Details of Subjects with Diabetes

Subject	Sex	Age (yrs)	Duration of Diabetes (yrs)	Insulin Dose (units)	Complications
DM	F	27	5	28	-
RG	M	28	16	36	*BR
CC	M	26	14	45	BR
RC	M	32	29	110	BR
GR	F	32	7	44	-
DA	F	23	1	32	-
RH	M	32	7	85	-
JB	F	45	22	32	BR
GS	M	40	2	38	-
WS	M	34	17	52	-
RP	M	34	20	44	-
DS	M	42	29	36	BR
KH	F	31	22	47	BR
CM	M	33	17	37	-
FR	F	27	11	36	-
RC	M	38	23	37	-
JT	F	30	15	44	-
BH	M	27	21	93	BR
KH	F	30	6	32	-
JD	M	33	4	50	-
JF	F	22	15	78	BR
RD	M	27	2	62	-
DD	F	18	2	60	-
Mean		30.9	13.3	50.3	
SD		6.4	8.8	21.7	

*BR- background retinopathy

TABLE 3.2

Clinical Details of Diabetic Subjects -cont'd.

Subject	Blood Glucose (mmol/l)	HbA1 (%)	Ankle BP (mmHg)	Brachial BP (mmHg)	Vibration threshold (Volts) R	L
DM	5.2	8.4	120	100/70	8	7
RG	12.3	8.8	170	140/80	6	8
CC	10.5	7.3	130	110/75	10	8
RC	5.6	11.8	125	115/80	10	8
GR	7.6	11.2	118	105/65	12	10
DA	8.4	8.4	100	95/50	4	6
RH	5.1	8.5	130	110/70	5	5
JB	20.1	8.6	160	120/80	10	11
GS	13.2	7.1	140	125/85	4	6
WS	4.5	9.1	134	100/70	14	10
RP	15.7	10.1	160	130/85	10	11
DS	7.5	9.0	134	120/75	8	6
KH	7.5	9.0	134	120/75	5	6
CM	4.2	9.2	140	125/80	10	12
FR	12.8	8.5	100	100/60	9	10
RC	11.5	-	140	110/50	6	9
JT	8.0	7.2	120	120/80	8	9
BH	7.4	9.6	120	110/80	12	13
KH	9.6	8.6	140	140/70	8	7
JD	7.3	8.8	125	130/75	7	9
JF	5.2	10.1	110	100/70	6	6
RD	9.7	11.3	120	120/85	8	6
DD	9.7	10.3	115	105/70	6	9
Mean	9.1	9.1	129.8	115.3/73.0	8.1	8.4
SD	3.9	1.3	17.7	12.8/9.7	2.7	2.1

TABLE 3.3

Clinical details of Control Subjects

Subject	Sex	Age (yrs)	Ankle BP (mmHg)	Brachial BP (mmHg)
BM	M	40	130	120/80
RR	M	25	125	115/75
SW	F	30	120	105/70
TC	M	29	140	130/80
MH	F	30	120	100/60
JJ	F	30	120	100/75
JN	F	28	135	110/65
GR	M	31	145	130/80
AR	F	29	110	95/60
SW	M	26	125	115/70
NG	M	20	130	120/70
NW	M	20	-	120/70
SR	M	32	110	105/65
JL	M	36	140	125/85
JS	M	35	130	120/65
JW	F	26	120	110/70
MO	F	25	125	120/80
GJ	F	38	125	115/70
EE	F	30	135	120/75
JL	F	30	130	125/70
MP	M	34	145	130/70
Mean		29.7	128.3	115.7/ 71.8
SD		5.2	10.0	10.3/ 6.9

ensure good contact between the probe heater and skin, the probe was placed just proximal to the space between the first and second metatarsal heads where the skin is relatively flat.

At the end of the acclimatisation period, rest flow was recorded for two minutes. As described in Chapter 2, the rotating heater probe allows blood flow to be recorded from nine sites, the average being more reproducible than a single measurement. Rest flow was recorded from only the first of the nine possible sites because unlike flow following thermal injury it is affected by the rotation of the probe. Skin temperature was recorded during the acclimatisation period from the dorsum of the foot approximately 2 cm from the heater probe.

After recording rest flow the heater was switched on. To ensure that a temperature of at least 43°C was reached, the skin was heated to 44°C for thirty minutes prior to blood flow measurements. This temperature was chosen as there is now considerable experience on the safety of transcutaneous oxygen electrodes used at this temperature (Lancet; editorial 1984) and as previously mentioned blood flow is maximised (Johnson 1986). Following thermal injury, redness at the measurement site was invariable; this sometime persisted for several hours, however in none of the subjects was there any long term sequelae.

At the end of the heating period the foot was placed fifty centimetres below heart level and blood flow was recorded from each the nine sites for one minute. As described in Chapter 2, local heating abolishes microvascular reflexes. In these circumstances the increased hydrostatic pressure which occurs on lowering the leg distends the passive microvessels and results in an increase in blood flow. The average of the nine blood flow recordings was calculated and termed "maximum blood flow".

3.2.4 Results

Brachial blood pressure and ankle systolic blood pressures were not significantly different in the two groups, Tables 3.1 and 3.3. For diabetic and control subjects individual results of skin temperature, blood flow at rest and maximum blood flow in the dorsum of the foot are given in Tables 3.4 and 3.5, respectively.

Mean skin temperature for the group of subjects with diabetes was 30.1 ± 2.2°C (range 26.0 to 34.6°C). This was not significantly different from the mean value of 30.4 ± 1.5 °C (27.3 to 32.6°C) for the control subjects. Mean resting blood flow on the dorsum of the foot in the diabetic group was 15.7 ± 13.2 mV (range 3.0 to 60.5 mV). This was not significantly different from that of the control group in which the mean value was 17.5 ± 8.5 mV (range 4.4 to 36.0 mV). Rest flow from the dorsum of the foot was significantly correlated with skin temperature in both the diabetic ($p<0.04$; $r=0.45$) and control groups ($p<0.002$; $r=0.68$).

Maximum blood flow results for individual diabetic and normal subjects are also shown in Tables 3.4 and 3.5 respectively, and represented graphically in Figure 3.1. The mean value for the diabetic group was 0.53 ± 0.11 V (range 0.31 to 0.72 V). This was significantly lower ($p<0.001$) than that of the control group in which the mean was 0.72 ± 0.10 V (range 0.57 to 0.93 V).

Eight of the diabetic subjects had background retinopathy. When these subjects are removed from the original group, maximum blood flow in the remaining uncomplicated group was still significantly reduced (0.58 ± 0.08 V) when compared with the group of normal subjects ($p<0.001$). In the group with background retinopathy, thermal injury response was significantly lower (0.43 ± 0.11 V) than that in the group free from complications ($p<0.005$).

TABLE 3.4

Skin temperature, Rest flow and Maximum (thermal injury)
blood flow in Diabetic Subjects

Subject	Skin Temp (°C)	Rest flow (Arbitrary units-milli Volts)	Maximum flow (AU-Volts)
DM	26.0	7.0	0.59
RG	30.5	19.5	0.34 (0.38)*
CC	29.5	22.5	0.56
RC	28.0	4.0	0.36 (0.34)*
GR	31.3	12.5	0.51
DA	27.6	2.0	0.66
RH	30.5	6.5	0.64
JB	34.6	20.0	0.52
GS	31.4	16.9	0.60
WS	31.4	10.5	0.59
RP	31.2	7.0	0.45
DS	31.6	32.5	0.34 (0.35)*
KH	33.5	60.5	0.45
CM	28.1	27.0	0.57
FR	27.2	30.0	0.52
RC	31.7	9.5	0.72
JT	29.5	13.0	0.46
BH	30.0	3.0	0.31 (0.34)*
KH	30.5	13.0	0.63
JD	-	34.0	0.54
JF	32.0	16.0	0.59
RD	27.3	12.0	0.66
DD	28.0	10.5	0.59
Mean	30.1	15.7	0.53
SD	2.2	13.2	0.11

* Denotes repeat measurement- see text.

TABLE 3.5

Skin temperature, Rest flow and Maximum (thermal injury)
blood flow in Control Subjects

Subject	Skin Temp (°C)	Rest Flow (Arbitrary units-milli Volts)	Maximum flow (AU-V)
BM	29.3	8.6	0.93
RR	29.1	7.5	0.74
SWa	32.6	16.5	0.62
TC	31.5	32.3	0.65
MH	29.5	14.8	0.76
JJ	28.6	4.4	0.78
JN	27.3	5.4	0.65
GR	31.5	21.0	0.88
AR	32.5	26.5	0.62
SW	-	-	0.62
NG	29.5	14.0	0.92
NW	32.4	22.0	0.72
SR	29.6	17.5	0.76
JL	30.5	36.0	0.61
JS	31.5	31.0	0.74
JW	-	-	0.57
MO	31.5	19.4	0.65
GJ	30.0	13.4	0.63
EE	-	-	0.71
JR	29.6	16.6	0.93
MP	31.5	18.0	0.72
Mean	30.4	17.5	0.72
SD	1.5	8.5	0.11
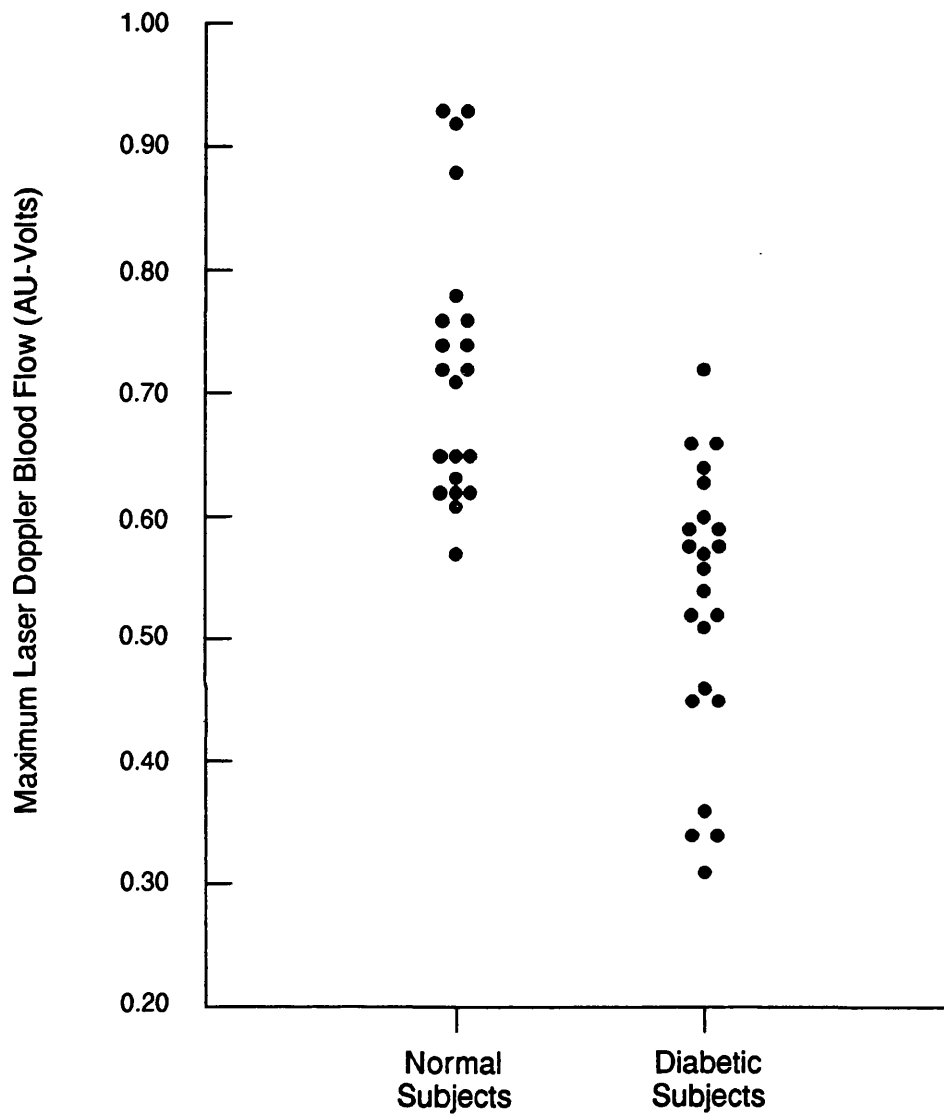
Figure 3.1

Maximum blood flow responses to thermal injury in
control and diabetic subjects (arbitrary units of volts).

Twelve of the twenty three diabetics had a maximum response which was less than the lowest normal response. Four of the diabetics with the lowest responses (<0.40 V) were restudied on another occasion within two weeks later of the original study. Virtually identical results were obtained, confirming that the previous findings were truly representative of maximum flow and not the result of poor technique. Each of these repeat measurements is denoted in Table 3.4 by an asterisk.

There was an inverse relationship between maximum blood flow and duration of diabetes (r = –0.61; p<0.002), Figure 3.2. Five of the six diabetics with the lowest responses had background retinopathy. When patients with background retinopathy are excluded from the analysis the relationship no longer holds (r = –0.22; p>0.4).

In the diabetic subjects there was no relationship between maximum blood flow and blood glucose or glycosylated haemoglobin (r = –0.03 and r = –0.17 respectively) and no relationship between maximum blood flow and vibration threshold (r = –0.24; p>0.1).

Maximum blood flow did not correlate with rest flow (r = –0.20, diabetic group and r = –0.37, normal group; p>0.1 for both groups) or skin temperature (r = –0.16, diabetic group and r = –0.33, normal group; p>0.1 for both groups). Maximum blood flow was not related to ankle systolic blood pressure (r = –0.25 diabetic subjects and r = 0.19 normal subjects; p>0.1 for both groups) or brachial systolic blood pressure (r = –0.29 diabetic subjects and r = 0.27 normal subjects; p>0.1 for both groups). Maximum blood flow was unrelated to age in the normal (r = 0.06) and diabetic groups (r = –0.03).
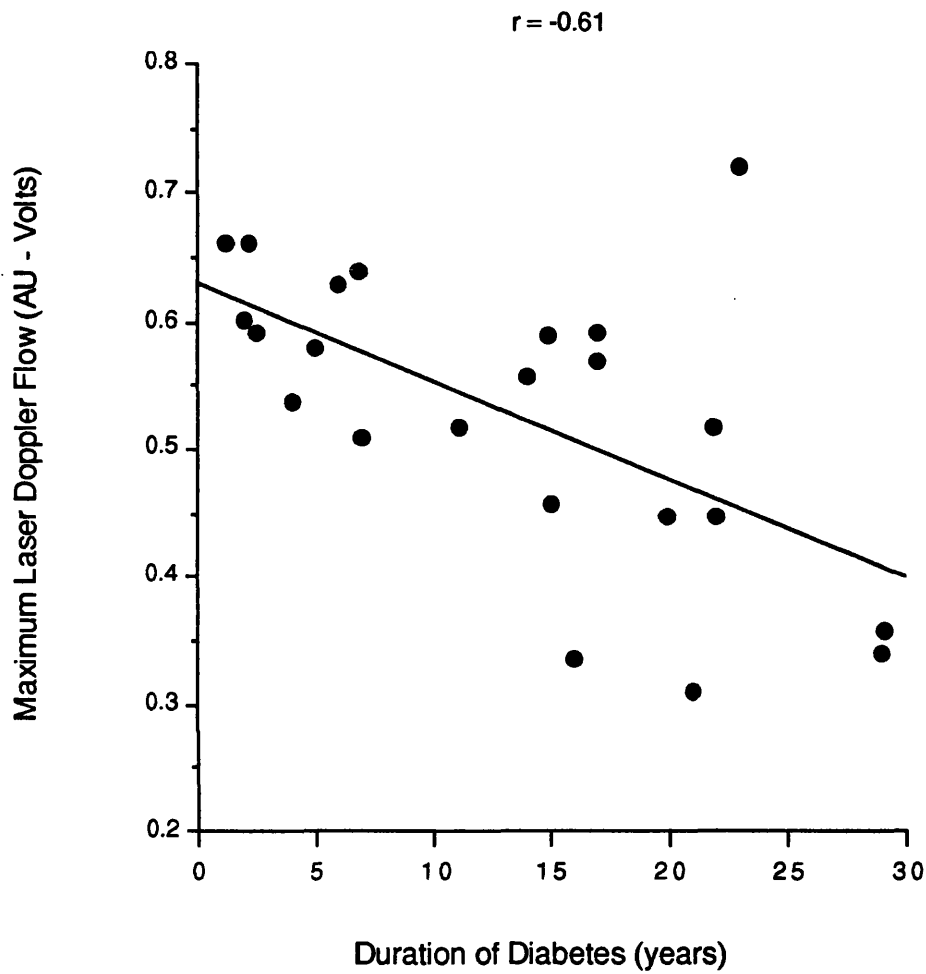
Figure 3.2

Relationship between maximum bloood flow response to
thermal injury and duration of diabetes. The solid line
represents the regression line.

3.3 THE MICROVASCULAR RESPONSE TO NEEDLE TRAUMA

3.3.1 Introduction

The aim of this study was to determine the skin blood flow response to mechanical injury in normal subjects and determine whether the response is abnormal in diabetic subjects. The form of mechanical injury chosen was that induced by needle stick to the abdominal skin since this being relatively minor and beong familiar to diabetic patients would be easily accepted.

3.3.2 Subjects and Methods

3.3.2.i Subjects

All diabetic and control subjects participating in this study had taken part in the previous study involving thermal injury (Tables 3.1 and 3.3). In each subject injection trauma was performed immediately after completing the thermal injury study.

3.3.2.ii Blood Flow Measurements

Blood flow was measured in the skin of the upper abdomen, approximately four centimetres below the costal margin, and eight to ten centimetres lateral to the mid-line (Figure 3.3). This area corresponds approximately to the T 10 dermatome. The standard laser Doppler probe holder was used to support the probe. Measurements were recorded from both the left and right sides of the abdomen. Ten minutes were allowed after attaching the laser probe before measurements were made. Initially, two minutes of rest flow were recorded. A 25 gauge needle was then introduced into the skin in the centre of the probe holder, penetrating to a depth of 0.5 cm. The depth was controlled by a depth guard on the needle (Figure 3.4). Immediately after pin prick, the site was examined. If any bleeding had occurred, a new area was investigated since stationary clotted blood was found to interfere
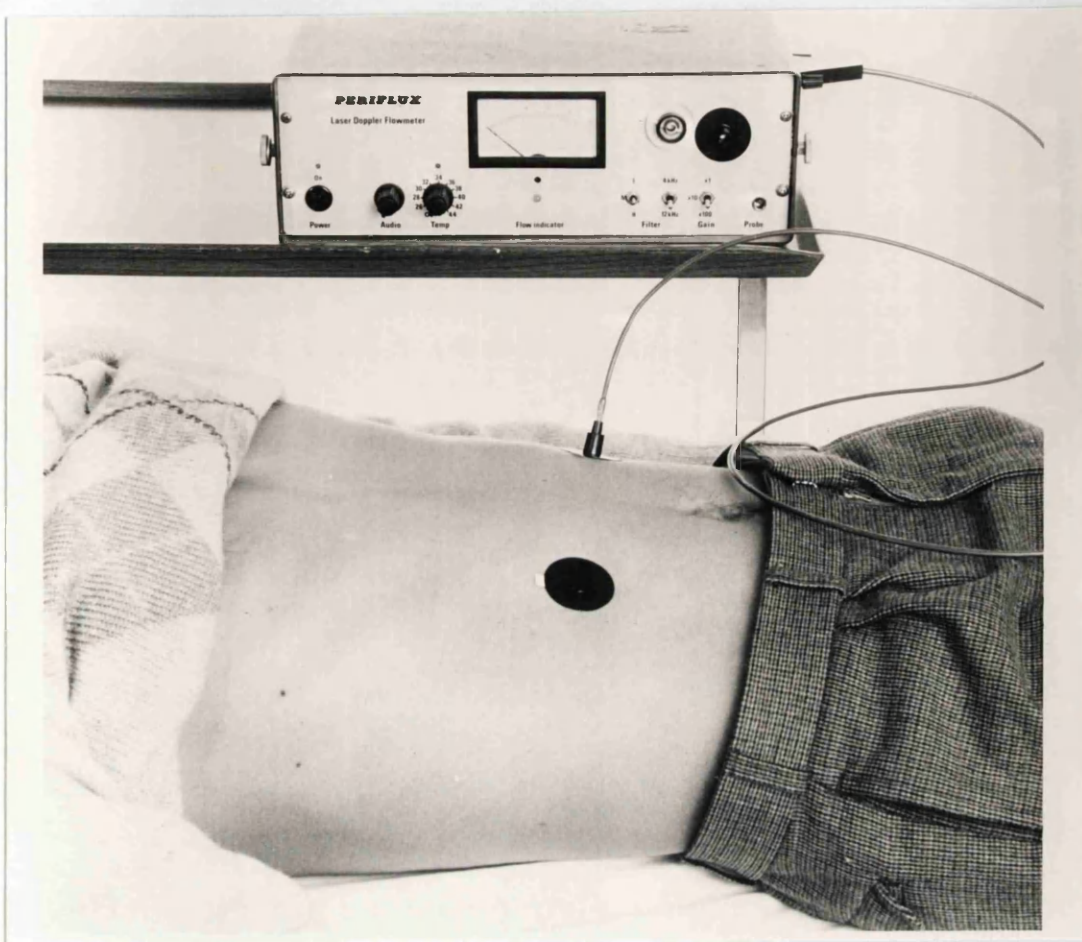
Figure 3.3: Photograph showing probe holders affixed to abdomen and blood flow measurement being taken from one of these sites.
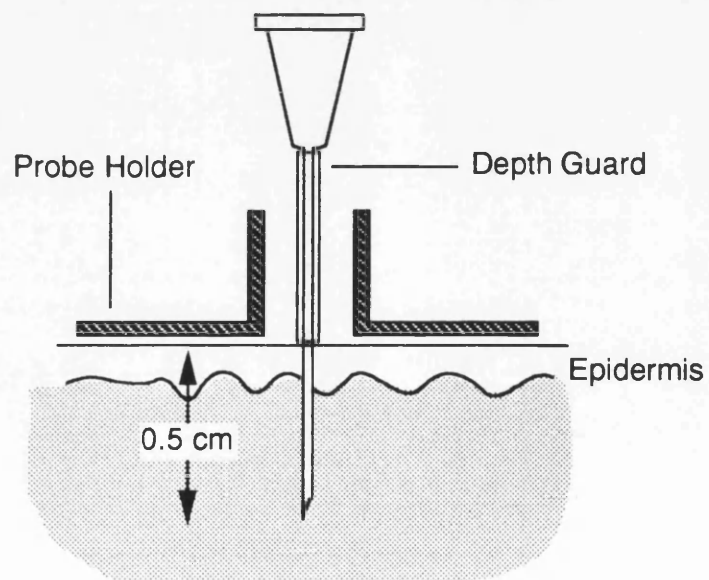
Figure 3.4 : Diagram showing needle inserted into centre of probe holder to a depth limited by depth guard.

with flow measurements. The probe was then returned to the probe holder and flow recorded continuously for fifteen minutes. Thereafter measurements were made for five minute periods at thirty and sixty minutes. Fifteen minutes after the first needle injury the manoeuvre was repeated at the opposite site. The coefficient of variation calculated from paired measurements in nine subjects was 8.6%. This data is presented in Table A 3.1. After the final measurement the probe holder was removed from the skin and the probe itself was pressed firmly against the skin at the injury site. This occluded the underlying vessels and a recording of the biological zero could be obtained. The blood flow values at equivalent time intervals for the two sites were averaged and are presented as a single result. The average of the maximum flow achieved at each of the two sites was calculated and is termed "peak blood flow".

3.3.4 Results

Skin temperature, rest blood flow, and peak blood flow for individual diabetic and control subjects are given in Tables 3.6 and 3.7 respectively.

Mean skin temperature was similar in the two groups (32.8 ± 0.9 °C, range 31.6 to 35.4 °C in the diabetic group; 33.2 to 1.0 °C, range 30.7 to 34.6 °C in the control group). Mean rest flow was not significantly different in the two groups (diabetics 16.8 ± 5.7 mV; range 9.0 to 31.0; controls 17.2 ± 6.0; range 9.0 to 35.0 V). There were weak correlations between abdominal skin temperature and rest flow in both diabetic ($r = 0.45$; $p<0.05$) and control groups.($r = 0.53$; $p<0.03$).

Individual blood flow results at the stated time points following injury for diabetic and control subjects are shown in Table A 3.2 and A 3.3 (Appendix). The time course of the hyperaemic response is illustrated in Figure 3.5. At all time points following injury, hyperaemic blood flow in the diabetic group was significantly lower than that in the control group (significance levels are shown in Figure 3.5). Peak blood flow occurred

TABLE 3.6

Abdominal skin temperature, Rest flow and Peak (injection trauma)
blood flow in Diabetic Subjects.

Subject	Skin Temperature (°C)	Rest Flow (Arbitrary Units-milli-Volts)	Peak Flow (AU Volts)
DM	32.2	16.0	0.31
RG	33.3	15.0	0.13
CC	34.7	20.0	0.30
RC	33.6	31.0	0.20 (0.22)*
GR	32.2	22.0	0.35
DA	32.6	13.0	0.44
RH	33.8	21.0	0.28
JB	35.4	20.0	0.30
GS	33.7	20.0	0.34
WS	33.7	10.0	0.23
RP	32.6	9.0	0.24
DS	31.6	-	0.18 (0.21)*
KH	32.1	11.0	0.17 (0.14)*
CM	33.1	16.0	0.15 (0.23)*
FR	32.9	12.0	0.22
RC	34.0	-	0.40
JT	32.6	16.0	0.34
BH	34.0	14.0	0.14 (0.19)*
KH	35.0	30.0	0.34
JD	33.2	21.0	0.21
JF	32.4	21.0	0.29
RD	-	12.0	0.45
DD	32.5	11.0	0.44
Mean	32.8	16.8	0.28
SD	0.9	5.7	0.10

* Denotes repeat measurement- see text.

TABLE 3.7

Abdominal skin temperature, Rest flow and Peak (injection trauma)
blood flow in Control Subjects

Subject	Skin Temp	Rest Flow	Peak flow
	(°C)	(Arbitrary Units-milli-Volts)	(AU-V)
BM	34.0	35.0	0.37
RR	32.6	16.0	0.56
SWa	32.1	17.0	0.44
TC	33.1	15.0	0.30
MH	-	12.0	0.53
JJ	33.3	12.0	0.38
JN	32.4	14.0	0.31
GR	31.4	9.0	0.44
AR	33.1	24.0	0.49
SW	32.1	15.0	0.30
NG	33.0	16.0	0.59
NW	32.5	16.0	0.43
SR	33.6	26.0	0.37
JL	33.3	14.0	0.39
JS	30.7	13.0	0.37
JW	32.9	15.0	0.50
MO	32.4	15.0	0.52
GJ	-	19.0	0.33
EE	32.5	-	0.44
JR	33.2	15.0	0.39
MP	34.6	17.0	0.28
Mean	33.2	17.2	0.42
SD	1.0	6.0	0.09

within five to fifteen minutes of injury in both groups. Individual results for diabetic and control subjects are shown in Figure 3.6. In the diabetic group peak blood flow was 0.28 ± 0.01 V (range 0.13 to 0.45 V). This was significantly lower (p<0.001) than that of the control group (0.41 ± 0.09 V; range 0.28 to 0.59 V).

When the subjects with background retinopathy are removed form the original group, peak blood flow response in the remaining uncomplicated group was still significantly reduced (0.32 ± 0.09 V) when compared with the group of normal subjects (p<0.012). In the group with background retinopathy, peak blood flow response was significantly lower (0.21 ± 0.07 V) than that in the group free from complications (p<0.005).

Ten of the twenty-three diabetics had peak responses below the lowest response in the control subjects. The five diabetics with the lowest responses were restudied two weeks later. Very similar results were obtained confirming the previous findings. Each of these repeat measurements is denoted in Table 3.6 by an asterisk.

There was a significant inverse relationship between peak laser Doppler flow and duration of diabetes (r = –0.60; p< 0.002) Figure 3.7. Five of the six diabetics with the lowest responses had background retinopathy. When these subjects are excluded from analysis there is no longer a relationship between peak response and duration of diabetes (r = – 0.44; p>0.1).

In the diabetic group as with the thermal injury response, peak laser Doppler flow did not correlate with prevailing blood glucose (r = 0.17; p>0.4), or glycosylated haemoglobin (r = 0.03; p>0.9).

There was no relationship between peak response and brachial systolic blood pressure (r = –0.32 in the diabetic group and r = –0.28 in the normal subjects; p>0.1 in both groups). There was an inverse relationship between age and peak blood flow in the group of normal subjects (r = –0.53; p<0.02) but not in the diabetic group (r = –0.24; p>0.3).
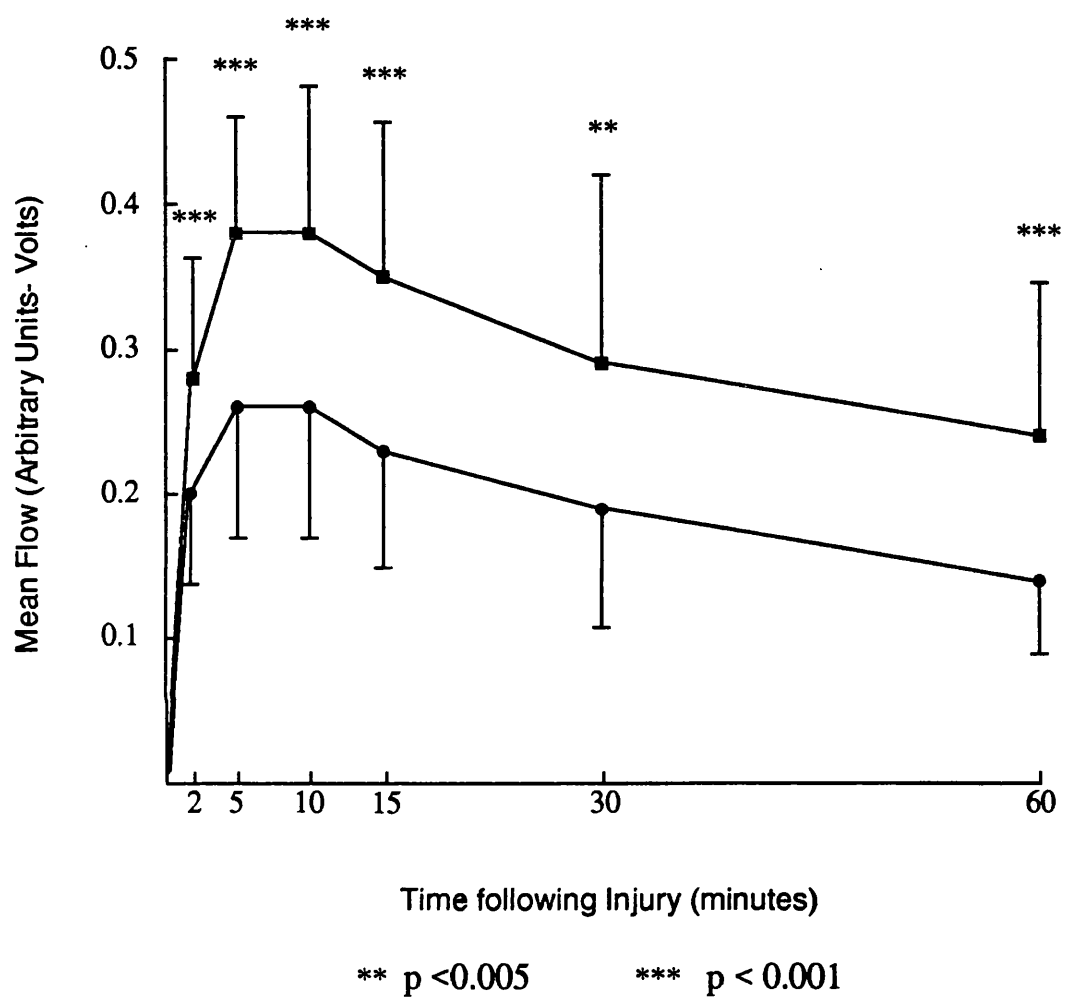
Figure 3.5

Time course of the peak blood flow responses to injection
trauma in diabetic (circles) and control (squares) subjects.
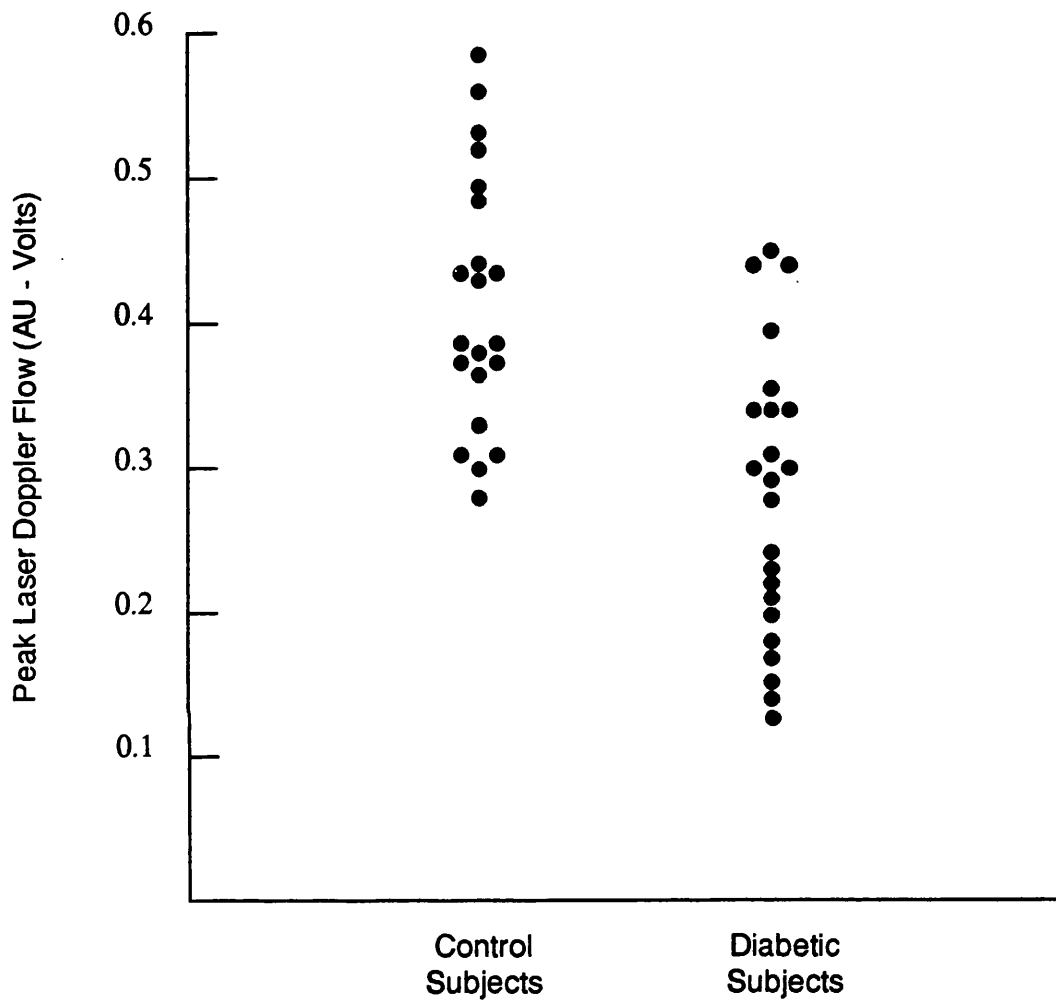
Figure 3.6

Peak laser Doppler blood flow (arbitrary units of volts)
following injection trauma in diabetic and control subjects
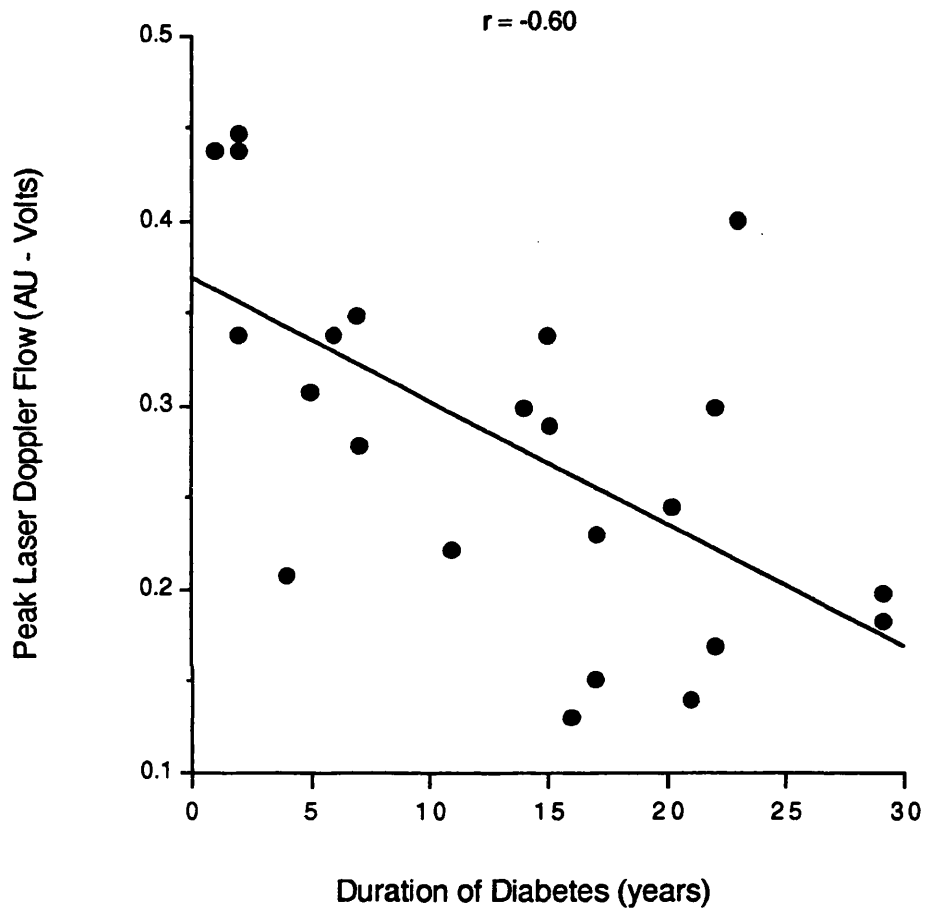
Figure 3.7

Relationship between peak blood flow response to injection
trauma and duration of diabetes. Regression line is shown.

3.4 RELATIONSHIP BETWEEN THERMAL AND MECHANICAL INJURY RESPONSES

As both the above studies involved the same subjects it is possible to compare the hyperaemic responses to the two different forms of injury in the two groups. In the diabetic subjects, maximum thermal injury response was significantly related to peak blood flow ($r = 0.72$; $p < 0.001$) Figure 3.8. In the control subjects, however, no such relationship was found ($r = 0.17$; $p > 0.4$).

3.5 THE EFFECT OF DIABETIC CONTROL ON HYPERAEMIC RESPONSES TO INJURY

In the previous studies no relationship was found between hyperaemic responses and prevailing glucose or glycosylated haemoglobin. This does not rule out an effect of changes in diabetic control on the hyperaemic response in individual subjects. To examine the effect of acute changes of blood glucose and the effect of insulin deficiency on thermal and needle injury responses, ten diabetics with glucose concentrations below 10 mmol/l when first studied, were restudied at between 9 am and 10 am on the morning after omitting of their pre-breakfast insulin. Mean blood glucose value for the first study was 7.1 ± 2.1 mmol/l and in all subjects the blood glucose values in the second study was higher; the mean value for the group was 17.6 ± 3.6 mmol/l (Table 3.8). Mean maximal thermal injury response during hyperglycaemia (0.48 ± 0.12 V) was not significantly different from that in the first study (0.47 ± 0.12 V) Table 3.8 and Figure 3.9 (a). Similarly there was no significant difference between mean peak flow response to needle injury in the hyperglycaemic state (0.23 ± 0.07 V) compared with that when the blood glucose was below 10 mmol/l (0.24 ± 0.7 V) Table 3.8 and Figure 3.9 (b).
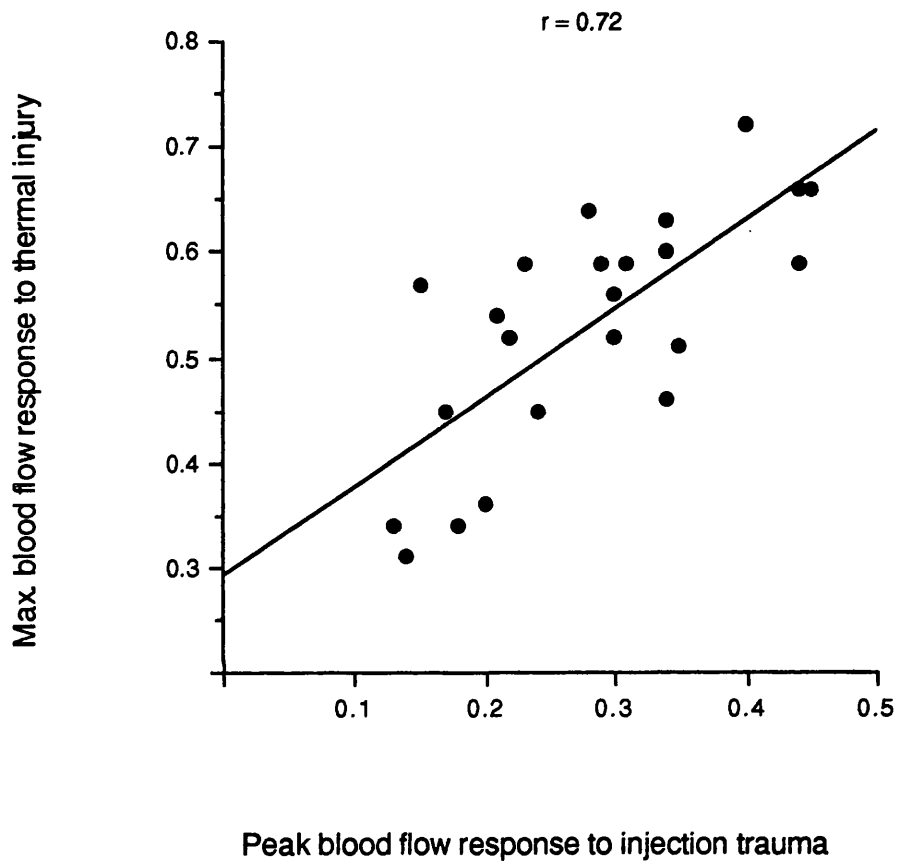
Figure 3.8

Relationship between maximum blood flow response to heat trauma and
peak blood flow response to injection injury in the diabetic group.
Regression line is shown.

3.6 THE EFFECT OF LOCALLY INJECTED INSULIN ON THE HYPERAEMIC RESPONSE TO NEEDLE INJURY

Insulin infusion has been shown to increase microvascular blood flow in the resting state (Tooke 1985). To determine whether local changes in insulin concentration influence hyperaemic responses, blood flow was measured at the point of insulin injection.

This study immediately followed that in which the effect of hyperglycaemia on blood flow was studied. Seven of the previous subjects agreed to take part. All were insulin deficient at the start of the study. Ten microlitres (1 unit) of Actrapid Novo (100 units/ml) was injected into the abdominal skin, to a depth of 0.5 cm using a 25 gauge needle and Hamilton precision syringe (Hamilton Ltd, Reno, Nevada, USA). Blood flow was measured as described in the needle injury studies. To ensure that any differences were not solely due to increased interstitial pressure resulting from the injected fluid, the results were compared with those obtained after injecting 10 microlitres of saline into an adjacent area of skin.

Compared with the response during hyperglycaemia (0.24 ± 0.07 V) neither insulin (0.24 ± 0.05 V) nor saline injection (0.22 ± 0.09 V) had a significant effect on peak blood flow (Table 3.9).

Subject	Glucose (mmol/l)		Maximum Flow (AU-V)		Peak Flow (AU-V)	
	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2
BH	7.4	20.1	0.31	0.34	0.18	0.19
DS	8.1	12.0	0.34	0.35	0.18	0.21
DM	5.2	15.4	0.59	0.51	0.31	0.28
JT	8.0	12.7	0.46	0.54	0.33	0.26
KHe	9.6	18.0	0.63	0.52	0.33	0.23
RC	5.6	23.4	0.36	0.34	0.20	0.22
CM	4.0	22.3	0.57	0.56	0.15	0.23
KH	7.5	15.4	0.45	0.55	0.17	0.14
JF	5.2	14.9	0.59	0.58	0.29	0.42
JD	7.3	17.6	0.54	0.45	0.21	0.21
Mean	7.1	17.6	0.48	0.47	0.23	0.24
±SD	2.1	3.6	0.12	0.10	0.07	0.07

TABLE 3.8: Effect of glycaemia on maximum (thermal injury) and peak (injection trauma) blood flow: Glucose in study 1< 10 mmol/l, and study 2 >10 mmol/l.
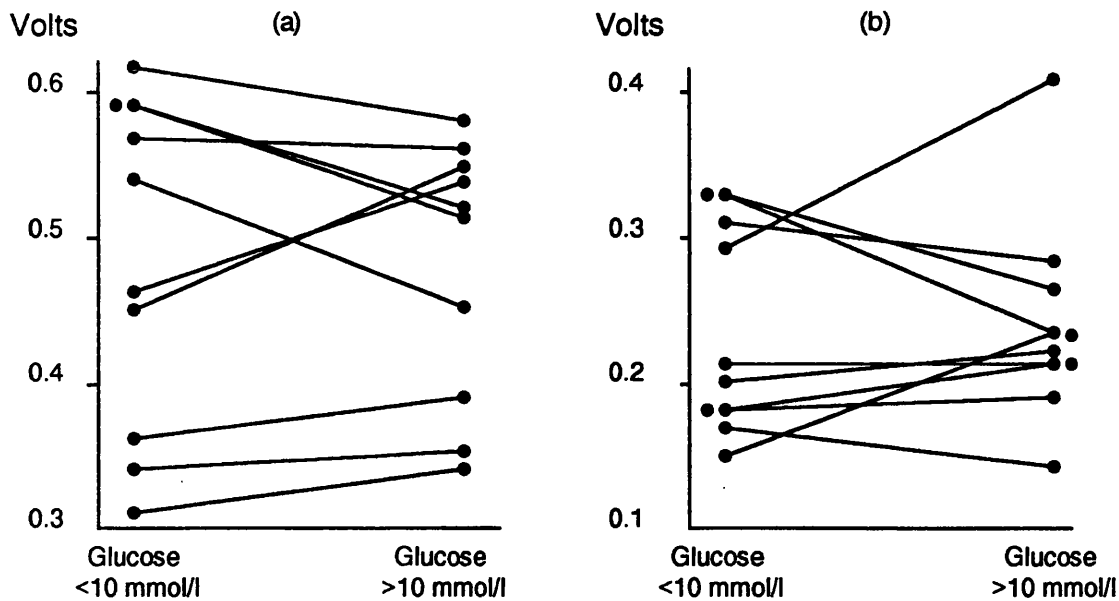
FIGURE 3.9 a,b: Flow following thermal (a) and injection trauma (b), with the glucose above and below 10 mmol/l.

TABLE 3.9

Peak blood flow responses following needle injury during
hyperglycaemia, and after the injection of 10 μl of normal saline and
10 μl of soluble insulin (100u/ml).

Subject	Peak Flow (AU-V)		
	Hyperglycaemia	Normal Saline	Insulin injection
BH	0.19	0.23	0.19
DS	0.21	0.17	0.17
DM	0.28	0.21	0.25
KHe	0.23	0.26	0.17
KH	0.14	0.15	0.12
JF	0.42	0.28	0.38
JD	0.21	0.24	0.27
Mean	0.24	0.22	0.22
SD	0.07	0.05	0.09

3.7 CAPILLARY DENSITY AND HYPERAEMIC RESPONSES TO THERMAL INJURY IN DIABETIC SUBJECTS

As described in Chapter 2, measurements obtained by the laser Doppler flowmeter are influenced by capillary density, being higher in areas where dermal capillary density is greatest. To determine whether the impaired responses in the diabetics result from capillary rarefaction, capillary density was measured in an area on the dorsum of the foot in sixteen diabetic subjects and fourteen control subjects all of whom had taken part in the previous studies.

A television microscope system was used to visualise superficial dermal capillary loops though the intact skin as described in Chapter 2. The images were recorded on to video tape and later analysed by two independent observers to derive the mean number of capillaries per mm^2 of skin surface.

Individual results are shown in Table 3.10 (a) and (b) respectively. Mean skin capillary number in the diabetic group was 52.7 ± 8.7 per mm^2 skin surface. This was not significantly different from that of the control group (50.7 ± 5.7 per mm^2 skin surface). There was no relationship between maximum blood flow and capillary density in either the diabetic or control groups ($r=0.14$ and $r=-0.28$, respectively).

Diabetic Subjects — Capillary Density loops/mm^2

Diabetic Subjects	Area 1	Area 2	Mean
DM	55	47	51.0
RG	52	54	53.0
CC	50	52	51.0
RC	59	62	60.5
BB	59	58	58.5
WS	48	37	42.5
DS	45	42	43.5
KH	49	53	51.0
CM	50	38	44.0
JT	50	50	50.0
BH	43	49	46.0
KHe	70	72	71.0
JD	74	62	68.0
JF	62	59	60.5
RD	46	46	46.0
DD	47	44	45.5
Mean			52.6
± SD			8.7

3.10 (a)

Control Subjects — Capillary Density loops/mm^2

Control Subjects	Area 1	Area 2	Mean
BM	55	54	54.5
TC	64	58	61.0
RR	40	42	41.0
MH	55	56	55.5
GR	24	24	48.0
AR	48	54	51.0
SW	62	58	60.0
NG	51	52	51.5
NW	54	40	47.0
SR	48	46	47.0
JL	56	44	50.0
JS	43	46	44.5
EO	46	60	53.0
JR	42	50	46.0
Mean			50.7
± SD			5.7

3.10 (b)

TABLE 3.10 (a,b)
Capillary density in diabetic (a) and control subjects (b).

3.8 DISCUSSION

These studies demonstrate impairment in laser Doppler measured microvascular blood flow following thermal and mechanical injury in diabetic skin. Before concluding that the findings truly represent reduced hyperaemic blood flow responses it is important to exclude other factors which could reduce the laser Doppler signal. One such factor is an increase in epidermal thickness. Under these circumstances light penetration to the deeper vascular dermal tissue will be limited, the volume of vascular tissue within the measurement hemisphere will be less and an apparent reduction in hyperaemic response will be observed. Since a two to three fold increase in epidermal thickness was shown to reduce the signal by only 10% (Chapter 2), epidermal thickness in the diabetics group would need to be increased by at least three fold to account for the 30% lower responses found in this group. Such an increase was not apparent on capillary microscopy, the apices of capillaries being as easily visible in diabetic as in control subjects. Furthermore, as later described in Chapter 4 epidermal thickness is not increased in diabetic subjects.

Another factor which could give rise to reduced laser Doppler signals despite normal microvascular responses in individual vessels, is a reduction in capillary density. However, capillary microscopy revealed no reduction in apical capillary density in the diabetic group. These findings are in keeping with the observations made in the finger nailfold by Tooke (1985). However, since the video-microscopy is limited to visualization of the most apical capillaries and the laser Doppler penetrates to a greater depth, capillary rarefaction at a deeper level could explain the reduced laser Doppler signal. This suggestion is not supported by the histological studies of Katz (1989) and Ajjam (1985) in which capillary density in the forearm was not reduced. Furthermore, in the study described in Chapter 4, no

reduction in capillary density was found in skin biopsies taken from the feet of diabetics.

Previous studies have shown that poor diabetic control (Christensen 1970; Gundersen 1974) and peripheral neuropathy (Scarpello 1980; Edmonds 1982; Archer 1984) are associated with an increase in resting blood flow and skin temperature. That there was no increase in either of these parameters in the diabetic group is not surprising as diabetic control was fairly good and none of the subjects had peripheral neuropathy. In addition, blood flow was measured in the dorsum of the foot and the abdominal wall, areas relatively devoid of shunt vessels (Grant 1931), whereas the increased flow is believed to occur through shunt vessels (Partsch 1977).

Acute insulin withdrawal has been shown to be associated with an increase in resting blood flow (Gundersen 1974). It was not possible to examine the effect of insulin withdrawal and subsequent hyperglycaemia on resting blood flow because of the large temporal and spatial variation in laser Doppler measured blood flow (Tenland 1982). Hyperaemic responses because of their good reproducibility do not present this problem. Acute changes in blood glucose due to insulin deficiency had no effect on this response, furthermore, neither had local insulin injection. The lack of an effect of diabetic control and local insulin on the hyperaemic response, would be consistent with the hypothesis that the abnormality is related to a fixed structural defect.

Since the publication of this study (Rayman 1986) several investigators using similar techniques have also reported impaired hyperaemic responses in diabetic subjects. Walmsley (1989) examined the hyperaemic response to injection trauma in the foot skin and found a significant reduction in diabetic patients with microvascular complications when compared with a control group of normal subjects. Unlike the present study, mean hyperaemic response although lower in the diabetic group free

from complications, was not significantly different from that in the control group. As the authors point out, failure to find a reduction in this group may relate to their use of a less potent stimulus and the failure to abolish sympathetic tone.

As with the present study Rendell (1989) examined the skin blood flow responses to thermal injury induced by heating the skin to 44°C. Compared with a group of normal subjects, the diabetic group had significantly reduced hyperaemic responses in the feet as well as in several other areas of skin. Only limited clinical details were presented so it is not possible to determine whether there were any differences between those with and without complications. In this study the TSI laser Doppler flowmeter (model BPM, St Paul MN, USA) was used. This allowed red cell velocity and the volume of moving cells to be independently assessed. An interesting finding was that both parameters were reduced. This suggests that the volume of vascular tissue is reduced in diabetic subjects. Since capillary density was not reduced in the present study this finding may suggest a reduction of vessel size.

Shore (1989) using a multi-sampling heating probe based on that described in this chapter, examined skin microvascular responses to thermal injury in diabetic children free from complications. As with the present study reduced hyperaemic responses were detected in subjects without clinical complications. Sandeman (1990) also using the multi-sampling heating probe found reduced hyperaemic responses in subjects with type II diabetes.

The pathophysiology of the reduced hyperaemic response in diabetic subjects is unclear. Proximal large vessel disease will reduce hyperaemic responses (Ranft 1986) but this would not explain the present findings as subjects with ankle/brachial systolic blood pressure ratios of less than 1.0 were specifically excluded and relatively young people were chosen on the pretext that they were less likely to have large vessel disease. Although

medial arterial calcification can give rise to falsely elevated ankle blood pressure readings (Edmonds 1982), few if any of the relatively young and uncomplicated diabetic subjects taking part in this study are likely to have had this complication. That the abnormality was also found in skin on the upper abdominal wall, also argues against the involvement of arterial disease which would be expected to exhibit a more distal predominance.

Since perfusion pressures (mean brachial and ankle pressures) were not reduced in the diabetic group, the abnormal responses must be the result of higher vascular resistance in the diabetic skin. Functional abnormalities such as increased levels of the potent vasoconstrictor endothelin-1 or reduced levels of the endogenous vasodilator EDRF (nitric oxide) might account for the findings. Elevated endothelin-1 levels have been recently described in diabetic subjects (Takahashi 1990) as has impairment of endothelium-dependent relaxation in animal studies (Katsuo 1989), however the relationship between these endothelial factors and injury responses has as yet to be determined in normal and diabetic subjects. Similarly, reduced prostacyclin synthesis or release could be involved. Impaired release of other vasoactive mediators such as Substance P and CGRP from local tissue or nerves, or an inability of the vessels to respond to such agents either due to a structural or functional abnormality could offer other theoretical explanations.

The possibility that the reduced response represents an abnormality of the axon reflex and not a microvascular defect needs to be considered. Several studies (Starr 1930, Ajjam 1984) have demonstrated reduced histamine flare in diabetic skin and have assumed that this represents a defect in the skin vasculature. Although this may be the so, the absence of the histamine flare in diabetes may be due to degeneration of the nociceptive C fibres as occurs in post ganglionic lesions of the brachial plexus (Bonney 1954), and familial dysautonomia (Smith 1963). In support of this, Parkhouse (1988) using iontophoresis of acetylcholine demonstrated reduced

axon reflex vasodilation measured by laser Doppler flowmetry in diabetics with peripheral neuropathy but not in those with an intact peripheral nervous system. Since the microvascular vasodilator response to direct stimulation induced by firmly stroking the skin was found to be normal in all diabetic groups, the authors concluded that the inability to produce a flare response was not vascular but the result of degeneration of nociceptive C fibres. In relation to the present study and those described above [Walmsley (1989), Rendell (1989) Shore (1989), Sandeman (1990)], that the microvascular response to direct stimulation was normal is surprising. The most likely explanation for this difference is a failure of the method used by Parkhouse to produce maximum vasodilatation. This is confirmed by the fact that the stimulus used caused only a 3-4 fold rise in blood flow compared with the 30-50 fold increase found in the present and other studies (Hassan 1987). In the present study hyperaemic responses to both thermal and mechanical injury were measured directly at the point of injury, they do not depend on the axon reflex but represent the direct vascular response to tissue damage; the first part of Lewis' triple response. That this response is neurologically independent was shown by Lewis (1924) in denervated skin. This is confirmed by the study described in Chapter 4 in which local anaesthesia did not diminish the response. In addition in the present study the response was impaired in subjects free from neuropathy, in keeping with the findings of Walmsley (1989) and Shore (1989). There was also no association between the hyperaemic response and vibration sensory threshold. Finally the response to injection trauma was equally blunted on the abdominal skin, whereas a distal predominance would have been expected if the defect was related to neuropathy.

The present study examined thermal responses in an area of skin with no arteriovenous anastamoses. Stevens (1990) in a recent study using a TSI laser Doppler flowmeter, examined the microvascular response to thermal injury in the big toe of subjects with and without peripheral neuropathy. He

made the interesting observation that whereas heating increased flow in the non-neuropathic subjects there was a paradoxical fall in diabetic subjects with neuropathy. Similar paradoxical responses were found in non-diabetic subjects with traumatic neuropathy. These findings suggest that this particular abnormality, which unlike the present study relates to arteriovenous blood flow regulation, is not due to microangiopathy but to neuropathy.

Increased vascular resistance due to a structural abnormality such as basement membrane thickening would provide an attractive explanation for the functional microvascular abnormality, as this would fit with the finding of greater reduction in hyperaemic responses in those diabetics with background retinopathy in whom the basement membrane would be expected to be increased. However, the finding of abnormal responses to both thermal injury and injection trauma in diabetics free from clinical complications would not be compatable with this hypothesis unless structural microvascular changes preceed clinically detectable microvascular disease. Alternatively this functional abnormality may precede and may in some way be implicated in the later structural changes. The relationship between structure and function is examined in Chapter 4.

CHAPTER 4

RELATIONSHIP BETWEEN THE MICROVASCULAR RESPONSE TO TISSUE INJURY AND CAPILLARY ULTRASTRUCTURE

4.1 INTRODUCTION

In the previous chapter the microvascular response to tissue injury was found to be impaired in subjects with Type I diabetes, those with background retinopathy appearing to have the greatest abnormality. It was postulated that this abnormality may be caused by impaired microvascular vasodilatation due to a fixed structural microvascular defect.

The aims of this study were, a) to examine in greater detail the relationship between the microvascular response to injury and the severity of diabetic complications, and b) to undertake a more detailed examination of foot skin capillary structure than has been attempted in previous studies, so as to determine the relationship between skin microvascular hyperaemic responses and capillary structure in diabetic patients. Thus, the response to tissue injury was measured in the foot skin of subjects with and without diabetic complications, and in non-diabetic control subjects; and in the same individuals detailed light and electron microscopy was performed on skin biopsied from adjacent skin.

4.2 SUBJECTS

Subjects with diabetes were recruited from the Diabetes Centre at the Ipswich Hospital. Smokers, and those with a history of foot lesions were excluded, as were those receiving medications other than insulin and thyroxine replacement therapy. Subjects with either systolic or diastolic

brachial blood pressures greater than 170 mmHg and 100 mmHg respectively, were also excluded. Ankle systolic blood pressure was determined by ultrasound Doppler in all subjects. Those with an evidence of lower limb occlusive vascular disease (ankle/brachial systolic pressure ratio of less than 1.0), were not included in further study (Yao 1969). Fundi were assessed by standard ophthalmoscopy through dilated pupils. All subjects with proliferative retinopathy were receiving or had received laser photocoagulation.

Twenty-eight insulin-dependent diabetics (fourteen male and fourteen female) were studied. Diabetic subjects were separated into three groups. Group I consisted of subjects without clinical evidence of complications. Clinical details are shown in Table 4.1. The group comprised twelve subjects, 5 females and 7 males. Mean age was 40.1 ± 11.4 years (range 23 to 62 years) and mean duration of diabetes 20.1 ± 11.5 years (range 8 to 48 years). Group II comprised those with background retinopathy alone, Table 4.2. There were six subjects, 2 females and 4 males. Mean age was 46.6 ± 8.1 years (range 34 to 58 years) and mean duration of diabetes 27.7 ± 7.4 years (range 20 to 38 years). Group III subjects were those with severe complications. Patients were considered to have severe complications if they had proliferative retinopathy alone or background retinopathy together with one or more of the other complications. Clinical details are shown in Table 4.3. There were ten subjects in this group, 5 females and 5 males. The mean age of the group was 44.3 ± 13.3 years (range 24 to 61 years) and mean duration of diabetes 27.4 ± 14.3 years (range 12 to 53 years). Five subjects had proliferative retinopathy, five background retinopathy, three had albustix positive proteinuria but normal plasma creatinine and six had peripheral neuropathy (vibration sensory threshold above the 90th centile for age).

The control group consisted of seventeen healthy volunteers from the staff at the Ipswich Hospital. Their clinical details are given in Table 4.4.

TABLE 4.1

Clinical details of Group I Diabetic Subjects.

Subject	Sex	Age	Duration	Insulin Dose units/d	Vibration Threshold (Volts)	Systolic BP mmHg	Diastolic BP mmHg	Ankle BP mmHg
GC	M	28	17	64	6	130	90	130
PC	F	30	24	63	10	145	95	150
LP	F	41	8	20	7	110	60	135
RS	F	43	28	37	8	120	70	160
JG	F	32	20	42	12	110	70	132
MS	M	23	9	51	8	140	80	155
ME	M	37	10	44	12	120	80	160
DK	M	37	9	56	8	110	75	140
DP	F	62	26	72	6	135	80	180
RC	F	53	26	56	7	160	100	180
GS	M	43	16	62	10	130	80	140
AR	F	52	48	40	10	150	80	160
Mean		40.1	20.1	50.6	8.9	130.0	80.0	151.0
SD		11.4	11.5	11.6	2.1	16.7	11.1	17.1

TABLE 4.2

Clinical details of Group II Diabetic Subjects

Subject	Sex	Age	Duration	Insulin Dose units/d	Vibration Threshold (Volts)	Systolic BP mmHg	Diastolic BP mmHg	Ankle BP mmHg
M B	M	50	34	30	14	120	80	130
L A	F	34	30	52	6	170	80	170
M G	F	42	38	34	6	170	70	170
G C	M	47	22	74	9	120	80	145
G D	M	58	20	54	10	150	85	160
B C	M	49	22	52	18	120	90	150
Mean		46.6	27.7	49.3	10.5	141.7	80.8	154.2
SD		8.1	7.4	15.8	4.7	24.8	6.6	15.6

TABLE 4.3
Clinical details of Group III Subjects

Subject	Complications	Sex	Age	Duration	Insulin Dose units/d	Vibration Threshold (Volts)	Systolic BP mmHg	Diastolic BP mmHg	Ankle BP mmHg
JM-S	PR	F	41	40	37	10	125	70	140
GS	BR, PN	M	61	47	50	32	170	100	180
PB	PR, PN	M	55	53	76	45	130	80	160
ET	BR, PN	F	45	23	45	32	160	80	160
CB	PR	F	27	14	62	7	130	70	130
DS	BR, PN	M	50	17	44	28	130	75	155
JM	BR, Alb	M	58	20	46	20	150	80	180
HH	BR, Alb, PN	M	52	21	72	30	130	90	140
TB	PR, PN	F	24	12	58	14	130	80	140
KS	PR, Alb	F	30	27	29	10	110	70	110
Mean			44.3	27.4	51.9	22.8	136.5	79.5	149.5
SD			13.3	14.3	14.9	12.5	18.0	9.6	21.9

PR= Proliferative retionpathy
PN= Peripheral Neuropathy
Alb= Albustix + proteinuria

TABLE 4.4: Clinical details of Control Subjects

Subject	Sex	Age	Vibration Volts	Systolic BP mmHg	Diastolic BP mmHg	Ankle BP mmHg
R M	M	22		120	80	120
G R	M	36	5	125	80	130
A B	F	44		120	70	130
McL	M	42	6	115	80	120
K F	M	49	7	130	90	145
P J	F	58	16	130	90	155
Yv T	F	49	12	130	85	140
B B	F	44	10	150	70	150
P B	M	38		140	85	145
J Mt	M	63		150	90	170
T G	M			130	80	150
P L	F	42		125	80	140
A L	M	42		130	80	145
M Mc	F	42		120	80	125
R B	F	21		100	70	110
M R	F	44		125	75	130
G D	M	42		160	90	170
Mean		42.9		129.4	80.9	139.7
SD		10.6		14.3	6.9	16.8

There were 9 men and 8 women. Their mean age was 42.6 ± 10.4 years (range 21 to 63 years). The exclusion criteria were the same as for the subjects with diabetes.

Written consent was obtained from all subjects and the study was approved by the District Ethical Committee of the East Suffolk Health Authority.

4.3 METHODS

4.3.1 Blood flow response to thermal injury

The protocol followed and methods used to measure the thermal injury response were as described in Chapter 3. Briefly, measurements were made on the dorsum of the foot, in skin that had been subjected to mild thermal injury. This was induced by heating the skin to 44 °C for 30 minutes using a specially constructed heating probe (Chapter 2). To maximally dilate the microvessels, measurements were made with the foot placed fifty centimetres below heart level. Readings were taken from nine sites and the mean was used for analysis.

The only differences between the blood flow methods in this and the previous study concerned the environmental temperature and model of laser Doppler flowmeter used. Because of the constraints of the Ipswich Hospital heating and cooling system it was more practical to use a environmental temperature which was controlled to between 22 and 24 °C; one degree higher than had used in the previous study. Blood flow was measured using a Periflux PF 2B flowmeter. The instrument is identical to the Periflux PF 1 except for the use of thinner fibre optic light guides to reduce fibre movement artefacts. The instrument was calibrated against a new calibration standard supplied by the manufacturers and hence the results are not comparable with those obtained with the Periflux PF 1.

4.3.2 Tissue biopsy

Skin biopsies were performed on a separate visit to that on which thermal injury responses were measured. Subjects were rested for ten minutes during which time blood glucose was measured. The skin was then anaesthetised by injection of 0.5ml of 2% lignocaine into an area overlying the 1st and 2nd metatarsals. A single biopsy was then taken from the dorsum of the foot approximately 2 cm distal to the injection site and also distal to the area from which thermal injury responses had been previously measured. Each biopsy was performed using a fresh disposable 3mm diameter Steifel biopsy punch (Stiefel Laboratories Ltd., Bucks, England, UK). The biopsy site was closed using a 4/0 braided silk suture (Mersilk 4/0 Ethicon Ltd., UK). The suture was removed at 5 days. No complications arose in any of the subjects as a result of the biopsy.

4.3.3 Blood flow response to mechanical injury

After suturing, the blood flow response to the trauma induced by the biopsy was measured in the skin immediately adjacent to the site of injury. Measurements were made at each of four points located approximately 1 mm from the centre of the cross formed by the the margins of the biopsy site and the crossing of the suture, Figure 4.1. The tip of the laser probe was supported over the measuring point using a specially moulded support. From each of these points recordings were made for one minute rotating between sites. Recordings were continued for 20 minutes. The average of the four peak blood flow measurements was used for analysis.

The skin temperature from an area of foot skin adjacent to the biopsy site was measured at the time of the blood flow recordings using a Comark Electronic Thermometer Type 1625 (Comark Electronics Ltd., Sussex, UK).

Fig. i Components of capillary wall

Basement Membrane

Endothelial Cell

Pericyte

Nucleus
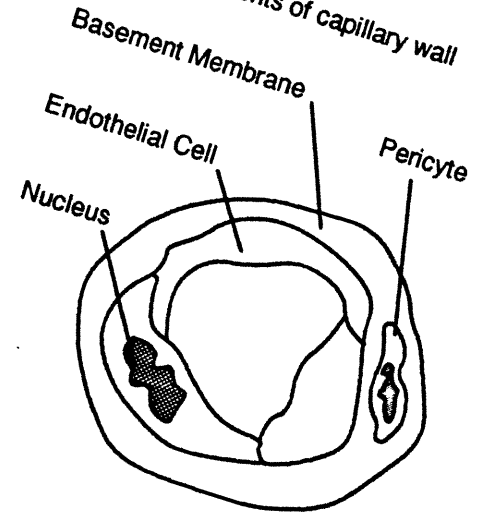
Fig ii. Grid Overlay
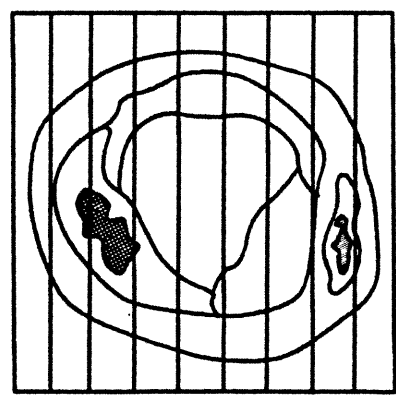
Fig iii. Points of intersection
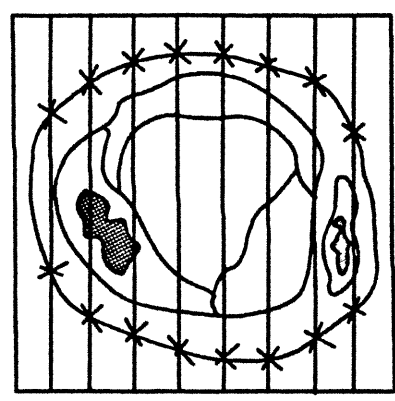
Fig iv. Tangents
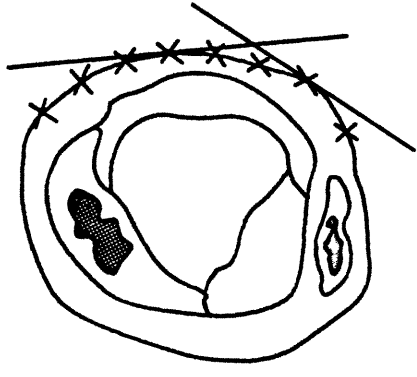
Fig v. Orthogonal
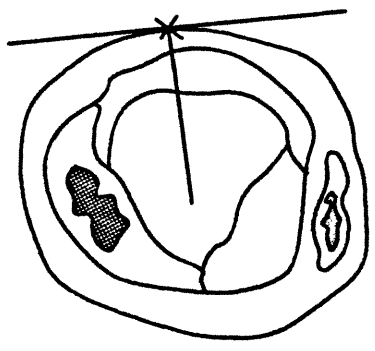
Fig vi. Parameters measured

Basement Membrane thickness

Capillary wall thickness

Endothelial Cell thickness
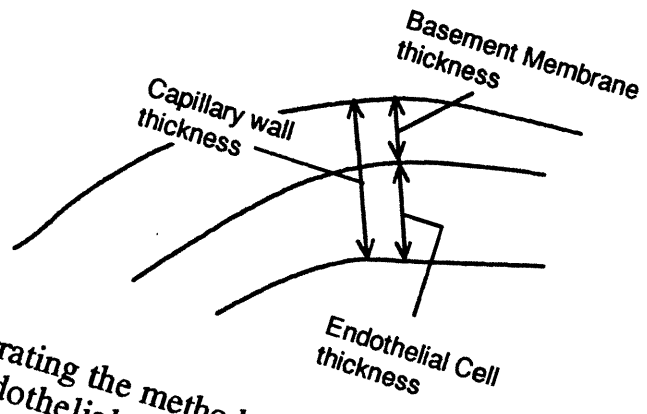
Figure 4.2 i-vi. Series of figures illustrating the method used to determine basement membrane thickness, endothelial cell and capillary wall thickness.

4.3.4 Fixation and processing

Immediately after biopsy the tissue was fixed in gluteraldehyde fixative (2.5%) in cacodylate buffer for five hours at room temperature. The tissue was then washed 6 times in cacodylate buffer containing 10% sucrose and left overnight. Secondary fixation was then carried out in 1% aqueous osmium tetroxide for five hours. After washing 9 times in distilled water the tissue was dehydrated in a graded series of ethanol, progressing from 2 changes of 5 minutes in 15% ethanol, 2 changes of 10 minutes in 30% ethanol, 2 changes of 15 minutes in 50% ethanol, 2 changes of 15 minutes in 70% ethanol, 2 changes of 30 minutes in 90 and 95 % ethanol and finally and finally 6 changes of 30 minutes in 100% ethanol, at room temperature. The tissue was then infiltrated with catalysed epon using propylene oxide as an intermediary and finally polymerised for 2 days at 60 °C in an oven.

4.3.5 Histological procedures

For light microscopy, semi-thin (0.75 µm) longitudinal sections were cut on a Reichart-Jung Ultracut OM-U4 mechanical advance microtome. The sections were stained with Toludine blue.

For electron microscopy, ultra-thin sections (60-90 nm) were cut, floated on to distilled water and flattened with trichlorethylene vapour. The sections were collected onto uncoated copper grids of mesh size 300, which had been previously cleaned in acetone and washed in distilled water. The sections were stained by placing in dimple tiles containing methanolic uranyl acetate for 20-25 minutes which was covered to exclude light. The sections were then washed through descending concentrations of methanol (100%, 75%, 50%, 30%) down to distilled water and counterstained with lead citrate in a dimple tile surrounded by sodium hydroxide pellets (to prevent lead citrate deposits forming on the section) for 3-4 minutes. The grids were then washed with distilled water and blotted on filter paper.

4.3.6 Microscopy and photography

Semi-thin sections were examined using a Vickers light microscope. Light micrographs of the sections were taken on Kodak Panatomic-X film, developed in Acutol and fixed in Ilfospeed hyperfixer. Each photograph was enlarged 3 times (x 835) on a Durst M670 enlarger and printed up on fine grain photographic paper. A standard graticule was included in the photograph to enable exact magnification to be calculated.

Ultra-thin sections were examined using a Phillip's High Resolution Transmission Electron Microscope (EM 201C). All microvessels without a complete layer of cells (pericyte or smooth muscle) surrounding the endothelial cells were considered to be capillaries and were photographed on Eastman Kodak 5302 film at a final magnification of x10,000. The film was developed and fixed in Ilfospeed developer and Ilfospeed fixer respectively.

4.3.7 Morphometric procedures

All capillaries within view and in which the ratio of the major to minor axes was less than 2:1 were analysed. This ratio was used to avoid analysis of capillaries which had been sectioned obliquely. For each biopsy specimen between 20 and 30 capillaries were examined. The number of endothelial cell nuclei, pericyte nuclei, and endothelial cell profiles (equivalent to the number of intercellular junctions) were counted directly from the micrographs.

The method used to obtain linear measurements is described in the series of diagrams shown in Figures 4.2 i-vi. A transparent acetate sheet bearing horizontal grid lines (1 cm apart) was placed on each capillary electron micrograph and the points of intersection of each line with the outer border of the basement membrane was marked (Figures 4.2 i-iii). From these points of intersection tangents were drawn (Figure 4.2 iv). Orthognals were then drawn from the points of intersection to the capillary

lumen (Figure 4.2 v). Lengths indicating the thickness of the basement membrane, the endothelial cell thickness and the capillary wall thickness (Figure 4.2 vi) were measured using a magneto-strictive digitiser interfaced to a BBC Microcomputer system (PSM Instruments Ltd., Maidenhead, Berks., UK). Approximately 30 measurements for each parameters were made for each of the 20-30 capillaries assessed. Luminal, outer endothelial cell and vessel perimeters were determined by tracing around the luminal and abluminal endothelial membranes and the outer vessel wall, respectively.

Epidermal thickness was derived from approximately 30 randomly derived intercept lengths. The method used was similar to that described above. A transparent overlay bearing parallel and equidistant lines 1 cm apart was superimposed on each light micrograph. Points of intersection between test lines and the outer aspect of the epidermis provided starting points for measuring random intercept lengths. Tangents to the surface were drawn through intersection points and orthogonals drawn from these to the dermis. All such distances were measured using a magneto-strictive digitiser interfaced to a BBC Microcomputer system (PSM Instruments Ltd., Maidenhead, Berks., UK).

Capillary density was assessed by counting the number of vessels without a smooth muscle coat and other previously described criteria (Yen 1976), in tissue lying within a standard depth of 500 μm below the dermo-epidermal junction. The area within which the count was made was digitized and the density calculated.
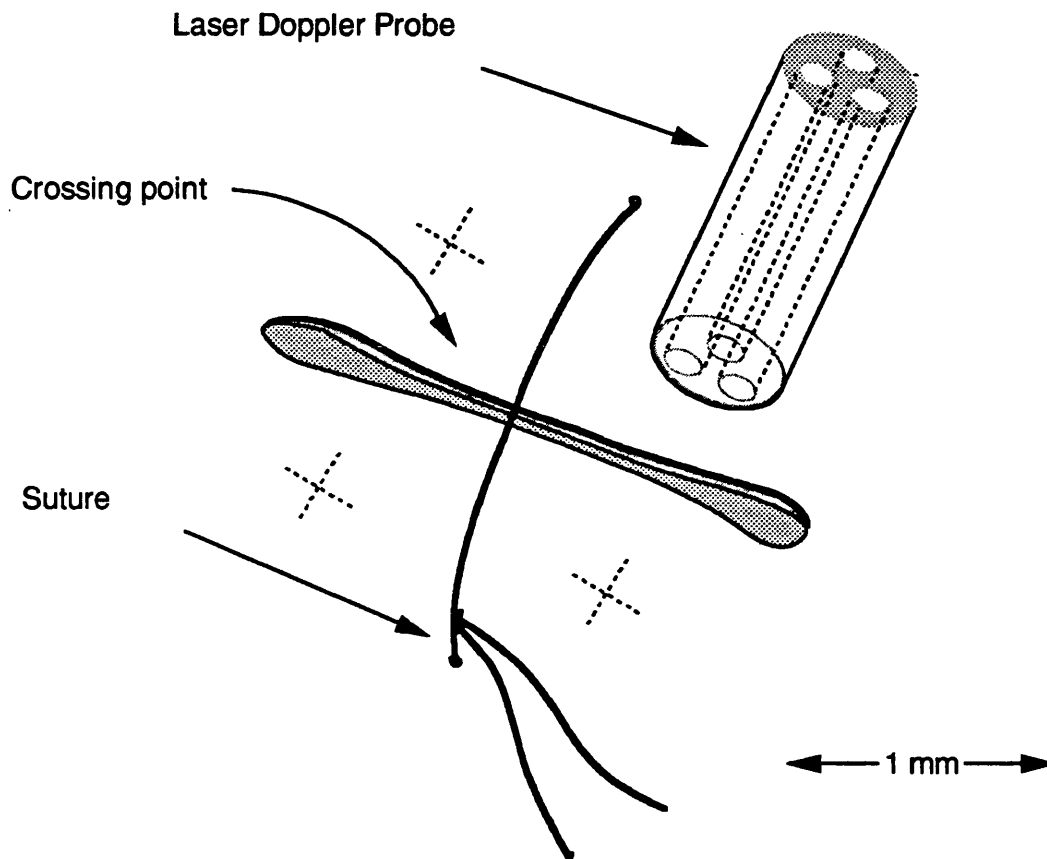
Figure 4.1: Diagram illustrating sites from which blood flow was measured. In the diagram the laser Doppler probe is shown located approximately 1mm from the crossing point of the suture and the edges of the closed wound. Each of the other measurement points are shown by an X.

4.4 RESULTS

4.4.1 Clinical status

There were no significant differences in ages, and brachial and ankle systolic blood pressures between the various groups. In the diabetic groups there were no statistical differences in disease duration. This data is summarised in Table 4.5 and individual subject data is shown in Tables 4.1 to 4.4.

Blood glucose measured at the time of the study (Table 4.5), though lower in the uncomplicated group (9.0 ± 4.9 mmol/l) was not significantly different from that in the other two groups (Group II- 13.3 ± 6.1; Group III- 13.3 ± 4.3 mmol/l). Individual subject glucose values are shown in Table A 4.1.

Skin temperature from the dorsum of the foot was not significantly different between the groups (Control subjects- 30.4 ± 1.7 °C; Group I- 31.2 ± 1.6 °C; Group II- 30.9 ± 2.0 °C; Group III- 32.3 ± 2.4 °C), Table 4.5. Individual skin temperatures are shown in shown in Table A 4.2.

4.4.2 Thermal injury responses

Hyperaemic blood flow responses to thermal injury in the various groups are shown in Table 4.6 and Figure 4.3. Compared with the control subjects (1.88 ± 0.35 V, range 1.23 to 2.45 V), thermal injury responses were significantly lower in Group I (1.34 ± 0.24 V, range 0.90 to 1.63 V; $p<0.001$), Group II (1.14 ± 0.19 V, range 0.90 to 1.34 V; $p<0.001$) and Group III (0.74 ± 0.17 V, range 0.45 to 1.00 V; $p<0.001$). There was a significant inverse relationship between the severity of complications and the blood flow response ($p<0.001$). Thermal injury responses were not related to age, systolic blood pressure, or ankle pressure in either diabetic or non-diabetic subjects and in the diabetics there was no relationship with

TABLE 4.5

Clinical details of control and diabetic groups*

	Controls	Group I	Group II	Group III
Sex	9 m 8 f	5 m 7 f	4 m 2 f	5 m 5 f
Age	42.6±10.4	40.1±11.4	46.7±8.1	44.3±13.3
Duration	-	20.1±11.5	27.7±7.4	27.4±14.3
Blood Pressure	129.4±14.2	130.0±16.7	141.7±24.8	136.5±18.0
mmHg	80.3±6.4	80.0±11.1	80.8±6.6	79.5±9.6
Ankle Pressure mmHg	139.7±16.8	151.0±17.1	154.2±15.6	149.5±21.9
Glucose mmol/l	-	9.0±4.9	13.3±6.1	13.1±4.3
Skin Temperature °C	30.4±1.7	31.2±1.6	30.9±2.0	32.3±2.4

* Values are means ± SD

TABLE 4.6

Thermal injury responses (maximum blood flow) in Control Subjects
Group I, Group II and Group III Subjects- arbitary units of volts

Control Subjects		Group I		Group II		Group III	
Subject	Thermal Injury response AU-Volts	Subject	Thermal Injury response AU-Volts	Subject	Thermal Injury response AU-Volts	Subject	Thermal Injury response AU-Volts
RM	-	GC	1.56	MB	0.90	JM-S	0.82
GR	2.45	PC	1.18	LA	1.34	GS	0.81
AB	2.31	LP	1.60	MG	1.34	PB	0.64
McL	1.62	RS	1.42	GC	1.20	ET	0.54
KF	1.66	JG	1.63	GD	0.94	CB	0.45
PJ	2.16	MS	1.40	BC	1.09	DS	1.00
YvT	1.51	ME	1.39			JM	0.69
BB	2.14	DK	1.60			HH	0.90
PB	1.71	DP	0.90			TB	0.74
JMt	1.92	RC	1.27			KS	0.81
TG	1.90	GS	1.17				
PL	1.23	AR	1.01				
AL	1.65						
MMc	1.52						
RB	2.26						
MR	1.81						
GD	2.29						
Mean	1.88		1.34		1.14		0.74
SD	0.35		0.24		0.19		0.17
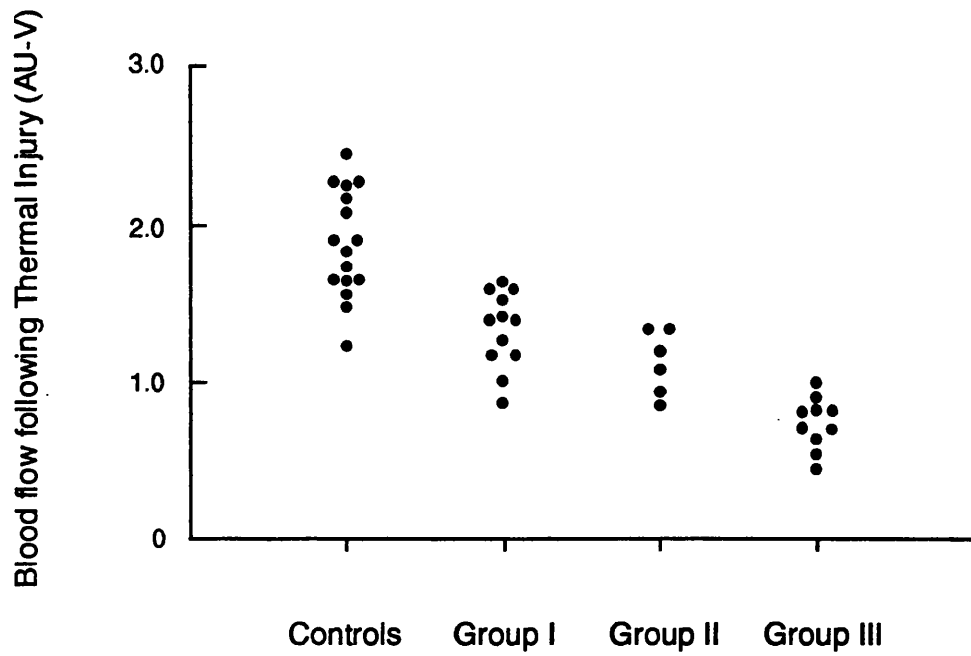
Figure 4.3: Laser Doppler blood flow following themal injury in control subjects and diabetic groups- arbitary units of volts
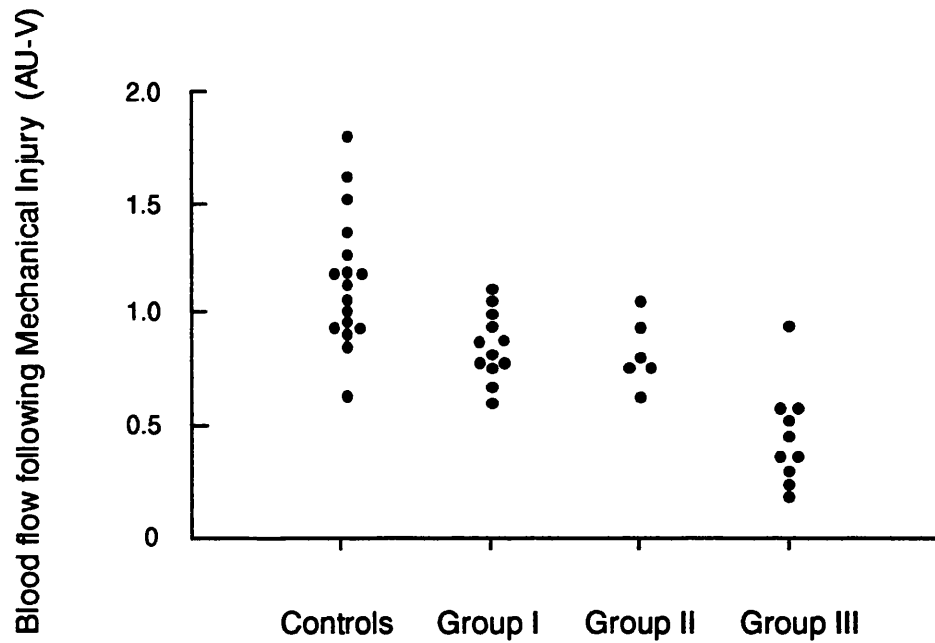
Figure 4.4: Laser Doppler blood flow following mechanical injury in control subjects and diabetic groups- arbitary units of volts

TABLE 4.7

Correlations *(r)* between selected clinical, functional and structural
parameters in diabetic patients

	Thermal injury response	Biopsy injury response	Basement membrane	Luminal perimeter
Age	-0.32	-0.10	0.01	0.04
Duration	-0.35	-0.20	0.24	0.12
Systolic BP	-0.20	-0.17	0.17	0.07
Ankle BP	-0.08	-0.10	-0.11	0.04
Glucose	-	-0.16	0.28	-0.04
Skin Temp.	-	-0.51**	0.16	-0.36
Category	-0.80***	-0.68***	0.56**	0.15
Retinopathy status	-0.74***	-0.65***	0.47**	0.07
Vibration threshold	-0.53**	-0.31	0.17	0.13
Nephropathy status	0.33	0.42*	0.20	0.09
Thermal injury response	-	0.76***	-0.53**	-0.19
Biopsy injury response	0.76***	-	-0.57**	0.34
Basement membrane thickness	-0.53**	-0.57**	-	0.39
Luminal perimeter	-0.19	0.03	0.39*	-
Capillary Density	-0.11	-0.22	0.04	-0.44*

* p <0.02   ** p <0.01   *** p <0.001

Category = Group I, II, and III.

Retinopathy status = none (1), background (2), proliferative (3).

Nephropathy status = none (0), albustix positive (1).

duration of diabetes. Correlations for the diabetic subjects are shown in Table 4.7.

In Group III, hyperaemic responses in subjects with neuropathy did not differ from those without neuropathy, nor did the responses differ in subjects with and without nephropathy. Although subjects with proliferative retinopathy had significantly reduced responses compared to those with background retinopathy alone, the responses were not significantly different when compared with subjects who had background retinopathy together with another complication.

4.4.3 Mechanical injury responses

Responses to mechanical injury are shown in Table 4.8 and Figure 4.4. Compared to the control group (1.16 ± 0.3 V, range 0.63 to 1.62 V) this response was also significantly reduced in all diabetic groups (Group I- 0.86 ± 0.15 V, range 0.60 to 1.10 V; p<0.01; Group II- 0.85 ± 0.15 V, range 0.66 to 1.08 V; p<0.02; Group III- 0.46 ± 0.22 V, range 0.23 to 0.96 V; p<0.001). There was a significant trend for the response to be decrease from Group I to Group III (p< 0.001), but this effect was mainly due the lower values in Group III since values in Group I and II were not different.

Hyperaemic responses following mechanical injury did nor correlate with blood glucose (r= - 0.16). As with thermal injury, the hyperaemic response to mechanical injury, was not related to age, systolic blood pressure, ankle pressure or duration of diabetes, Table 4.7.

In Group III, hyperaemic responses in subjects with neuropathy did not differ from those without neuropathy, nor did the responses differ in subjects with and without nephropathy. Although subjects with proliferative retinopathy had significantly reduced responses compared to those with background retinopathy alone the responses were not significantly different from those with background retinopathy and another complication.

TABLE 4.8

Biopsy injury responses (peak blood flow) in control subjects, Group I, Group II and Group III subjects- arbitary units of volts (AU-V).

Control Subjects		Group I		Group II		Group III	
Subject	Biopsy Injury response AU-Volts	Subject	Biopsy Injury response AU-Volts	Subject	Biopsy Injury response AU-Volts	Subject	Biopsy Injury response AU-Volts
RM	1.52	GC	1.10	MB	1.08	JM-S	0.23
GR	1.37	PC	0.85	LA	0.97	GS	0.53
AB	1.03	LP	1.07	MG	0.78	PB	0.59
McL	1.62	RS	1.00	GC	0.78	ET	0.34
KF	0.95	JG	0.91	GD	0.66	CB	0.47
PJ	0.96	MS	0.80	BC	0.83	DS	0.96
YvT	1.04	ME	0.80			JM	0.29
BB	1.19	DK	0.82			HH	0.59
PB	1.17	DP	0.60			TB	0.26
JMt	1.00	RC	0.78			KS	0.38
TG	0.63	GS	0.94				
PL	0.88	AR	0.67				
AL	1.16						
MMc	1.27						
RB	1.81						
MR	0.88						
GD	1.20						
Mean	1.15		0.86		0.85		0.46
SD	0.30		0.15		0.15		0.22

4.4.4 Relation between thermal and mechanical injury responses

In diabetic subjects the microvascular responses to the two forms of injury were significantly correlated (r=0.75, p<0.001) as shown in Figure 4.5. No such relationship was found in the control subjects (r=0.26, p>0.3).

4.4.5 Effect of Local Anaesthesia on Injury Response

To determine whether local anaesthesia may have influenced the hyperaemic response, thermal injury responses were measured in forearm skin and also in an adjacent area of skin which had been anaesthetised by the injection of 2 ml of 5% lignocaine. Eight separate comparisons were made. The results of each of these studies is presented in Table A 4.3. Mean maximal thermal injury response in un-anaesthetised skin was 1.66 ± 0.11 V; this which was not significantly different to that in the anaesthetised skin, 1.65 ± 0.15 V.

4.4.6 Light Microscopy

Group data relating to epidermal thickness and capillary density are shown in Table 4.9 and data for individual subjects are shown in Table A 4.4

Mean epidermal thickness in the control group was 73.5 ± 17.3 μm (range 43.7 to 104.0 μm). This was not significantly different when compared with that in Group I (85.3 ± 18.2 μm, range 57.0 to 116.3 μm), Group II (79.4 ± 9.4 μm, range 67.0 to 95.0 μm) and Group III subjects (68.8 ± 18.8 μm, range 38.5 to 89.9 μm). Between the diabetic groups there were no significant differences in epidermal thickness nor was there a trend to either increasing or decreasing thickness with severity of complications.

Capillary density in the control group was 59.0 ± 23.6 capillaries/mm^2. This was not significantly different to that in any of the
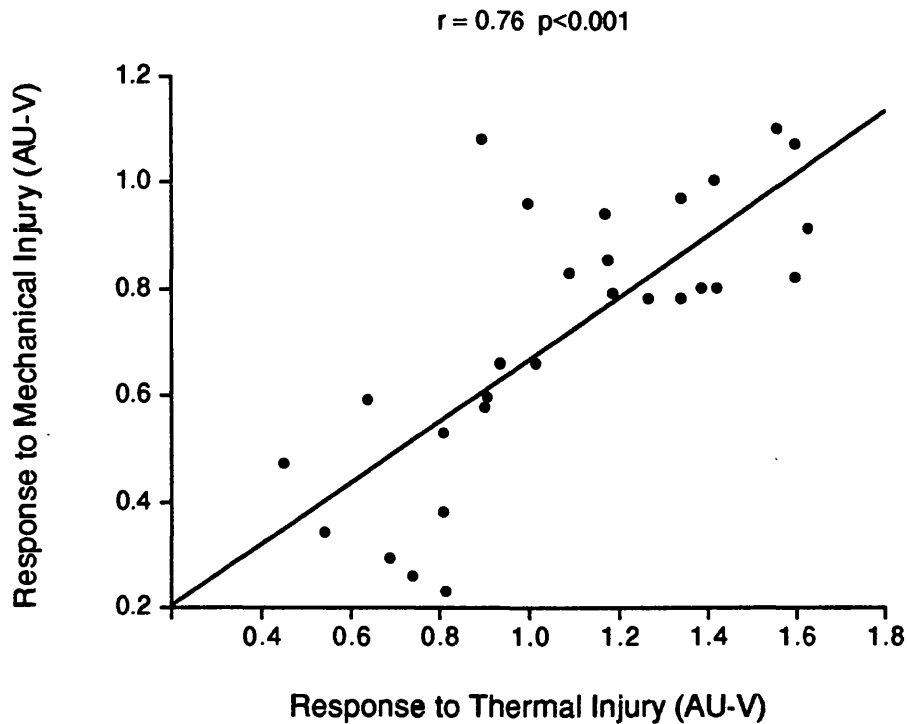
Figure 4.5: Relationship between mechanical and thermal injury responses.

Table 4.9: Summary of data relating to capillary density, epidermal thickness, and cellular components of the capillary wall.

	Controls	Group I	Group II	Group III
Capillary density caps/mm^2	59.0 ± 23.6	62.5 ± 21.7	56.8 ± 15.3	71.9 ± 24.4
Epidermal thickness µm	73.5 ± 17.3	85.3 ± 18.2	79.4 ± 9.4	68.8 ± 18.8
Endothelial cell profile No.	5.18 ± 0.84	4.81 ± 0.50	5.18 ± 0.93	4.78 ± 0.61
Endothelial cell nuclear No.	1.53 ± 0.44	1.33 ± 0.23	1.42 ± 0.49	1.39 ± 0.46
Pericyte Nuclear No.	1.02± 0.37	1.06 ± 0.17	0.95 ± 0.16	1.08 ± 0.47
Endothelial/Pericyte nuclear ratio	1.67 ± 0.66	1.27 ± 0.23	1.53 ± 0.54	1.41 ± 0.48

diabetic groups (Group I subjects - 62.5 ± 21.7 capillaries/mm^2; Group II- 56.8 ± 15.3 capillaries/mm^2; Group III- 71.9 ± 24.2 capillaries/mm^2). There were no significant differences between groups, nor was there a trend with the severity of complications.

4.4.7 Electron Microscopy

Cellular components of capillary wall

Regarding the cellular components of the capillary wall, between the various groups there were no significant differences in endothelial cell profile number, endothelial cell nuclear number, pericyte nuclear number and endothelial/pericyte cell nuclear ratios, nor was there a significant change in any of these parameters with severity of complications. For clarity the data is not included in the text but summarised in Table 4.9. Individual subject data is shown in Tables A4.5 - A4.8. Electron micrographs of representative vessels from control and diabetic subjects are shown in Figure 4.6.

Perimeter measurements

Compared with the normal subjects, mean luminal perimeter (31.3 ± 6.0 μm) was significantly reduced in Group I (23.5 ± 4.6 μm; p<0.001) and Group III (25.0 ± 5.0 μm; p<0.012), Table 4.10 and Figure 4.7.

Endothelial cell outer perimeter was also significantly reduced in Group I (31.4 ± 4.4 μm; p<0.007) and Group III (31.6 ± 2.9 μm; p<0.007) compared with the control group (39.6 ± 9.0 μm), Table 4.11 and Figure 4.8. In Group II both luminal perimeter and endothelial cell outer perimeter were reduced (24.9 ± 6.0 μm and 31.4 ± 4.4 μm) compared with the control group but because of the small size of the group neither reached significance. Neither capillary luminal perimeter nor endothelial cell outer perimeter related to age, sex, or skin temperature and in the diabetics there was no relationship with duration of diabetes and blood glucose.
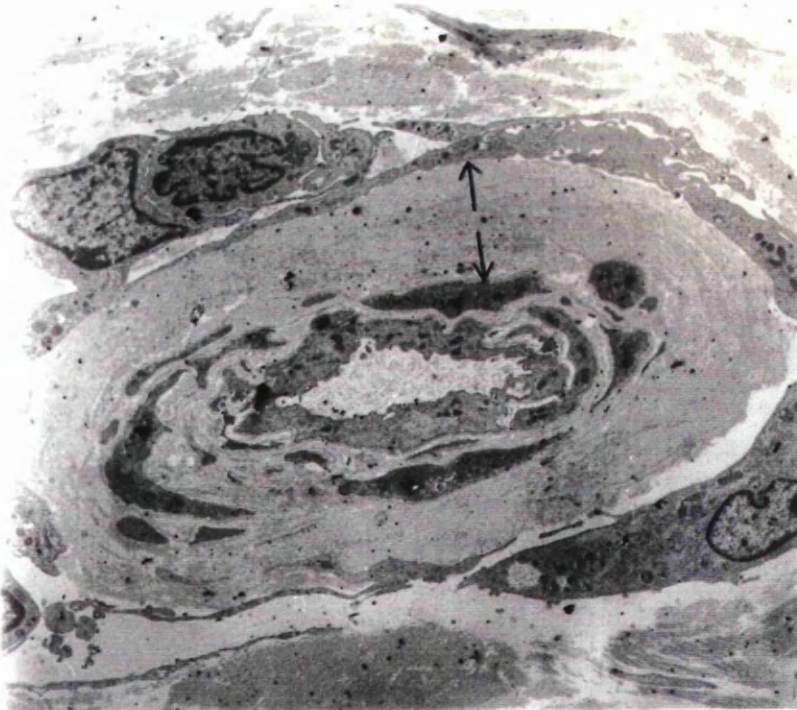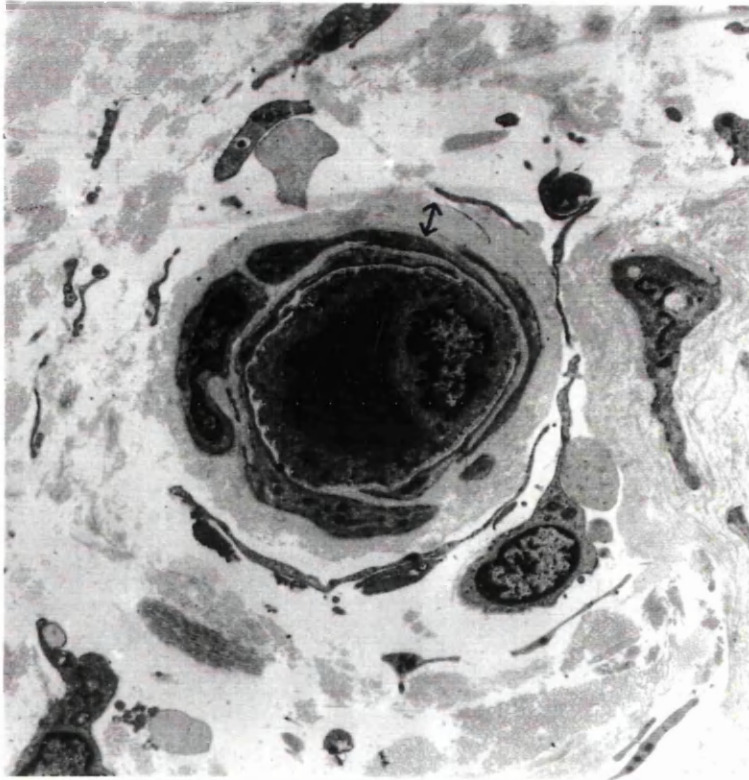
Figure 4.6: Electron micrographs from a non-diabetic (upper print) and diabetic subject with severe complications (lower print) demonstrating the marked difference in basement membrane thickness (basement membrane shown by the arrows).

TABLE 4.10

Luminal perimeters in control subjects, Group I, Group II and Group III subjects.

Control Subjects		Group I		Group II		Group III	
Subject	Luminal Perimeter μm	Subject	Luminal Perimeter μm	Subject	Luminal Perimeter μm	Subject	Luminal Perimeter μm
RM	30.51	GC	21.71	MB	21.69	JM-S	20.04
GR	28.69	PC	29.54	LA	19.67	GS	25.98
AB	42.77	LP	22.90	MG	30.82	PB	22.27
McL	23.12	RS	23.47	GC	25.95	ET	24.68
KF	31.01	JG	25.81	GD	18.47	CB	30.79
PJ	28.15	MS	21.67	BC	32.87	DS	34.43
YvT	28.15	ME	20.02			JM	28.71
BB	42.39	DK	18.48			HH	18.80
PB	26.11	DP	21.42			TB	22.55
JMt	29.56	RC	16.19			KS	21.75
TG	38.00	GS	26.84				
PL	30.45	AR	32.29				
AL	38.86						
MMc	28.00						
RB	25.13						
MR	35.00						
GD	25.87						
Mean	31.28		23.36		24.91		25.00
SD	5.99		4.59		5.98		4.99

TABLE 4.11

Endothelial cell outer perimeters in control subjects,

Group I, Group II and Group III subjects.

Control Subjects		Group I		Group II		Group III	
Subject	endothelial cell outer perimeter μm	Subject	endothelial cell outer perimeter μm	Subject	endothelial cell outer perimeter μm	Subject	endothelial cell outer perimeter μm
RM	33.76	GC	28.42	MB	29.79	JM-S	31.10
GR	36.27	PC	38.80	LA	26.44	GS	32.06
AB	61.05	LP	37.87	MG	34.67	PB	27.97
McL	31.96	RS	30.66	GC	35.27	ET	30.99
KF	34.66	JG	28.43	GD	22.38	CB	35.08
PJ	30.39	MS	32.99	BC	39.99	DS	35.24
YvT	37.27	ME	25.67			JM	32.67
BB	45.41	DK	30.50			HH	25.83
PB	30.02	DP	34.55			TB	32.67
JMt	37.71	RC	26.15			KS	32.78
TG	44.00	GS	27.94				
PL	38.67	AR	34.39				
AL	41.93						
MMc	58.10						
RB	33.00						
MR	45.60						
GD	32.70						
Mean	39.56		31.36		31.42		31.64
SD	9.02		4.36		6.45		2.91
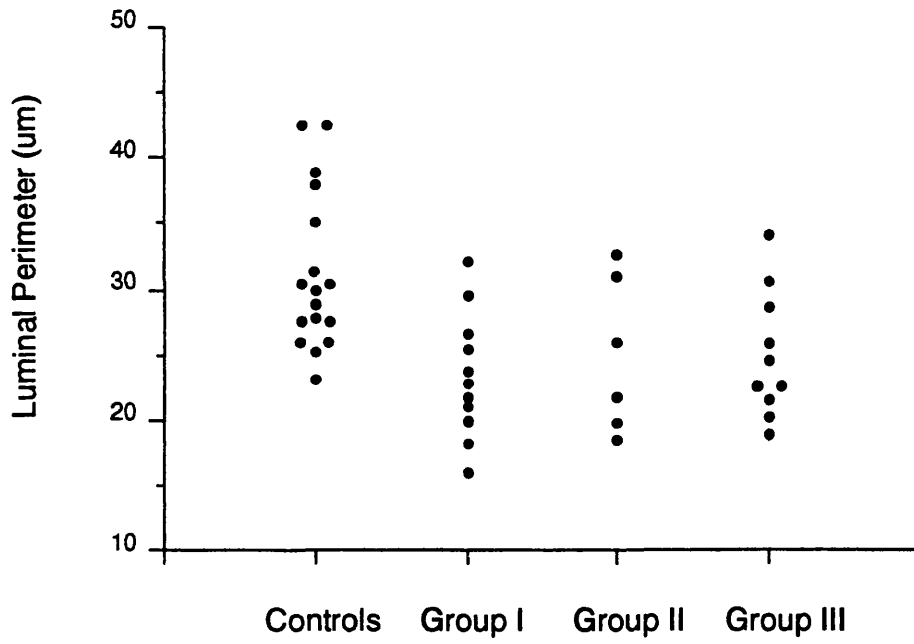
Figure 4.7: Luminal perimeters in control subjects and each of the diabetic groups.
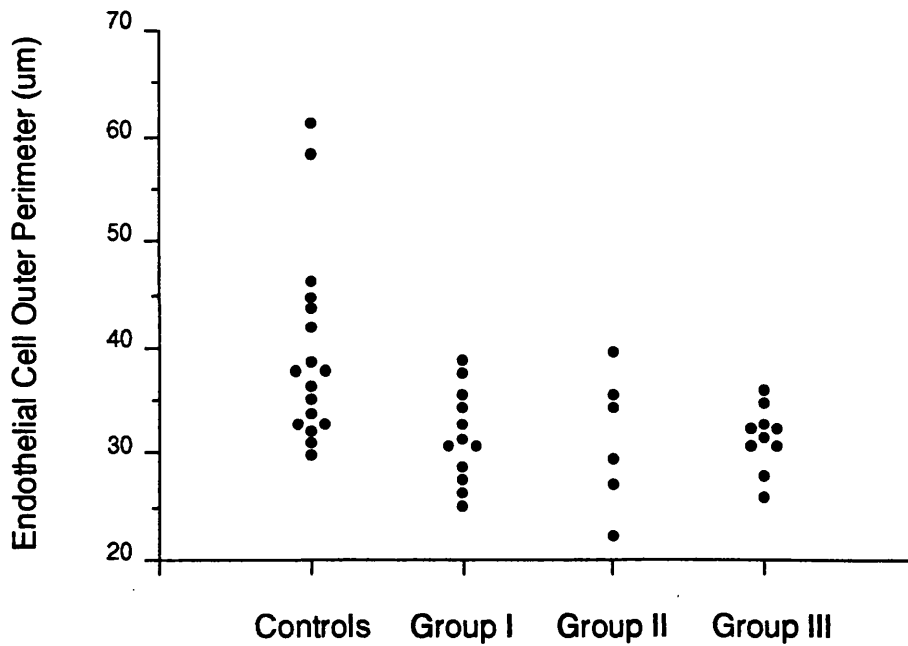
Figure 4.8: Endothelial cell outer perimeters in control subjects and each of the diabetic groups.

Capillary wall measurements

Data relating to basement membrane thickness is shown in Table 4.12 and Figure 4.9. In the diabetic groups there was a progressive increase in basement membrane thickness with severity of complications (Group I- 1.63 ± 0.58 μm; Group II- 1.81 ± 0.57 μm and Group III- 2.51 ± 0.59 μm: $p<0.006$). Compared with the control group (1.29 ± 0.28 μm) basement membrane thickness was significantly increased in Group II ($p<0.02$) and Group III ($p<0.001$).

Data relating to capillary wall thickness is shown in Table A 4.9. There was a trend to increasing capillary wall thickness with severity of diabetic complications (Group I- 5.07 ± 1.10 μm; Group II- 5.53 ± 0.95 μm and Group III- 6.23 ± 1.00 μm: $p<0.05$) mainly accounted for by Group III in which this was significantly greater compared with control (5.31 ± 0.86 μm: $p<0.02$) and Group I subjects ($p<0.02$).

Endothelial cell thickness was not significantly different between any of the groups. This data is shown in Table A4.10.

4.4.8 Relationship between structural and functional parameters

In the diabetic group there was a significant inverse correlation between basement thickness and blood flow response to thermal injury ($r=-0.53$; $p<0.004$). This is depicted in Figure 4.10 (a). There was a similar inverse relationship between basement membrane thickness and blood flow response to mechanical injury ($r=-0.57$; $p<0.002$), shown in Figure 4.10 (b). No such relationship was found in the control group.

There was no relationship between hyperaemic blood flow and any of the other morphological parameters, in particular capillary density, epidermal thickness, luminal perimeter and endothelial cell outer perimeter.

TABLE 4.12

Basement membrane thickness in control subjects, Group I,

Group II and Group III subjects.

Control	Subjects	Group I		Group II		Group III	
Subject	Basement Membrane Thickness μm	Subject	Basement Membrane Thickness μm	Subject	Basement Membrane Thickness μm	Subject	Basement Membrane Thickness μm
RM	1.51	GC	1.17	MB	1.68	JM-S	3.16
GR	0.92	PC	2.18	LA	1.37	GS	2.49
AB	1.49	LP	2.39	MG	2.74	PB	1.23
McL	1.15	RS	1.60	GC	2.25	ET	3.27
KF	1.72	JG	1.40	GD	1.29	CB	3.05
PJ	1.04	MS	1.14	BC	1.53	DS	2.18
YvT	1.21	ME	1.12			JM	2.23
BB	1.39	DK	1.36			HH	2.61
PB	1.92	DP	1.31			TB	2.52
JMt	1.05	RC	1.46			KS	2.31
TG	1.40	GS	1.44				
PL	1.11	AR	2.99				
AL	0.95						
MMc	1.26						
RB	1.18						
MR	1.59						
GD	1.11						
Mean	1.29		1.63		1.81		2.51
SD	0.28		0.58		0.57		0.59
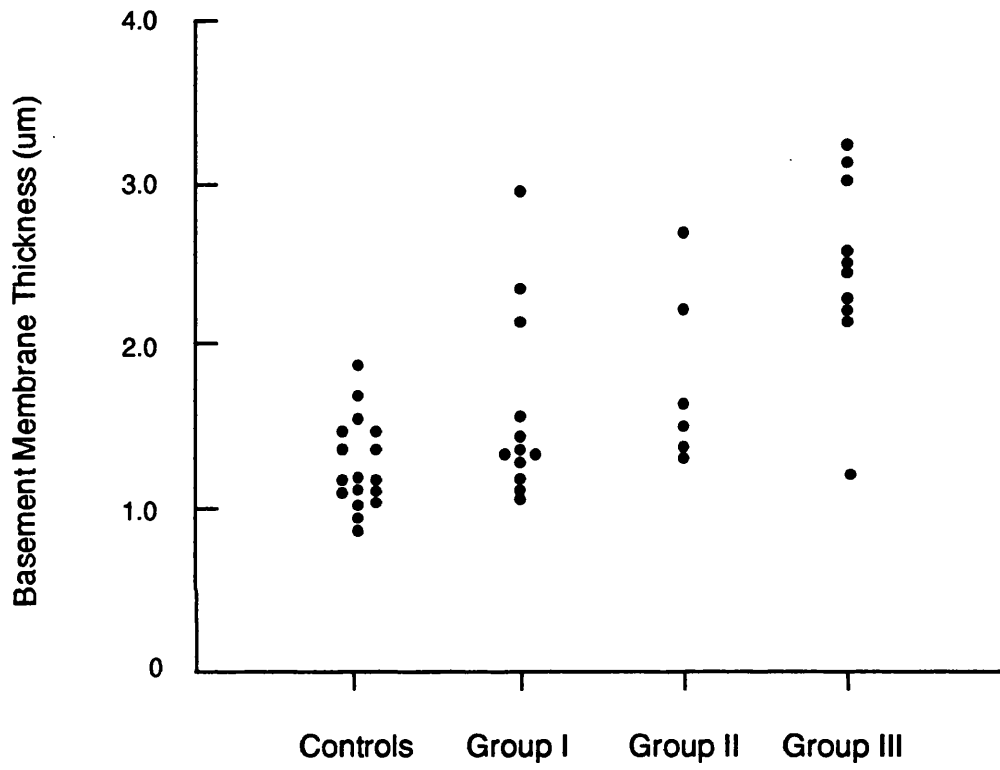
Figure 4.9: Basement membrane thickness in control subjects and each of the diabetic groups
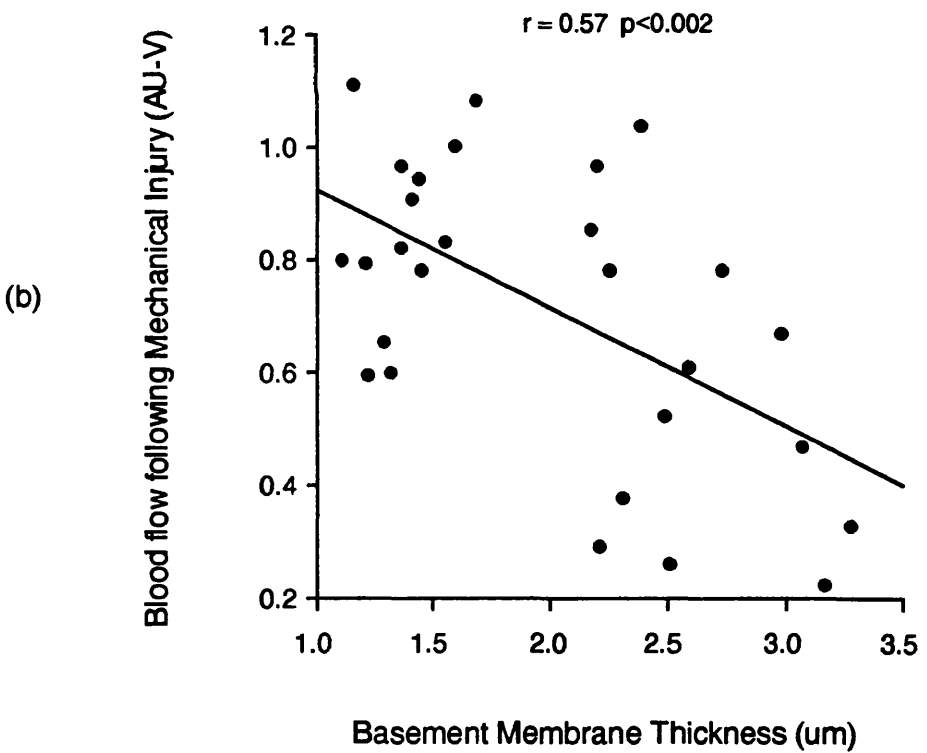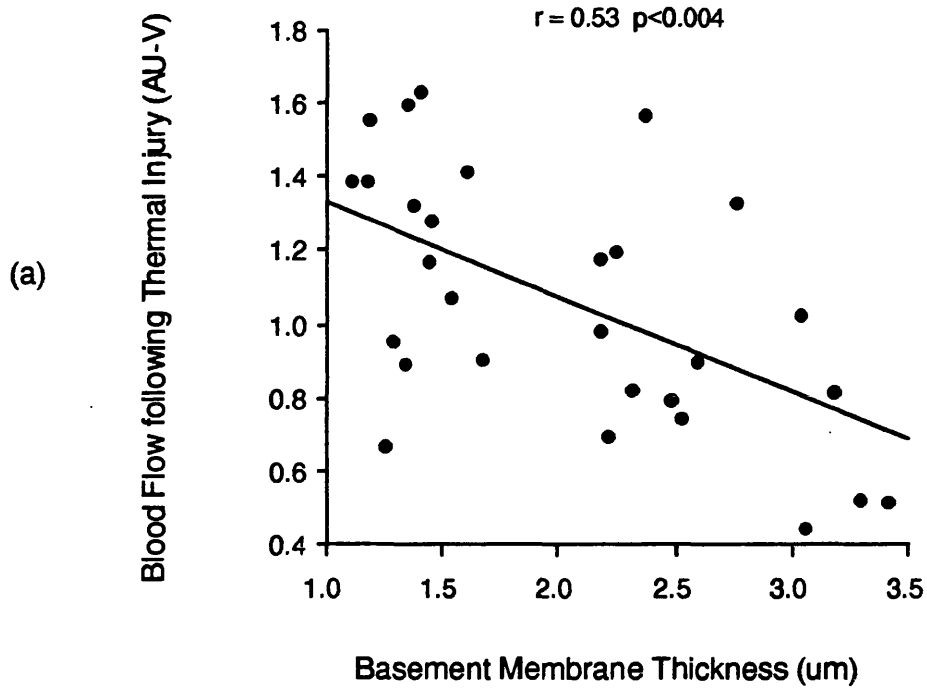
Figure 4.10 (a) and 4.10 (b): Relationship between basement membrane thickness and blood flow response to thermal (a) and mechanical (b) injury.

4.5 DISCUSSION

Regarding blood flow, this study shows that hyperaemic responses are impaired in diabetic subjects, in agreement with the study described in Chapter 3. The additional finding is of a progressive impairment in the injury response with the severity of microvascular disease. This is consistent with the suggestion that the abnormality is linked to a structural microvascular defect. The presence of reduced responses in those free from complications would imply that either this functional abnormality precedes the development of structural microvascular disease elsewhere or that it precedes clinically detectable structural microvascular complications; rather in the way microalbuminuria may precede overt microangiopathy. In the group with severe complications the responses were less than half those of the normal subjects with only one of the diabetics having a response above the lowest of the normal responses. By deliberate exclusion none of the subjects in this group had foot ulcers; a direct relationship between ulceration and impaired hyperaemic responses cannot therefore be made, however an association is suggested in that those with the severest abnormalities, Group III subjects, would be described as being 'at risk' of ulceration according to the criteria put forward by Young (1987). A limited study undertaken by the author, Boolell and Tooke (Boolell 1986) has confirmed that hyperaemic responses to thermal injury are more severely impaired in diabetic patients with foot ulcers.

In agreement with the study described in Chapter 3 and that reported by Walmsley (1989), there was no relationship between the blood flow abnormalities and blood glucose. This is to be expected if the major limiting factor is a fixed defect. However, even in the presence of 'structural locking', rheological factors such as blood viscosity, red cell and leucocyte flexibility may be potentially reversible components. These were not investigated; however, in the study described in Chapter 3, a change in

diabetic control which is known to alter rheology did not influence hyperaemic blood flow.

As with the previous study, in the diabetic groups there was a good correlation between the blood flow responses to the different forms of injury suggesting a common underlying abnormality. Thermal injury responses were however consistently lower than those following mechanical injury. This is not unexpected for several reasons. Firstly, the former was measured in the dependent foot, whereas the latter was measured with the foot at heart level. Passive distension of the microvessels resulting from the increased transmural pressure in the dependent foot would certainly account for some of the difference. Secondly, thermal injury responses were measured directly at the point of injury whereas mechanical injury responses were measured from a site adjacent to the point of injury. Finally, heating to 44°C may simply be a more potent stimulus than mechanical trauma. The use of local anaesthetic does not account for the difference as the injury response did not differ between anaesthetised and un-anaesthetised skin.

As previously shown, epidermal thickness may alter the laser Doppler signal, but the effect is small for a relatively large change (Chapter 2). Even so, as there was no significant difference in epidermal thickness between any of the groups this would not explain the differences in hyperaemic laser Doppler blood flow. In the normal subjects the mean epidermal thickness was 73.5 ± 17.3 μm which is similar to the mean of 72.0 ± 12.0 μm reported by Whitton (1973) for skin from the ankle.

In the previous study (Chapter 3) no reduction in capillary density was found in subjects with diabetes. However, since only superficial capillaries were visualized and as the majority of the subjects in that study were free from complications, a difference in capillary density could still account for the differences between the various diabetic groups in the present study. Thus, capillary density to a depth of 0.5 mm below the

dermo-epidermal junction was assessed and a relationship between capillary density and severity of complications was specifically sought. Capillary density was not reduced in any of the diabetic groups nor was there a relationship with either diabetic complications or hyperaemic blood flow.

With regard to previous studies of dermal vascular structure, as discussed in the introductory chapter, only a limited number have been performed with quite often inadequate numbers of patients. The great majority have employed light microscopic techniques, which provide an insufficient assessment of detailed pathological changes. The methods for assessing microvascular abnormalities have been semi-quantitative or purely qualitative. Furthermore in the majority of studies, biopsies have been taken from sites other than the foot and therefore have no direct relevance to the problem of foot ulceration.

In the present study detailed quantitative ultrastructural light and electron-microscopic examination of foot skin was performed in a large number of Type I diabetic patients. A marked increase in the thickness of the basement membrane was found in subjects with severe complications, confirming previous observations in foot skin (Banson 1964). However, the mean basement membrane thickness for both control and diabetic subjects in the present study was approximately twice that reported by Banson. Methodological differences and the fact that Banson's study was performed 2-12 hours post mortem or post amputation may explain these conflicting results. The skin capillary basement membrane thickness in the normal subjects is much as four times greater than the values quoted for muscle capillaries in the foot (Clough 1987, Tilton 1985). Such marked differences between tissues at the same anatomical level but subserving different functions have been previously described; for example Malik (1989) reported that at the mid-calf, the mean basement membrane thickness of capillaries supplying the sural nerve (1.10 ± 0.20 µm) was more than five times that of muscle capillaries (0.19 ± 0.01 µm).

This study failed to find endothelial cell hypertrophy or proliferation, pericyte loss or change in the endothelial/pericyte cell nuclear ratio. Such abnormalities are well recognised features in diabetic retinal (Kohner 1989, Engerman 1989), and neural vasculature (Powell 1985, Dyck 1985, Malik 1989). It is not clear why capillaries at this site should differ from those at other sites. Tilton (1985) has also been unable to find a reduction of pericytes in skeletal muscle capillaries in the feet of diabetic subjects, but he suggested that this was because pericyte turnover was increased; cellular debris within the basement membrane was considered to represent pericyte degeneration. Since such debris is assessed subjectively and it is by no means certain that it truly represent pericyte degeneration no attempt was made to quantify it in this study.

An unexpected finding was the marked reduction in luminal perimeter and endothelial cell outer perimeter in all diabetic groups, even those without complications. These findings suggest that in diabetic patients the skin capillaries are smaller and have a reduced luminal diameter. Ajjam (1985) also found reduced microvascular dimensions in diabetic subjects; but this study related to forearm skin, and capillaries could not be specifically examined since the assessment was by light microscopy. In contrast to these histological studies, in-vivo studies in which capillaries were directly visualized by microscopy, report either no difference (Pazos-Moura 1990) or increased skin capillary luminal size in diabetic subjects (Landau 1960, Karlander 1985, Flynn 1988).

There are a number of possible reasons why capillary diameter measured by the in-vivo and histological methods might differ. The in-vivo studies measure erythrocyte column width rather than the true capillary luminal size. As mentioned in the introductory chapter and as pointed out by Flynn (1988) the increase in red cell column width found in diabetic subjects on capillary microscopy may merely reflect a reduction in the marginal layer of plasma sheathing the erythrocytes rather than an increase in luminal

diameter. In addition, Fagrell (1977) has shown that the red cell column width increases as skin temperature and capillary blood flow increase. Thus, the greater red cell column width in diabetic subjects reported in some studies may simply reflect increased skin temperature and perfusion. This suggestion is supported by Flynn's (1988) study in which both skin temperature and capillary blood flow were increased in the diabetic group in whom the red cell column width was increased.

In the present histological study, very accurate measurements were possible as in each subject a large number of capillaries was assessed and the measurements were obtained using a digitizing pad. However, these measurements may not fully reflect the in-vivo dimensions since, a degree of vessel recoil may be expected as capillary pressure falls following the biopsy (the vessels could not be fixed under pressure), and fixation may result in a minor degree of tissue shrinkage (Hayat 1981). However, as these changes should be common to both diabetic and non-diabetic groups they would not explain the large differences in capillary diameter found, unless there was an alteration in the elasticity of the vessel wall in the diabetic subjects. However, there was no obvious change in the vessel wall in the uncomplicated diabetic group yet the capillary diameter was reduced, suggesting that this is an unlikely explanation.

Why the capillary size at the time of biopsy should be reduced in the diabetic groups, particularly in the uncomplicated group is not clear. Alterations in basal vascular tone due to increased circulating levels of endothelin-1 or reduced production of EDRF (nitric oxide) are theoretical explanations and as mentioned in the previous chapter recent work would support either of these suggestions (Takahashi 1990, Katsuo 1989). Another possibility relates to the trauma of the biopsy. Although the sections were fixed as soon as possible following biopsy, several minutes will have elapsed between performing the punch removing the section from the dermis and its blood supply and placing the specimen in the fixative. During this time

vasoactive substances will have been released as a result of the tissue trauma and will alter microvascular tone. Impaired release or response to such vasoactive mediators in the diabetic subjects may thus explain the difference in capillary size between diabetic and non-diabetic subjects and may also explain why the in-vivo and histological methods for assessing capillary diameter give different results.

This is the first study which attempts to relate microvascular function in foot skin with detailed histomorphometric analysis of skin capillaries visualized by electron microscopy. Kastrup (1987) found a significant inverse relationship between the vascular distensibility and the degree of arteriolar hyalinosis in foot skin. However, the latter was based on a crude subjective scoring system of the quantity of PAS positive staining in the vessel wall, no quantitative assessments of vascular dimensions were undertaken, and as discussed in the introductory chapter, the use of histamine to produce maximal microvascular relaxation may by itself have given rise to the apparent differences in distensibility. Ajjam (1985) in addition to finding reduced forearm skin vascular dimensions, described reduced vasodilator responses to injected histamine and topically applied *Transvasin* (ethyl nicotinate), assessed by tracing around the areas of redness. There was no mention of the relationship between the functional and structural abnormalities, presumably none was found which may not be surprising in view of the limitations of the methods used to assess vascular responses. Katz (1989) also examined forearm dermal capillaries, but unlike Ajjam found no significant structural changes on electron microscopy and unlike the present study found no relationship between microvascular structure and function. This may in part relate to patient selection as the diabetic group consisted solely of Type II patients free from complications. It may also relate to the choice of forearm skin where the capillary pressure is not as great as in the dependent foot, a factor proposed to explain the exaggerated difference in muscle capillary basement membrane thickness in

capillaries from upper and lower parts of the body in diabetics patients (Vracko 1970).

Blood flow in the microcirculation is regulated at the level of the resistance vessels, however, when functional vascular tone is abolished structural influences dominate in determining vascular resistance (Folkow 1971). Reduced capillary density, or capillary luminal size (due to smaller capillaries or reduced capillary distension) could account for a reduction in hyperaemic flow, however the former can be excluded as capillary density was not reduced, in keeping with previous observations (Tooke 1985). With regard to capillary structure, an association was observed between the degree of basement membrane thickening and the injury response, supporting but not proving a cause/effect relationship. The basement membrane has a Young's elastic modulus comparable to that of collagen and is believed to provide structural support for the endothelial cells (Murphy 1975). It distends with increasing capillary pressure (Swayne 1989, Bouskela 1989); doubling its width has been estimated to approximately halve its distensibility (Murphy 1975). Thus, reduced capillary compliance due to encasement in thickened basement membrane may explain the observed inverse relationship between basement membrane thickness and hyperaemic responses. This however, cannot explain the impaired responses in subjects free from complications in whom the basement membrane width was not increased. The only substantial structural abnormality in these subjects was the marked reduction in capillary size (luminal perimeter and endothelial cell outer perimeter). Assuming that the capillary wall compliance is comparable with that in the control group, basement membrane thickness being similar, then the capillary diameter when maximally distended should also be smaller than that in the control group. Since blood flow is related to the fourth power of the luminal radius such changes would readily account for the reduction in hyperaemic responses observed in these patients. On the other hand between the various diabetic

groups there were no differences in capillary diameters yet hyperaemic responses differed. This may be explained if, as previously suggested, the large differences in basement membrane thickness and hence capillary compliance in this group of subjects dominate in determining the fully vasodilated capillary diameter.

In summary this study demonstrates marked impairment in the hyperaemic response to minor injury in diabetic skin. This abnormality has been related to an increase in basement membrane thickness. In addition to the effect of these structural alterations on hyperaemic blood flow it is likely that the reduction in capillary filtration surface area consequent upon the reduced capillary size, together with the increased diffusion distance resulting from the thickening of the basement membrane will also impair nutrient exchange across the vessel wall. Impaired nutrient exchange and hyperaemic microvascular responses to tissue injury are likely to be of considerable importance in both the development and subsequent repair of tissue injury in the diabetic foot, particularly in patients with macrovascular disease.

CHAPTER 5

THE EFFECT OF CHANGE IN POSTURE ON SKIN BLOOD FLOW IN THE FEET OF DIABETIC SUBJECTS

5.1 INTRODUCTION

On placing the foot below heart level there is a pronounced fall in subcutaneous, skeletal muscle and skin blood flow (Henriksen 1977). These changes are believed to be brought about by an increase in pre- to post capillary resistance. Henriksen suggested that this vasoconstrictor response involves a local sympathetic axon reflex since it is abolished by chronic sympathectomy and local anaesthesia but is unaffected by acute spinal blockade. This he termed the veno-arteriolar reflex.

Several studies have demonstrated increased foot blood flow in diabetic subjects with neuropathy (Archer 1984, Edmonds 1982) and have postulated that this may result from sympathetic nerve failure. Since sympathetic nerves are believed to be involved in the veno-arteriolar reflex, this study sought to determine whether this postural change in blood flow is altered in diabetic subjects, particularly in those with neuropathy.

5.2 SUBJECTS

Three groups of subjects were studied; diabetics with peripheral neuropathy, diabetics without neuropathy, and normal control subjects. Diabetic subjects were recruited from the Diabetic Clinic at Charing Cross Hospital. The normal volunteers were recruited from medical and technical staff at the Charing Cross Hospital and Medical School and visitors to the Charing Cross Hospital. Patients on medications other than insulin or oral hypoglycaemic agents were excluded, as were subjects with brachial systolic

blood pressures of greater than 170 mmHg. Fundi were assessed by standard ophthalmoscopy through dilated pupils. The study was approved by the local Ethical Committee and informed consent was obtained from all subjects.

Subjects were classified as having neuropathy if ankle reflexes were absent and vibration sensory thresholds, determined with a biothesiometer (Biomedical Instrument Co., Ohio, USA) were above the 90th centile for age when compared with published centile charts for normal subjects of varying ages (Bloom 1984). The neuropathic group consisted of 13 subjects (9 males and 4 females). Subject details are summarised in Table 5.1. The mean age of the group was 59.5 ± 11.6 years (range 35 to 72 years) and the mean duration of diabetes was 12.8 ± 11.3 years (range 2 to 31 years). Eight subjects had had a previous amputation or foot ulcer. Vibration sensory threshold results for individuals in the groups are shown in Table 5.2 and Figure 5.1. Seven had evidence of cardiac autonomic neuropathy Table 5.2. Four had mild unexplained ankle oedema in the absence of overt cardiac or renal impairment. All subjects had ankle/brachial systolic blood pressure ratios of greater than 1.0, Table 5.3.

The non-neuropathic diabetic group consisted of 13 subjects (7 males and 6 females) whose mean age was similar to that of the neuropathic group (55.2 ± 12.5 years; range 32 to 75 years), Table 5.4 The mean duration of diabetes for the group was slightly greater but not significantly different from that of the neuropathy group (15.5 ± 8.1 years; range 3 to 29 years). All subjects had normal autonomic function tests, intact ankle reflexes, and vibration sensory thresholds within the 90th centile, Table 5.5 and Figure 5.1. None of the subjects had a history of foot ulceration. Ankle/brachial blood pressure ratios were greater than 1.0 in all subjects, Table 5.6.

Details of the control group of seven males and six females are shown in Table 5.7. Their mean age was 59.5 ± 11.6 years (range 33 to 79 years); not significantly different from that of the either of the diabetic groups.

TABLE 5.1

Clinical details of the Subjects with Neuropathy

Subject	Diabetes Type	Sex	Age	Duration	Retinopathy	Foot Complications*
JJ	NIDDM	M	61	3	BR	TA
SR	IDDM	M	65	25	PR	FU
GM	IDDM	M	52	30	BR	BKA
FB	NIDDM	M	72	2	-	FU
FM	NIDDM	M	54	3	-	TA
VD	IDDM	F	55	20	BR	-
MT	NIDDM	M	64	2	-	FU
MJ	NIDDM	F	75	3	BR	FU
MB	NIDDM	M	71	5	-	-
GL	IDDM	F	35	31	PR	-
J K	IDDM	M	43	23	-	-
JL	IDDM	M	67	9	BR	FU
FJ	NIDDM	M	60	10	-	-
Mean	-	-	59.5	12.8	-	-
SD			11.6	11.3		

* FU = foot ulcer; TA = toe amputation; BKA = below knee amputation

BR = background retinopathy; PR = proliferative retinopathy

TABLE 5.2

Vibration thresholds and Autonomic function tests in the Neuropathic Group

Subject	Vibration Threshold		E:I Ratio	30:50 Ratio	Valsalva Ratio	Change in Systolic BP on
	R	L	NR >1.10	NR >1.03	NR >1.20	standing
JJ	35	35	1.04	1.00	1.08	-36
SR	>50	>50	1.03	0.97	1.09	-25
GM	-	35	1.32	1.20	1.22	0
FB	>50	>50	1.16	1.02	1.24	-15
FM	25	24	1.17	1.10	1.40	5
VD	42	45	1.03	1.00	-	-15
MT	33	37	1.12	1.00	1.22	-10
MJ	45	>50	1.13	1.04	1.18	-20
MB	42	42	1.00	1.00	1.14	-66
GL	30	35	1.06	1.00	1.03	-40
JK	45	40	1.24	1.13	1.50	0
JL	>50	35	1.10	0.98	-	-14
FJ	>50	>50	1.00	1.00	1.00	-18
Mean	40.6	41.4	1.11	1.03	1.19	-20.3
SD	8.1	8.7	0.10	0.07	0.15	18.3
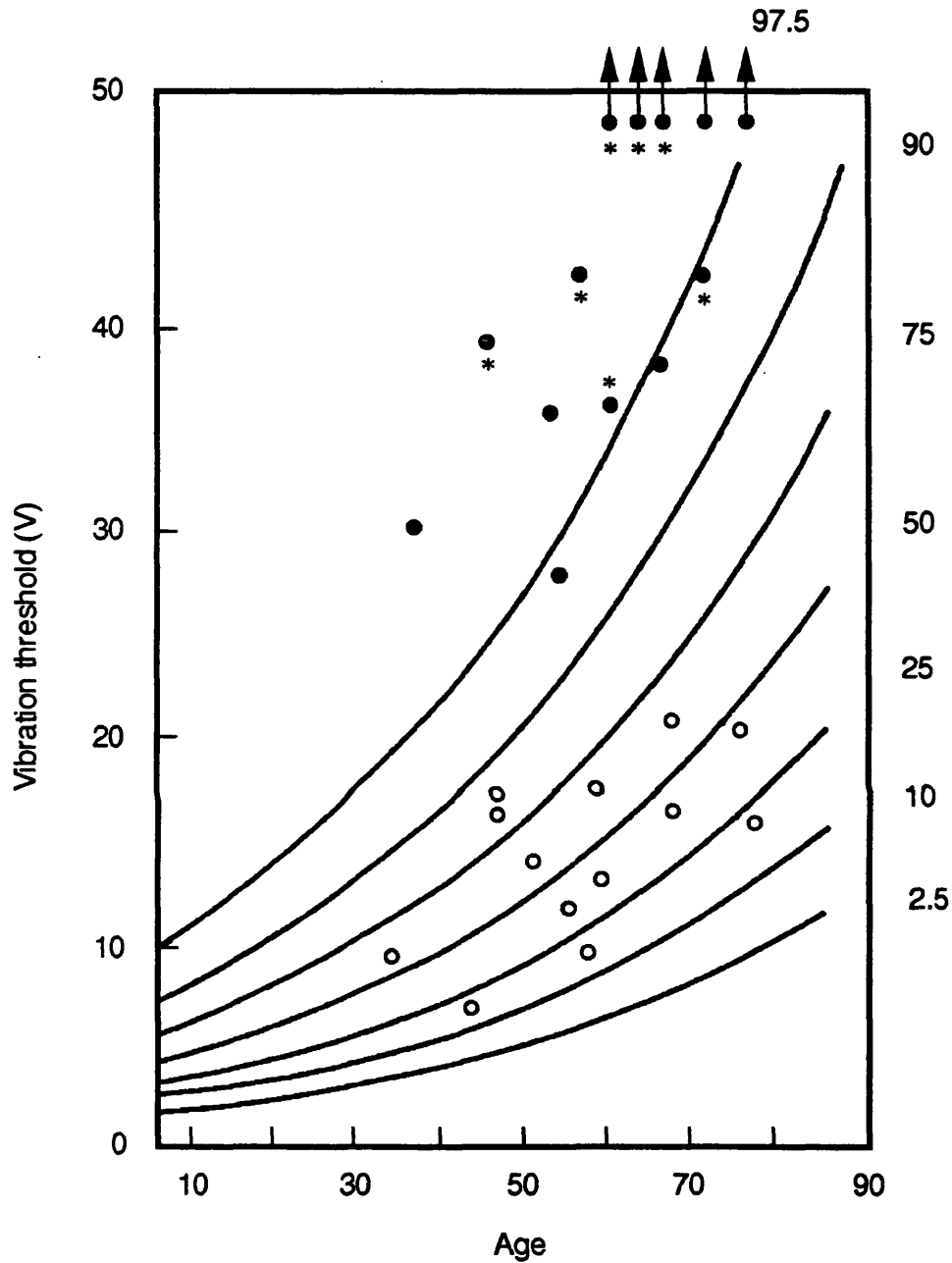
Figure 5.1: Centile chart relating vibration threshold in the big toe with age in normal individuals (Bloom 1984). Closed and open circles represent values in diabetics with and without neuropathy respectively. Asterisks indicate patients with autonomic neuropathy.

TABLE 5.3

Ankle / Brachial Pressure Ratios in the Neuropathic Group

Subject	Ankle Systolic BP (mmHg)	BrachialSystolic BP (mmHg)	Ankle/ Brachial Pressure Ratio
JJ	140	128	1.09
SR	270	170	1.59
GM	170	150	1.13
FB	180	140	1.29
FM	180	120	1.50
VD	200	130	1.54
MT	200	140	1.43
MJ	200	170	1.18
MB	185	145	1.28
GL	200	160	1.25
JK	168	130	1.29
JL	195	158	1.23
FJ	185	158	1.17
Mean	190.2	146.1	1.31
SD	29.5	16.4	0.16

TABLE 5.4

Clinical details of the Subjects without Neuropathy

Subject	Diabetes Type	Sex	Age	Duration	Retinopathy
MH	IDDM	F	46	17	BR
AS	NIDDM	F	75	7	-
FB	NIDDM	M	58	12	-
SP	IDDM	M	49	13	-
MF	IDDM	F	53	23	BR
EP	IDDM	M	65	24	BR
CB	NIDDM	F	64	3	-
CF	NIDDM	M	74	10	-
JB	IDDM	F	45	22	PR
GR	IDDM	F	32	7	-
DS	IDDM	M	42	29	BR
RC	NIDDM	M	57	11	-
FR	IDDM	M	58	23	-
Mean	-	-	55.2	15.5	-
SD			12.5	8.1	

BR = background retinopathy PR= Proliferative retinopathy

TABLE 5.5

Vibration thresholds and Autonomic function tests in the
Non-Neuropathic Group

Subject	Vibration Threshold		E:I Ratio	30:50 Ratio	Valsalva Ratio	Change in Systolic BP on
	R	L	NR >1.10	NR >1.03	NR >1.20	standing
MH	17	16	1.24	-	1.45	–12
AS	11	15	1.20	1.20	1.50	10
FB	8	8	1.18	1.16	1.49	0
SP	15	13	1.50	1.09	1.52	–10
MF	10	10	1.20	1.14	1.42	– 5
EP	21	20	1.10	1.13	1.65	10
CB	12	13	1.25	1.28	1.28	–15
CF	20	20	1.10	1.20	1.40	5
JB	16	16	1.40	1.26	1.65	8
GR	10	8	1.45	1.18	1.75	10
DS	8	6	1.35	1.24	1.43	6
RC	12	13	1.20	1.10	1.78	14
FR	13	16	1.24	1.10	1.56	10
Mean	13.4	13.3	1.26	1.17	1.53	2.4
SD	4.4	4.2	4.2	0.06	0.15	9.8

TABLE 5.6

Ankle / Brachial Pressure Ratios in the Non-neuropathic Group

Subject	Ankle Systolic BP (mmHg)	Brachial Systolic BP (mmHg)	Ankle / Brachial Pressure Ratio
MH	140	120	1.17
AS	170	130	1.31
FB	155	120	1.29
SP	155	145	1.06
MF	140	130	1.08
EP	160	135	1.19
CB	145	145	1.00
CF	165	140	1.18
JB	165	130	1.27
GR	155	135	1.15
DS	135	115	1.17
RC	150	130	1.15
FR	185	160	1.16
Mean	155.4	133.5	1.17
SD	13.9	12.1	0.09

BR = background retinopathy; PR = proliferative retinopathy

TABLE 5.7

Details of the Normal Control Subjects

Subject	Sex	Age	Ankle Systolic BP (mmHg)	Brachial Systolic BP (mmHg)	Ankle/Brachial Pressure Ratio
MH	F	58	150	130	1.15
AL	F	79	160	120	1.33
VH	F	67	160	155	1.03
MF	F	58	150	145	1.03
MO	F	56	170	150	1.13
BB	M	41	150	110	1.36
LH	M	72	175	145	1.21
MM	F	33	120	110	1.09
AC	M	60	170	150	1.13
BP	M	56	140	120	1.17
GC	M	45	145	140	1.04
HD	M	70	175	160	1.09
AM	M	78	180	140	1.29
Mean		59.5	157.3	136.5	1.16
SD		13.8	17.0	16.9	0.11

5.3 METHODS

5.3.1 Skin Blood Flow and Temperature Measurement

Skin blood flow was measured in the plantar surface of the left big toe using a laser Doppler flowmeter (Periflux PF 1 D). In subjects in whom this toe or foot had been amputated the right great toe was used. The laser Doppler probe was affixed to the toe using the plastic holder supplied by the manufacturers. This had been heat moulded to a semi-curved shape to assist its fixture with double sided adhesive tape to the skin.

Skin temperature was recorded using a Comark electronic thermometer (type 1625) and copper-constantan thermocouple as described in Chapter 2. The thermocouple was placed between the adhesive disc affixing the laser Doppler probe holder and the skin. The temperature was thus recorded from skin immediately adjacent to that from which blood flow was measured.

5.3.2 Study Conditions and Experimental Protocol

The room temperature was controlled to between 21 and 23 °C and the environmental conditions were as described in Chapter 2. The studies were started at between 08.30 and 09.30, the subjects having had their usual breakfast and diabetic subjects their normal pre-breakfast insulin dose. Tea, coffee and tobacco were not permitted on the morning of the study and alcohol was not permitted from the previous evening. The subjects were acclimatised resting supine on a couch with their feet exposed. The couch had been constructed so as to allow either leg to be lowered while the subject remained supine, Figure 5.2. During the thirty minute acclimatisation period the various measuring probes were attached to the skin, and subject details, vibration threshold, blood glucose and blood pressure were recorded.
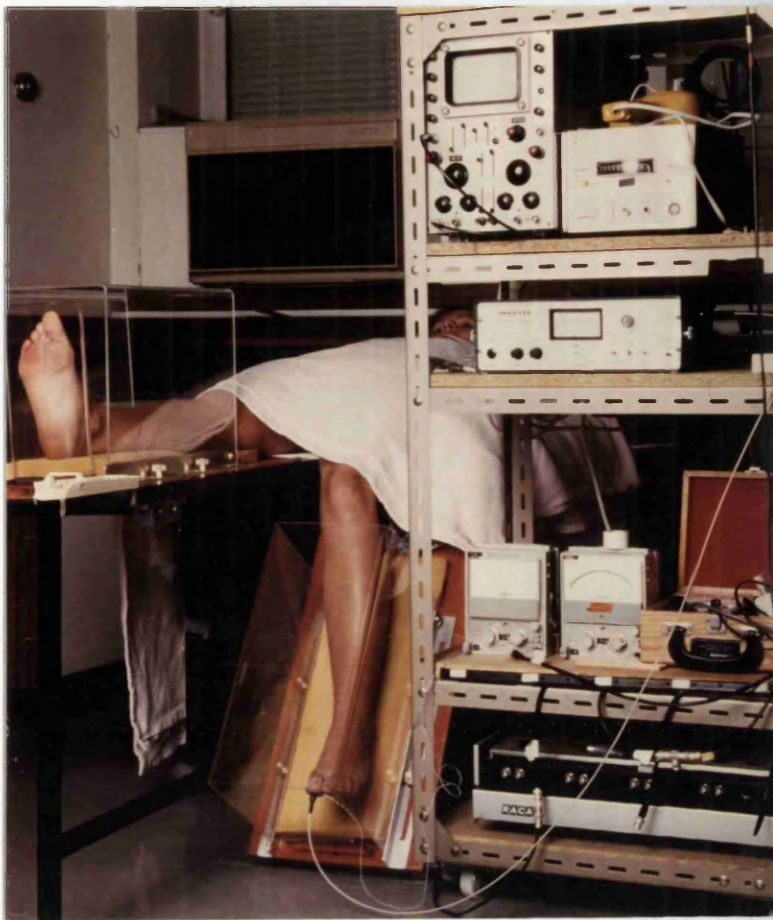
Figure 5.2: Photograph showing toe blood flow being measured in the dependent foot using the laser Doppler flowmeter.
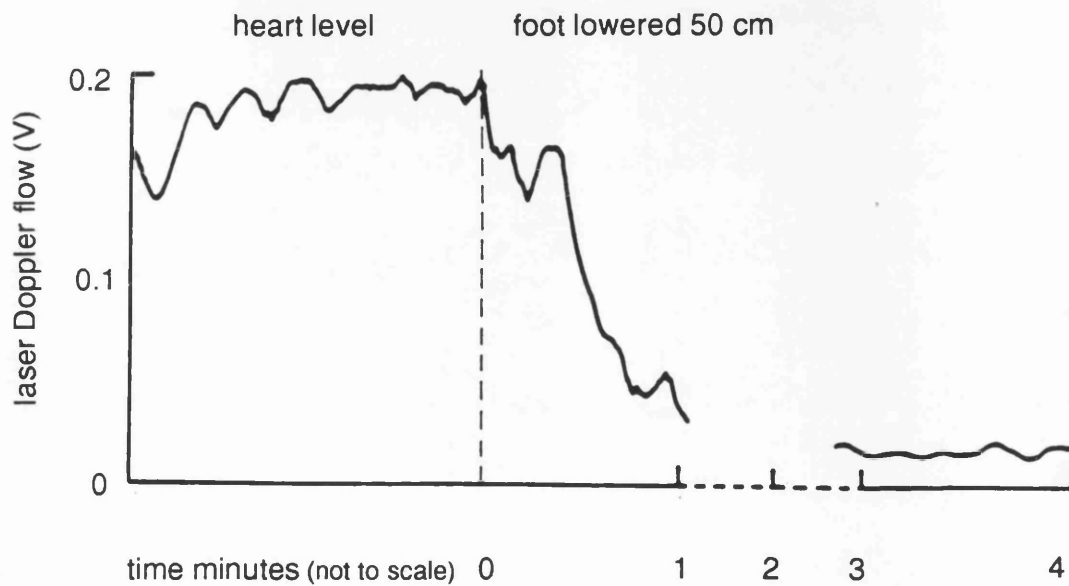
Figure 5.3: Drawing of an original trace showing the change in toe blood flow on lowering the foot in a normal subject. The dotted line marks the time at which the foot was lowered from heart level.

After acclimatisation, a reading of skin temperature was taken, and blood flow recorded for two minutes (this is referred to as rest flow). The foot was then placed fifty centimetres below the mid-axillary line and blood flow recorded during the fourth minute of dependency (referred to as dependent flow). The change in blood flow on lowering the foot was expressed as a percentage of the resting blood flow ie. (rest flow- dependent flow) / rest flow x 100. Figure 5.3 shows a typical trace of the blood flow change on dependency in a normal subject. The inter-individual coefficient of variation based on duplicate measurements on two separate days in six subjects was 10.4%. This data is presented in Table A 5.1.

5.4 RESULTS

5.4.1 Ankle and Brachial Blood Pressures and Pressure Ratios.

Brachial, ankle and ankle/brachial blood pressures are shown in Tables 5.3 (neuropathic subjects), 5.6 (non-neuropathic subjects) and 5.7 (non-diabetic subjects. As expected ankle systolic blood pressure and ankle/brachial pressure ratio were significantly increased in the neuropathic group (190 ± 29.5 mmHg and 1.31 ± 0.16 respectively) compared with those in the non-neuropathic (155.4 ± 13.9 mmHg; $p<0.002$ and 1.17 ± 0.09; $p<0.02$) and control groups (157.3 ± 17.0; $p<0.002$ and 1.16 ± 0.11; $p<0.02$).

5.4.2 Diabetic Control

Blood glucose and glycosylated haemoglobin results for the diabetic subjects are shown in Table 5.8. There were no significant differences in either measures of diabetic control in the two diabetic groups. Neither blood glucose nor glycosylated haemoglobin correlated with toe temperature, resting or dependent blood flow, and the percentage change in blood flow during dependency.

TABLE 5.8

Blood Glucose and Glycosylated Haemoglobin levels in Diabetic Subjects

Neuropathic Group			Non-neuropathic Group		
Subject	Glucose	HbA1	Subject	Glucose	HbA1
JJ	8.8	10.2	MH	12.2	7.1
SR	6.0	9.1	AS	10.7	11.4
GM	10.3	-	FB	3.4	-
FB	12.3	8.2	SP	7.6	9.4
FM	8.8	6.8	MF	9.6	-
VD	12.3	11.8	EP	7.9	9.1
MT	6.9	8.6	CB	8.5	8.4
MJ	5.1	7.0	CF	6.3	8.2
MB	6.8	9.4	JB	20.1	8.4
GL	12.6	11.1	GR	7.6	8.7
JK	12.5	9.4	DS	-	8.7
JL	6.5	7.6	RC	7.9	9.8
FJ	10.4	10.0	FR	12.5	8.5
Mean	9.2	9.1		9.5	8.9
SD	2.7	1.6		4.2	1.1

5.4.3 Skin Temperature

Toe skin temperatures for the three groups are shown in Tables 5.9-5.11.and Figure 5.4 The neuropathic group had a mean toe temperature of 32.2 ± 2.0 °C (range 29.3 to 34.5 °C). This was significantly greater than that of both the normal control group (27.7 ± 3.3 °C; range 23.2 to 32.7 °C; p< 0.002) and the non-neuropathic group of diabetics (28.7 ± 3.1 °C; range 24.0 to 33.2 °C; p< 0.002). The range of skin temperatures in the neuropathic group was less than that of other two groups and none of the subjects with neuropathy had a toe temperature below 29 °C. There was no significant difference in toe temperatures between the diabetics without neuropathy and the normal subjects.

5.4.4 Rest Blood Flow

Laser Doppler readings for the two minutes before dependency are shown in Tables 5.9 to 5.11 and Figure 5.5. As would be expected from the higher toe temperatures, mean resting blood flow in the diabetics with neuropathy (434 ± 229; range 113 to 775 mV) was significantly greater than that of the normal subjects (128 ± 112; range 18 to 340 mV; p<0.002). The mean blood flow for the non-neuropathic group (334 ± 373; range 27.5 to 1250 mV) was higher than that of the control group (128 ± 112 range 18 to 340 mV), however, as the data in the non-neuropathic group is skewed by two subjects (FB and JB) in whom blood flow was extremely high, there was no statistical difference between the two groups. Mean resting blood flow in the non-neuropathic group (334 ±373; range 27.5 to 1250 mV) was also not significantly different from that in the neuropathic group (434 ± 229; range 113 to 860 mV).

TABLE 5.9

Skin Temperature and Blood Flow results in the Neuropathic Group

Subject	Skin Temp °C	Rest Blood Flow (mV)	Dependent Flow (mV)	% fall in Blood Flow
JJ	30.8	240	96	60.0
SR	29.6	113	96	15.0
GM	35.0	318	104	67.3
FB	33.0	213	90	57.8
FM	32.5	434	157	63.8
VD	29.3	137	144	-5.1
MT	33.8	596	208	65.2
MJ	30.0	477	320	32.9
MB	34.1	533	303	43.1
GL	31.8	480	310	35.2
JK	34.5	474	147	69.0
JL	33.6	860	610	29.1
FJ	30.1	775	231	70.2
Mean	32.2	434	217	46.4
SD	2.0	229	145	23.7

TABLE 5.10

Skin Temperature and Blood Flow results in the Non-Neuropathic Group

Subject	Skin Temp °C	Rest Blood Flow (mV)	Dependent Flow (mV)	% fall in Blood Flow
MH	26.1	27.5	5.5	80.0
AS	25.2	116.0	23.5	79.7
FB	33.2	1250.0	427.0	65.8
SP	29.5	272.0	45.5	83.3
MF	27.7	79.5	7.5	90.6
EP	29.5	361.0	34.5	90.4
CB	31.5	251.0	51.0	79.7
CF	32.8	438.0	259.0	40.9
JB	31.5	990.0	504.0	49.1
GR	25.6	63.0	7.0	88.9
DS	25.6	160.0	102.0	36.2
RC	30.4	279.0	98.0	64.9
FR	24.0	63.0	16.0	74.6
Mean	28.7	334.6	121.6	71.7
SD	3.1	373.9	167.8	18.6

TABLE 5.11

Skin Temperature and Blood Flow results in Control Subjects

Subject	Skin Temp °C	Rest Blood Flow (mV)	Dependent Flow (mV)	% fall in Blood Flow
MH	26.4	45.5	2.0	95.6
AL	27.8	114.5	9.0	92.1
VH	29.5	101.0	18.5	81.7
MF	28.5	62.5	4.0	93.6
MO	32.7	279.0	87.0	68.8
BB	23.7	86.0	3.5	95.9
LH	25.5	44.0	10.0	77.3
MM	23.2	18.0	1.5	91.7
AC	28.0	41.0	14.0	65.9
BP	22.5	34.0	6.0	82.4
GC	31.0	198.0	31.0	84.3
HD	30.5	340.0	140.0	58.8
AM	31.0	300.0	60.0	80.0
Mean	27.7	128.0	29.7	82.2
SD	3.3	112.4	41.9	11.9
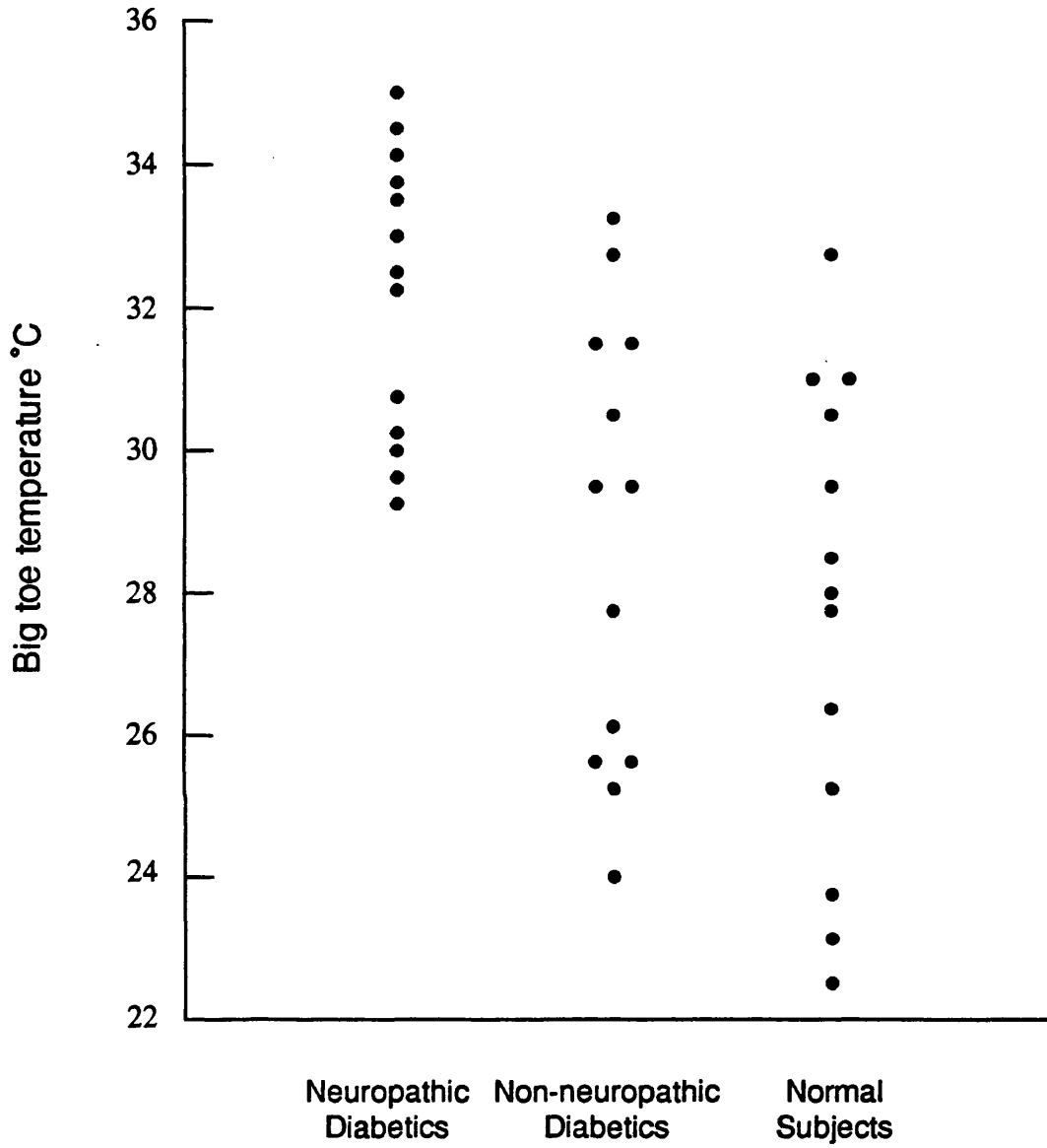
Figure 5.4

Skin toe temperatures in diabetic subjects with neuropathy,
diabetic subjects without neuropathy and non-diabetic subjects.
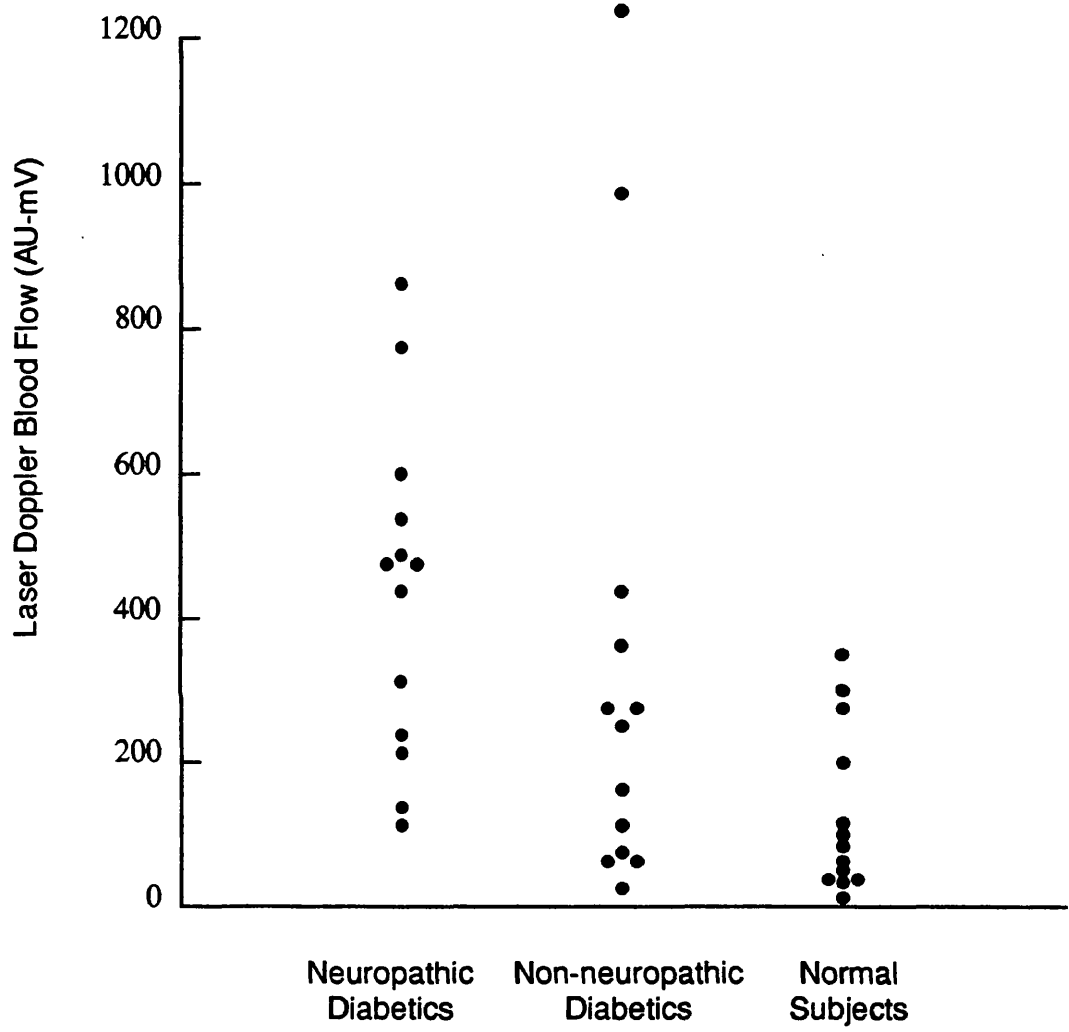
Figure 5.5

Resting toe blood flow in diabetic subjects with neuropathy, diabetic subjects without neuropathy and non-diabetic subjects. Blood flow is recorded in arbitary units of mV (AU-mV).

5.4.5 Dependent Blood Flow

Blood flow during the fourth minute of dependency is shown in Figure 5.6 and Tables 5.9 to 5.11. In the normal subjects lowering the foot substantially reduced blood flow to a mean value of 29.7 ± 41.9 mV (range 2.0 to 140 mV). This represented a mean fall in blood flow of approximately 100 mV. In the non-neuropathic group blood flow fell to 121.6 ± 16.8 mV (range 5.5 to 504 mV). Although the mean dependent blood flow in the non-neuropathic group was greater than that of the normal subjects, the results for the two groups were not statistically different; the higher mean value being largely accounted for by three individuals in whom flow on dependency remained very high (FB, JB, CF). In the neuropathic group mean blood flow during that fourth minute of dependency was 217 ± 145 mV (range 90 to 610 mV). This was significantly greater than that of both the non-neuropathic diabetics ($p<0.05$) and the normal subjects ($p<0.002$).

5.4.6 Postural change in blood flow

Results for the three groups are shown in figures Tables 5.9 to 5.11 and compared graphically in Figure 5.7. In the normal subjects blood flow fell by 82.2 ± 11.9% (range 58.8 to 95.6%). A similar fall was seen in the diabetic subjects without neuropathy, the mean value of 71.1 ± 18.6% (36.2 to 90.6%) being not statistically different from that of the normal subjects. In the neuropathic group the percentage fall was only 46.4 ± 23.7 % (range -5.1 to 70.2%). This is significantly less than that in the control ($p<0.002$) and the non-neuropathic groups ($p<0.02$).

The percent change in blood flow during dependency was was positively correlated with the Valsalva ratio ($r_s = 0.51$; $p<0.015$) and the E:I ratio ($r_s = 0.50$; $p<0.015$). There was a weak correlation with the change in systolic blood pressure on standing ($r_s = 0.45$; $p<0.025$).
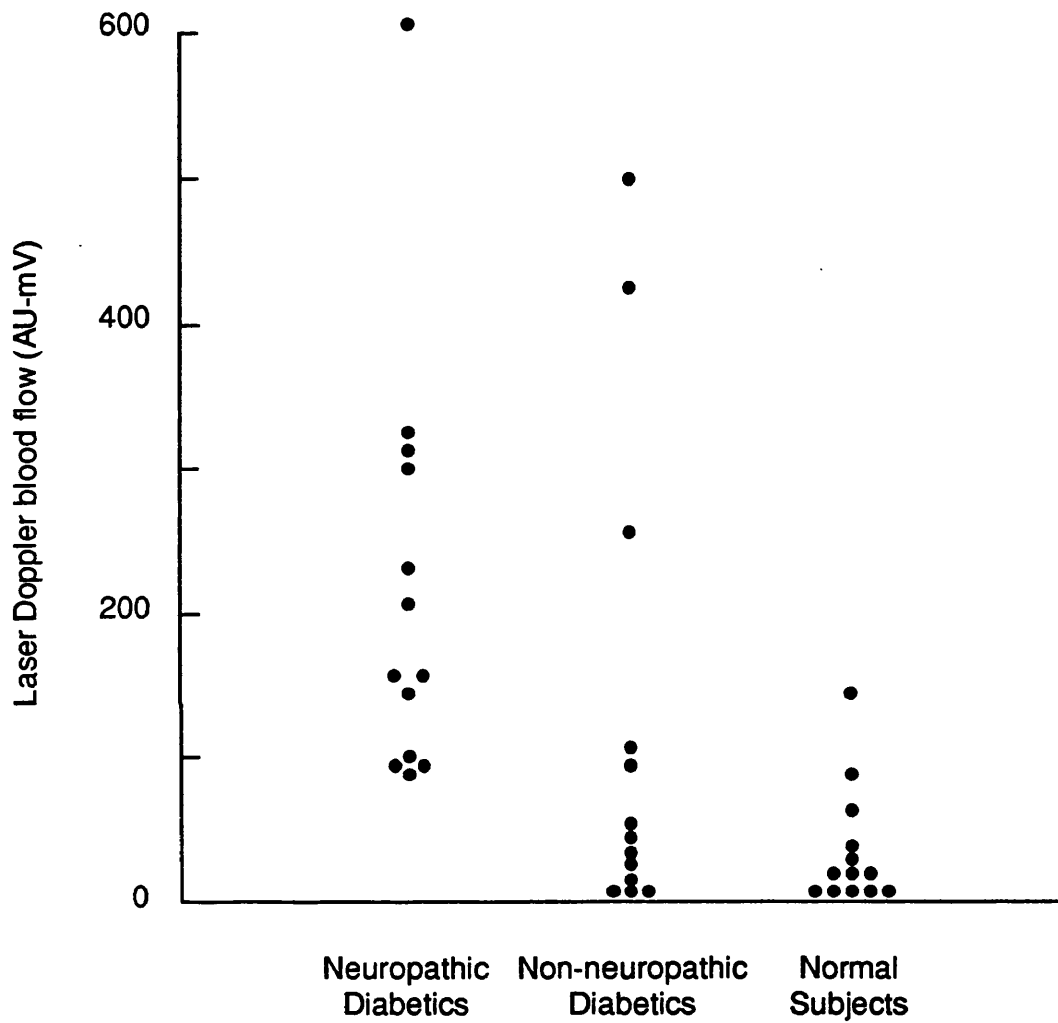
Figure 5.6

Toe blood flow during the fourth minute of dependency in the three groups
of subjects. Blood flow is shown in arbitary units of mV.
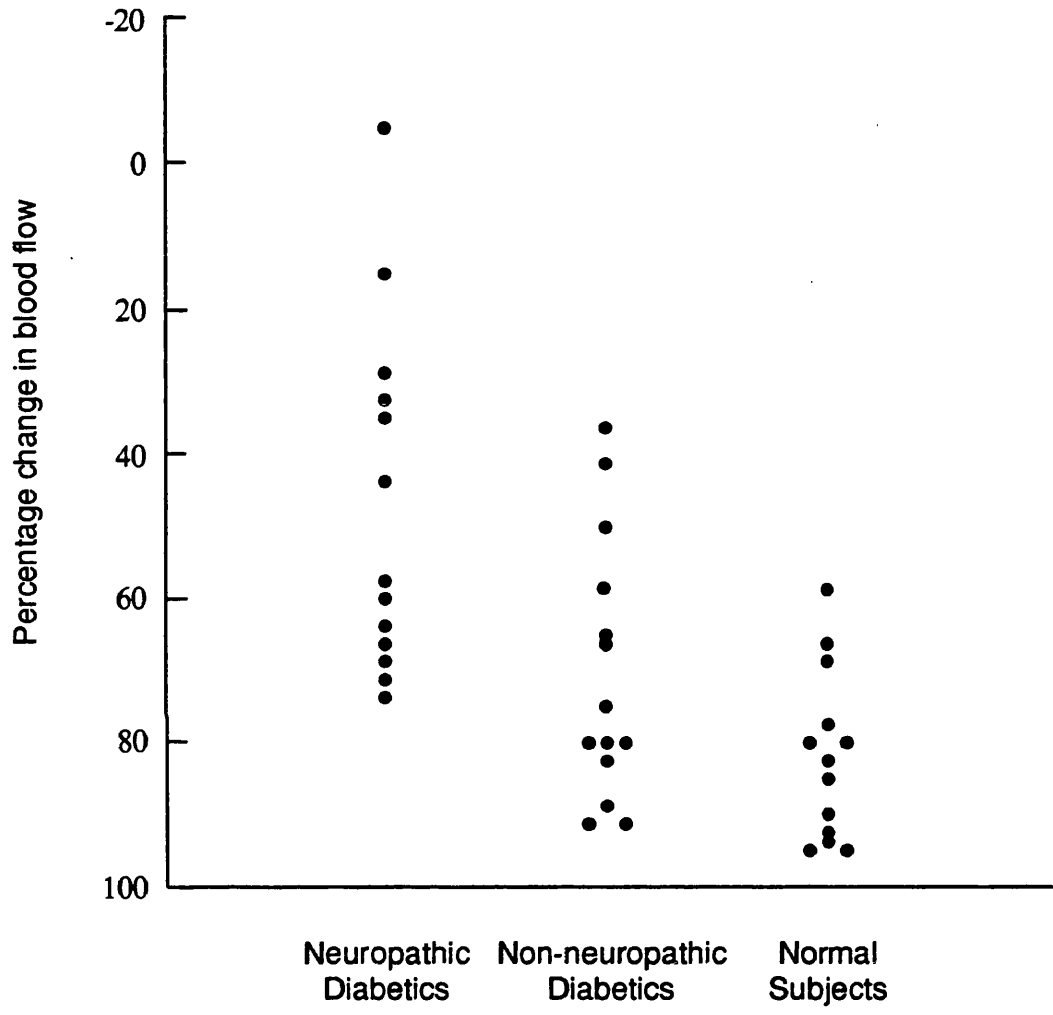
Figure 5.7

Percentage change in blood flow in diabetic subjects with
neuropathy, diabetic subjects without neuropathy and
non-diabetic subjects

(rest - dependent flow / rest flow x100)

5.5 DISCUSSION

The most striking finding in this study is the considerably elevated skin blood flow in diabetic subjects with neuropathy, with the abnormality being even more marked on dependency.

The increase in resting blood flow in patients with neuropathy is in agreement with previous studies. Edmonds (1982) and Corbin (1987) described abnormalities in Doppler ultrasound waveforms from the posterior tibial artery consistent with increased flow and reduced peripheral resistance. Archer (1984) using strain gauge plethysmography found toe blood flow in subjects with neuropathy to be five times greater than that of non-diabetic subjects and the mean toe temperatures in the two groups were 33.5°C and 25.8°C, respectively. In a recent study, Flynn (1988) using the laser Doppler technique reported a three fold higher toe skin blood flow in diabetics with neuropathy compared with normal subjects and mean skin temperatures of 32.6 °C and 27.1 °C in the two groups, respectively. The results of both these studies agree very closely with those of the present study, where blood flow in the toe when at heart level was three times higher in the neuropathic group compared with the control group and mean skin temperatures were 32.2 °C and 27.7 °C, respectively. Since the laser Doppler technique used on the pulp of the toe and venous occlusion plethysmography used in the digit measure predominantly shunt blood flow, and flow through arteriovenous shunts is regulated by sympathetic efferent nerve fibres, these findings are compatible with the suggestion that the increased blood flow is a result of sympathetic failure as suggested by Watkins and Edmonds (1983). Further support for this comes from the work of Hassan (1988) who demonstrated that sympathetic blockade by epidural anaesthesia produced a three fold increase in laser Doppler toe pulp blood flow (from 106.8 mV to 325.2 mV), and increase in skin temperature (from 25.4 to 31.6 °C); results remarkably similar to the differences in skin

temperature and blood flow between the diabetics with neuropathy and the normal subjects found in the present study.

In the non-diabetic subjects lowering the foot evoked a pronounced fall in laser Doppler flow to 18% of the resting flow value, a reduction of 82%. The results agree with those of Hassan (1988), who working under the same laboratory conditions and using a similar protocol found an 85% reduction in laser Doppler measured toe blood flow when the foot was lowered 50 cm. Similar changes were found in toe nailfold capillary blood flow measured using capillary microscopy; Hassan (1987) reported a 91% fall and Flynn (1988) found a 88% fall in toe capillary blood flow. As discussed in Chapter 2 these changes are considerably greater than the 50% reduction in blood flow recorded using the Xenon clearance technique, a difference which may be explained by the injection hyperaemia induced during Xenon injection.

In comparison with the responses in the normal subjects, the mean percentage reduction in blood flow in the neuropathic group was only 46%. Kastrup using the Xenon clearance technique was unable to confirm the findings of this study which were reported in the British Medical Journal (Rayman 1986). On the other hand Hilsted (1979) also using the Xenon clearance technique had previously reported impaired veno-arteriolar reflexes in diabetics with severe neuropathy and in agreement with the present study also found a relationship between the degree of abnormality and the degree of autonomic dysfunction. The difference between Kastrup's findings and those of Hilsted's may be explained by the difference in patient groups. Hilsted's patients had severe autonomic neuropathy and orthostatic hypotension whereas only one of the patients in Kastrup's study had hypotension, the remainder had only mild to moderate neuropathy. The patients in the current study also had more severe neuropathy than those in Kastrup's study nevertheless based on the present findings at least some of the neuropathic patients in that study might have been expected to have had

impaired postural responses compared to the normal control group. The failure to find such a difference may be explained partial failure of the postural vasoconstrictor response in the normal subjects due to the hyperaemia resulting from the injection of Xenon.

Hassan (1988) has also shown that abolishing central sympathetic tone by lumbar epidural anaesthesia partially attenuates the vasoconstrictor response; dependent blood flow in the shunt area of the toe fell by only 42%, very similar to the findings in the neuropathic group. Similarly, Hassan, Rayman and Tooke (1986) have demonstrated that indirect heating, which also releases of central sympathetic tone, also over-rides the vasoconstrictor response in normal subjects. Thus, the impaired vasoconstrictor response to dependency in diabetics with neuropathy may be related to either a reduction in central sympathetic tone and/or peripheral sympathetic nerve failure.

Several of the non-neuropathic diabetic subjects had high resting and dependent blood flows with impaired postural responses. Clinically there were no features which would distinguish these individuals from those in the same group with normal responses. It is conceivable that these patients may have sympathetic neuropathy without detectable sensory or cardiac autonomic neuropathy, supporting Watkin's (1983) suggestion that peripheral sympathetic neuropathy may be an early feature in patients with diabetes.

Since failure of pre-capillary vasoconstriction on dependency exposes the capillary bed to a greater hydrostatic load this may result in peripheral oedema. In this study blood flow was measured in a shunt flow area so it would incorrect to infer failure of pre-capillary vasoconstriction, nevertheless, since the shunts are in continuium with the venous end of the capillary, failure of shunt blood flow regulation will be associated with increased capillary pressure and oedema formation (Luckner 1955). In support of this is a study by Williams, Rayman, and Tooke (1989), in which

diabetic and non-diabetic hypertensive subjects treated with the calcium channel blocker nifedipine, were found to have elevated toe pulp blood flow on dependency which was associated with the development of leg oedema in 4 of the 10 of the diabetic and 2 of 17 non-diabetic subjects. In the present study 4 of the subjects with neuropathy had mild peripheral oedema in the absence of cardiac failure. Renal impairment cannot be completely excluded as creatinine clearances were not measured but plasma creatinine was normal and none of the subjects was albustix positive suggesting that diabetic nephropathy is unlikely.

Peripheral oedema resulting from diabetic neuropathy was first described by Pryce in 1893 and later noted by Rundles (1945) and Martin (1952). Though gross oedema is relatively uncommon, mild degrees of oedema if sought are not infrequent particularly in the elderly diabetic subjects with neuropathy. Martin (1952) reported oedema in one third of his patients with autonomic neuropathy, and Rundles (1945) found dependent oedema in 35 of 125 patients with neuropathy. In both studies other causes of oedema were excluded. Lithner (1984) found leg oedema in 64 % of his patients with foot ulceration, 28 % of whom had no other cause for the oedema. Apelqvist (1990) reported oedema in 38 % of patients with foot ulceration and although factors such as congestive cardiac failure and previous venous thrombosis were involved in many cases, oedema was more common in those with neuropathy. The high prevalence in Lithner's series led him to the suggestion that this may be a predisposing factor in ulceration. Apelqvist found that those with leg oedema were more likely to have had an amputation or have died. In a prospective study of relatively young diabetic patients between the ages of 15 to 50 yrs. Borssén (1990) reported that 20 % of Type I and 16 % of Type II patients had a history of leg oedema. Whether this was linked to neuropathy was not stated.

Edmonds (1983) put forward the hypothesis that neuropathic oedema may be explained by increased foot blood flow and arteriovenous shunting

resulting from sympathetic denervation. This study confirms that arteriovenous blood flow is elevated in the neuropathic foot, furthermore on dependency this abnormality is exaggerated. The latter provides and additional and perhaps more important explanation for the development of neuropathic oedema.

CHAPTER 6

A STUDY OF FACTORS GOVERNING FLUID FILTRATION IN THE DIABETIC FOOT

6.1 INTRODUCTION

In the previous study, evidence was provided for a disturbance of postural regulation of blood flow in diabetic subjects. The aim of this study was to further investigate this and other oedema preventing mechanisms using the relatively more severe stress of sitting with the leg almost fully extended.

On changing from the supine to the standing position, arterial and venous pressures in the foot increase in direct proportion to the change in height of the column of blood between the heart and foot. A similar increase in capillary pressure would rapidly result in interstitial oedema unless there were compensatory changes in either capillary pressure and/or the other determinants of fluid filtration. Levick and Michel (1978) demonstrated that the rise in capillary pressure in the toe nailfold on standing is less than would be predicted simply by the change in local arterial pressure, indicating an increase in pre- to post-capillary resistance. This suggestion is supported by the studies of Henriksen (1977) mentioned in the previous chapter, in which it was demonstrated that lowering the leg resulted in a fall in foot subcutaneous blood flow due to an increase in vascular resistance, the veno-arteriolar reflex. Furthermore, Serjersen (1981) demonstrated that on lowering the calf to 40 cm below the heart level the increment in transcapillary filtration rate was less than would have been expected if capillary pressure had changed by the predicted change in hydostatic pressure. Linked with these observations, Henriksen (1983) showed that in chronically sympathetic denervated limbs, in which the veno-arteriolar vasoconstrictor response is absent, venous pressure elevation to 40 mmHg caused a linear increase in capillary filtration rate, whereas in the opposite

intact limb where the veno-arteriolar reflex was unaffected the capillary filtration rate increased by only 66% of that predicted by the change in hydrostatic pressure.

Another important determinant of capillary filtration is plasma osmotic pressure. Youmans (1934) proposed that the effect on the filtration rate of a rise in capillary pressure might be counteracted by a concomitant increase in capillary plasma oncotic pressure. This suggestion is supported by Noddeland (1981) who demonstrated that in the dependant stationary foot, colloid osmotic pressure rises progressively, observations confirmed by Moyses and Michel (1984). Such an increase in colloid osmotic pressure can only occur if microvascular blood flow is low, as would occur with pre-capillary vasoconstriction. Thus posturally induced pre-capillary vasoconstriction by limiting the rise in capillary pressure, reducing microvascular blood flow and increasing plasma osmotic pressure appears to be central to the prevention of interstitial oedema in the dependent limb.

In this study capillary pressure, colloid osmotic pressure, capillary and shunt blood flow were measured in the feet of diabetic and non-diabetic subjects during a change in posture, to determine whether any of these factors governing fluid filtration are disturbed and reflected in an increased foot swelling rate in diabetic patients.

6.2 SUBJECTS

The studies were limited to male subjects, as the menstral cycle has been shown to have profound effects on peripheral blood flow (Keates 1969), and postural vasoconstriction (Hassan 1990). All subjects were non-smokers. Sitting still for fifty minutes was found to be relatively stressful and in a preliminary study a control subject fainted after thirty minutes. The study was therefore limited to relatively fit young persons, and diabetics with neuropathy were excluded because of possible postural hypotension.

The diabetic study group comprised twelve insulin dependent diabetics (Table 6.1). Their mean age was 32.3 ± 5.5 years (range 21 to 40 years) and mean duration of diabetes was 16.5 ± 6.4 years (range 5 to 29 years). Five subjects had background retinopathy; none had proliferative retinopathy or albustix positive proteinuria. Vibration sensory thresholds were within normal limits for age when assessed using centile charts (Bloom 1984). Cardiac autonomic function tests were normal in all subjects and none had significant falls in blood pressure on standing. All subjects had ankle/brachial systolic blood pressure ratios of greater than 1.0. Blood glucose was assessed just prior to and at the end of the study.

The control group consisted of ten normal subjects. They were volunteers from the medical and technical staff of the Charing Cross Medical School. Subject details are given in Table 6.2. The mean age of this group (34.9 ± 5.5 years; range 26 to 46 years) was not statistically different from the diabetic group (32.3 ± 5.5 years; range 21 to 40 years).

6.3 METHODS

6.3.1 Study Conditions

Studies were conducted in a temperature controlled environment as described in Chapter 2. In order to minimise flow through arteriovenous shunts, the room temperature was lowered to 21 °C and tightly controlled to within 0.5 °C. Radiant heat from sunlight was excluded by the use of heavy curtains and lighting provided by fluorescent lamps.

The studies were started at 9 am and at least one hour after the subject's breakfast. No alcohol was allowed on the evening prior to study. Diabetic subjects took their usual pre-breakfast insulin and all subjects had a normal breakfast. Tea and coffee were not permitted.

TABLE 6.1

Clinical Characteristics of the Diabetic Subjects

Subject	Age	Duration	Complications	Plasma Glucose mmol/l	Vibration Threshold R	L	Supine BP mmHg	Sitting BP* mmHg	Ankle BP mmHg	E:I Ratio	Valsalva Ratio
TW	33	10	BR	9.1	9	9	130/90	120/85	150	1.41	2.00
DH	38	11	-	5.6	6	5	120/80	130/80	130	1.10	1.62
RP	35	20	-	3.8	6	7	125/70	130/75	150	1.32	1.79
TH	21	18	BR	7.8	9	9	110/85	110/80	130	1.35	1.50
PT	31	15	-	6.1	5	6	105/70	110/70	120	1.35	1.50
CM	34	17	BR	4.9	10	11	120/75	110/75	120	1.20	2.10
WS	35	17	-	3.9	9	10	110/80	110/70	120	2.03	2.35
NM	23	16	BR	9.4	10	10	125/70	125/85	130	1.63	2.14
RW	40	25	-	15.6	8	8	130/70	130/80	135	1.66	2.00
GT	31	29	BR	12.1	6	7	120/85	115/90	125	1.62	1.90
JD	33	5	-	7.5	7	7	120/65	110/60	135	1.53	2.44
AH	33	15	-	24.3	9	6	120/70	135/70	120	1.42	2.00
Mean	32.3	16.5		9.1	7.8	7.9	119.6/75.8	119.6/76.6	130.4	1.47	1.95
SD	5.5	6.4		5.9	1.7	1.9	7.8 7.9	9.8 8.3	10.7	0.25	0.30

* Blood pressure after 50 minutes of sitting.

TABLE 6.2

Clinical Details of Control Subjects

Control Subjects	Age	Supine BP	Sitting BP*	Ankle Systolic BP
SR	32	115/75	110/70	120
KM	39	120/80	125/70	125
DN	34	115/65	120/70	125
SW	26	125/70	120/70	130
JS	35	130/85	115/80	130
BB	39	120/70	120/90	120
JG	46	110/85	110/70	120
JL	36	120/80	115/75	115
GR	32	115/75	110/75	120
TL	30	120/80	115/85	135
Mean	34.9	119.6/76.5	116.0/76.0	124.0
SD	5.5	5.7 6.7	5.2 7.0	6.1

* Blood pressure at the end of the sitting period

6.3.2 Experimental Protocol

Subjects were acclimatised for forty minutes before any blood flow measurements were made. During this time they lay on a couch which had been specially constructed to allow the leg sections to be lowered, either together, to allow the subject to move from the supine to sitting position without excessive body movement, or independently, and so permit one leg to be lowered while the subject remained supine.

At the start of the acclimatisation period, a vein on the dorsum of the right foot was cannulated. To assist cannulation, the veins were engorged by lowering the leg 50 cm for five to ten minutes. A 'butterfly' type cannula, with 21 gauge needle (Butterfly-21 INT; Abbott Laboratories Ltd., Kent, UK) was used. The dead space of the cannula was reduced to approximately 10 ul, by removing the hub and part of the cannula tubing, leaving a length of approximately 3 cm, Figure 6.1. A shortened teflon sleeve from a 16 gauge Abbocath- T cannula (Abbott Laboratories Ltd., Kent, UK) was wedged into place in the open tubing. From this device blood could be readily sampled using a 19 gauge B-D (Becton Dickenson, Oxford, UK.) needle. Between samples a 'bulldog' clip was used to occlude the tubing.

After cannula insertion, the subject returned to the supine position for the remainder of the acclimatisation period. During this period, probes for measuring skin blood flow, temperature and swelling rate were positioned on the foot.

After the acclimatisation period the subject sat up with the right foot placed in a relaxed state one meter below heart level (Figure 6.2). The level of the heart was taken to be 9 cm below the jugular notch in the seated position (Amoroso 1989). The measurements were made using a spirit level and meter rule. To help maintain this position and prevent the subject from slipping off the end of the couch, the left foot was supported on a perspex platform. Blood was sampled just prior to sitting and thereafter at ten
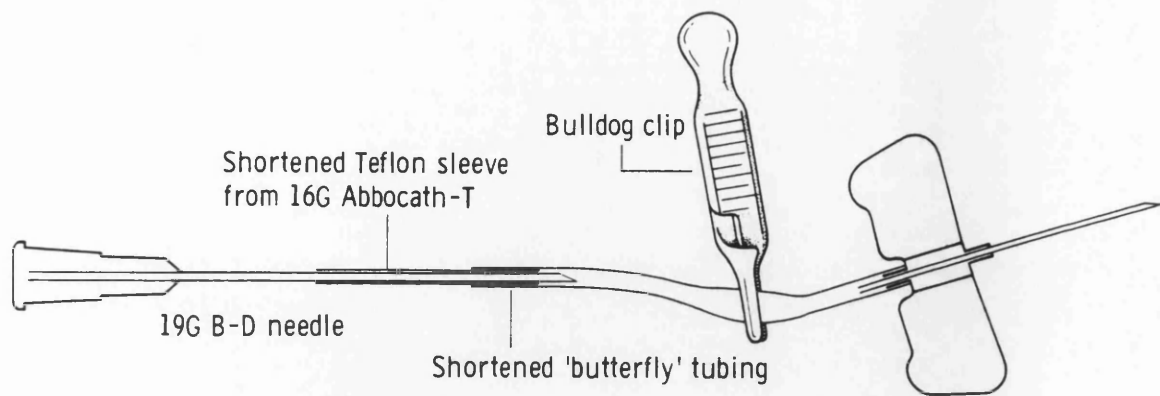
Figure 6.1: Modified 'butterfly' cannula used for venous sampling
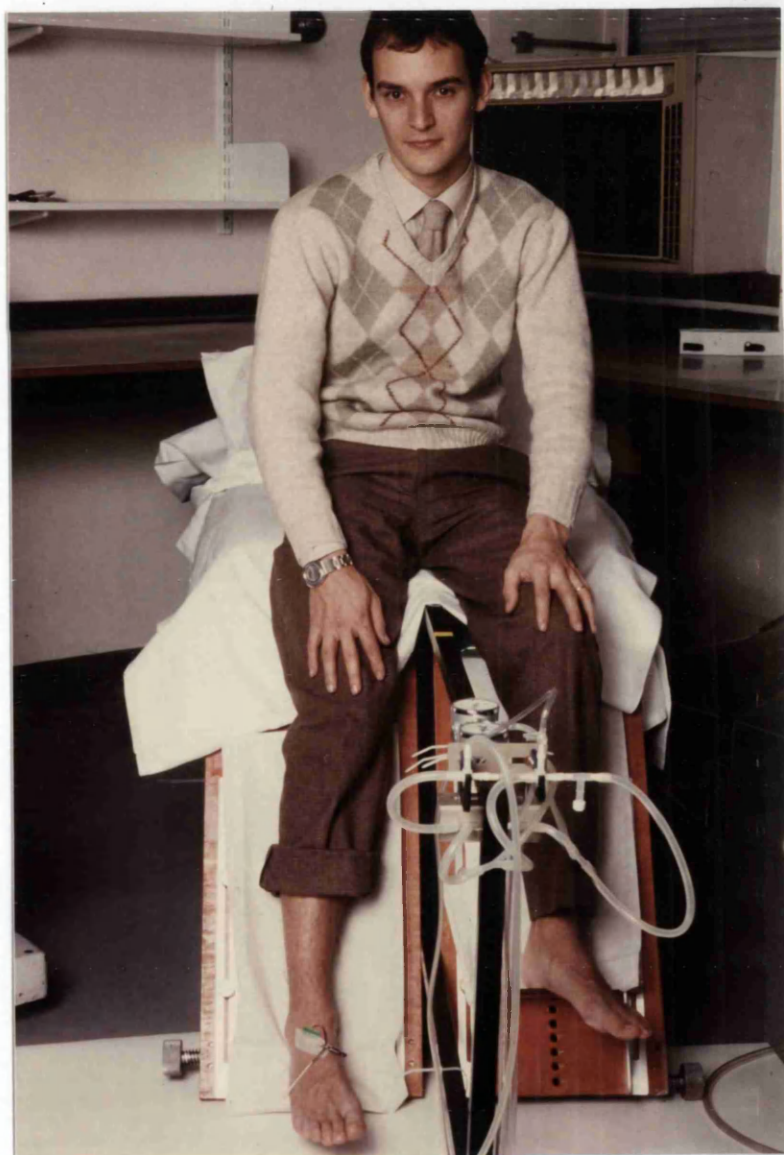
Figure 6.2: Photograph showing seated subject with cannula in place

minute intervals for fifty minutes. Time zero was taken from the time of first sitting. In five of the diabetic subjects (TW, NM, RW, GT, JD) and three control subjects (SR, DN, JG) samples were not obtained at the 10 and 30 minute points. The cannula was kept patent with a minimum quantity of heparin saline (approximately 0.25 ml of a 10 units per ml solution). Prior to each sampling, 0.5 ml of blood was withdrawn and discarded.

6.3.3 Blood Flow Measurements

Blood flow was recorded from the plantar surface of the big toe of the right foot and the dorsal surface of the same foot using a laser Doppler flowmeter (Periflux Model PF I, Perimed Ltd., Sweden). By using two separate fibre-optic lines and alternately inserting them into the laser Doppler flowmeter, it was possible to measure flow from two sites using the one laser Doppler flowmeter. Had only one fibre-optic line been used its' probe would have had to been moved from one skin site to another which would have disturbed the local blood flow.

Toe blood flow was measured immediately prior to sitting, for five minutes (time -5 to 0 minutes) and thereafter at five minute intervals just before each sampling period ie. at the time intervals of 5-10, 15-20, 25-30, 35-40, and 45-50 minutes. Blood flow was also recorded from the dorsum of the foot for five minutes, at ten minutes before sitting (time -10 to -5 minutes), and thereafter at the time intervals of , 0-5, 10-15, 20-25, 30-35, and 40-45 minutes.

6.3.4 Skin Temperature

Temperature was recorded from the skin on the dorsum of the foot and the plantar surface of the toe at ten minute intervals from the start of sitting using a Comark 1625 electronic thermometer and a copper-constantan thermocouple.

6.3.5 Foot Swelling Rate

Details of the method for measuring foot swelling are given in Chapter 2. The correct size of gauge for the size of the subject's foot was selected and placed around the foot, approximately mid-way between the metatarsal heads and the ankle, avoiding the site of cannula insertion. To prevent artefacts caused by the silastic tube of the gauge rolling or slipping as the foot expanded, it was taped to the skin at approximately 2 cm intervals using Micropore tape (3 M, St. Pauls, Minneapolis USA) cut into thin strips approximately 3 mm in width. The tape does not interfere with circumferential expansion. The swelling rate was calculated from the tissue swelling curve at between 10 to 20 minutes of sitting (Chapter 2).

6.3.6 Plasma Colloid Osmotic Pressure and Haematocrit

Details of the methods used for these measurements are given in Chapter 2. For both colloid osmotic pressure and haematocrit the results given represent the mean of two measurements for each sample.

6.3.7 Capillary Pressure

Capillary pressure was measured in the nailfold capillaries of the big toe using the Landis microinjection technique, as described in Chapter 2. It would have been preferable for the measurements to have been made immediately after the foot blood sampling part of the study without the subject having changed position. Unfortunately this was not possible, the capillary pressure apparatus having being located in a different part of the laboratory. This necessitated the subjects having to walk across the room. Capillary pressure was measured with the subject seated, however because of the constraints of the apparatus the leg could not be as fully extended as in the earlier part of the study. Capillary pressure was therefore measured

with the foot 93 cm below the jugular notch (Chapter 2; Figure 2.11). Because of the difficulty of the technique attempts at capillary pressure measurement were not always successful. In a number of subjects in whom capillary measurement failed, a successful cannulation was made when the subject returned on following day.

6.4 RESULTS

6.4.1 Toe Skin Temperature

Starting, 10 and 50 minute, toe skin temperature measurements, for diabetic and normal control subjects are shown in Table 6.3. Mean starting temperature although higher in the diabetic group (26.1 ± 3.4 °C) was not significantly different from that of the control group (23.8 ± 1.7 °C). Both normal and diabetic subjects showed a progressive fall in toe temperature. At the end of the study, toe temperatures in both control and diabetic subjects were close to room temperature (24.3 ± 3.7 °C in the diabetic group and 22.1 ± 0.7 °C in the control group). In some subjects foot temperature was actually below room temperature. This may be explained by the evaporation of 'cold sweat', sweating being occasionally observed in these subjects. One diabetic subject (TH) had a starting toe temperature above 30 °C which paradoxically rose towards the end of the study.

6.4.2 Toe Blood Flow

Starting toe blood flow measurements and those at between 15-20 minutes and 25-50 minutes, for diabetic and normal control subjects are shown in Tables 6.3 and Figure 6.3 and 6.4.

Resting toe blood flow although higher in the diabetic group (median 85.8 mV, range 18.8 to 500.0 mV) was not significantly different to that of the control subjects (median 39.7 mV, range 12.0 to 93.9 mV). Several of

TABLE 6.3

Skin Temperature and Blood flow in the pulp of the Big toe in Diabetic (upper panel) and Control (lower panel) Subjects

Subject	Skin Temperature (°C)			Laser Doppler Blood Flow (mV)		
	Starting	10 min	Final	Rest Flow	15-20 mins	25-50 mins
TW	30.7	29.3	27.3	351.0	72.0	62.0
DH	24.3	23.7	23.3	32.5	2.0	2.6
RP	22.6	21.2	21.1	18.8	6.9	11.2
TH	31.0	30.2	32.6	500.0	352.0	592.0
PT	24.0	23.0	21.7	59.0	3.2	1.1
CM	23.1	23.4	21.6	62.0	7.1	6.9
WS	23.5	23.4	23.0	19.3	3.5	2.9
NM	30.0	28.2	29.0	210.0	127.0	118.0
RW	25.4	30.2	26.0	164.0	188.0	114.0
GT	27.8	25.6	21.6	109.6	2.8	5.4
JD	21.8	21.3	21.0	42.0	6.7	9.3
AH	28.5	28.3	24.2	155.0	27.8	17.5
Median	24.9	24.7	23.2	85.8	7.0	10.3
Mean	26.1	25.7	24.4	143.5	66.6	78.5
SD	3.4	3.4	3.7	148.8	108.0	167.0

	Starting	10 min	Final	Rest Flow	15-20 mins	25-50 mins
SR	25.5	23.9	22.5	62.4	3.0	2.9
KM	26.0	23.0	21.8	93.9	0.0	0.0
DN	22.2	22.0	21.0	35.0	2.4	0.0
SW	23.5	23.1	22.5	30.0	5.5	2.2
JS	26.5	25.8	23.5	80.6	1.8	1.3
BB	21.7	21.7	21.5	22.0	1.0	0.0
JG	23.7	22.7	21.9	44.4	1.5	1.6
JL	22.9	22.1	22.4	51.5	1.8	2.7
GR	23.5	23.8	22.3	15.9	1.9	0.4
TL	22.2	21.9	22.0	12.0	2.3	8.0
Median	23.5	22.8	22.2	39.7	1.9	1.5
Mean	23.7	23.0	22.1	44.7	2.1	1.9
SD	1.7	1.3	0.7	27.5	1.4	2.4
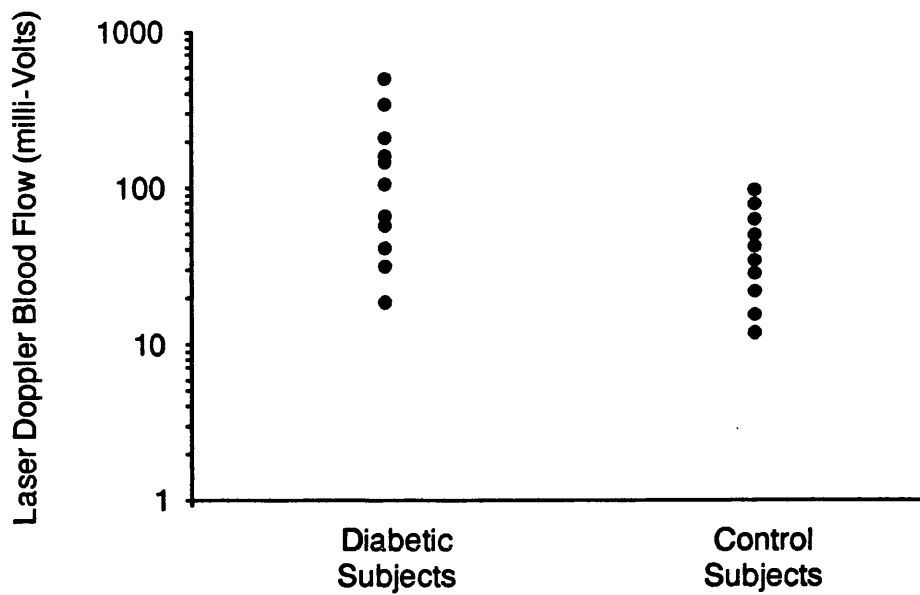
Figure 6.3 : Toe blood flow immediately prior to sitting in diabetic and control subjects- laser Doppler flow is plotted on a log scale.
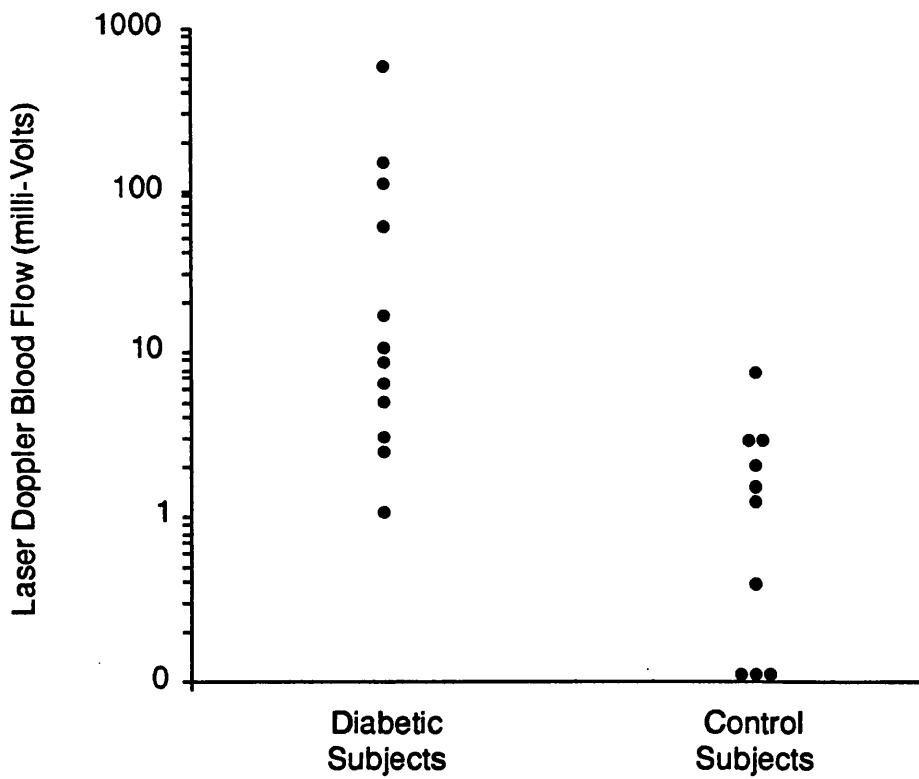
Figure 6.4 : Toe blood flow in diabetic and control subjects over the 25-50 minute period- laser Doppler flow is plotted on a log scale.

the diabetic subjects (TH, TW, NM, RW, AH) had resting blood flow values above the range for the normal subjects corresponding with their higher skin temperatures.

Toe blood flow recorded between 15 and 20 minutes of dependency was significantly higher in the diabetic group (median 7.0 mV, range 2.0 to 352.0 mV) than in the control group (median 1.9 mV, range 0.0 to 5.5 mV; $p< 0.002$) as was toe blood flow during the 25 to 50 minute period (diabetics- 10.3, range 1.1 to 592.0 mV; normals- 1.5, range 0.0 to 8.0 mV: $p< 0.002$) Figure 6.4. In three of the normal subjects toe blood flow was either zero or too low to be recorded with the laser Doppler flowmeter.

6.4.3 Blood Flow and Temperature on the Dorsum of the Foot

Starting, 10 minute and final skin temperatures, and starting, 10-15 minute and 25-45 minute blood flow measurements from the dorsum of the foot are shown in Tables 6.4. Skin temperatures were not significantly different in the two groups at any of the time points. Similarly blood flow from the dorsum of the foot was not significantly different at any of the time intervals when the two groups are compared, although towards the end of sitting, blood flow in six of the twelve diabetic subjects was higher than the highest flow recorded in the control group.

6.4.4 Colloid Osmotic Pressure and Haematocrit

Table 6.5 show individual colloid osmotic pressures and the percentage change above the starting value for the diabetic and normal subjects, respectively. Whereas there was a progressive rise in colloid osmotic pressure in the control group, colloid osmotic pressure reached a plateau at thirty minutes in the diabetic group, Figure 6.5. Mean colloid osmotic pressure was significantly higher in the normal subjects at both 40 minutes (diabetics- 36.2 ± 5.4 mmHg, range 28.5 to 42.9 mmHg; normals- 45.8 ±

TABLE 6.4

Skin Temperature and Blood flow on the Dorsum of the Foot in Diabetic
(upper panel) and Control (lower panel) Subjects

Subject	Skin Temperature (°C)			Laser Doppler Blood Flow (mV)		
	Starting	10 min	Final	Rest Flow	15-20 mins	25-50 mins
TW	29.5	30.0	28.6	9.2	2.6	1.2
DH	26.5	25.6	24.9	7.0	2.8	3.3
RP	26.9	26.0	24.7	5.2	3.3	1.8
TH	29.5	30.2	32.6	11.2	4.9	8.6
PT	28.0	24.7	23.4	20.5	2.2	1.8
CM	26.2	25.5	25.1	9.3	0.0	0.0
WS	25.8	24.5	25.1	5.3	0.0	0.0
NM	27.9	24.5	28.0	9.0	4.4	4.1
RW	28.6	28.7	26.0	22.4	2.4	1.7
GT	28.5	26.1	23.7	19.5	2.0	3.0
JD	27.5	26.7	26.1	13.0	5.5	4.8
AH	28.5	29.4	26.8	15.5	6.1	4.3
Median	27.9	26.1	26.6	10.3	2.0	2.4
Mean	27.8	26.8	25.3	12.3	3.0	2.9
SD	1.2	2.2	2.5	6.0	2.7	2.4

	Starting	10 min	Final	Rest Flow	15-20 mins	25-50 mins
SR	29.8	28.5	26.4	13.7	2.7	1.5
KM	28.0	27.8	26.4	13.7	0.0	0.0
DN	28.7	27.8	25.3	14.4	3.1	2.7
SW	28.2	27.4	26.3	15.0	2.9	2.3
JS	29.0	27.7	26.3	9.5	1.8	1.5
BB	25.0	24.0	22.8	5.2	0.0	0.0
JG	28.4	27.2	26.0	13.2	3.2	1.6
JL	28.7	28.0	26.7	7.9	1.7	1.9
GR	28.5	27.3	25.5	6.5	1.4	1.4
TL	24.5	24.5	24.2	7.7	3.3	1.8
Median	27.8	27.6	26.2	11.4	2.3	1.6
Mean	28.5	27.0	25.6	10.7	2.0	1.5
SD	1.7	1.5	1.2	3.7	1.3	0.9

TABLE 6.5

Colloid Osmotic Pressure and Percentage change in Colloid Osmotic
Pressure in Diabetic (upper panel) and Control Subjects (lower panel)

Subject	Colloid Osmotic Pressure (mmHg)						% change
	0 min	10 min	20 min	30 min	40 min	50 min	50 v 0 min
TW	23.8	-	27.5	-	30.1	31.5	32.3
DH	22.4	27.2	34.7	37.9	38.7	38.5	41.8
RP	25.2	27.8	34.0	36.3	36.7	36.7	45.6
TH	26.5	28.8	26.9	28.4	28.6	28.6	7.8
PT	25.8	31.4	39.1	42.4	40.6	40.1	55.4
CM	24.1	32.2	38.1	43.4	42.9	41.4	71.8
WS	23.9	26.7	31.6	36.4	38.4	35.6	49.5
NM	23.7	-	29.6	-	28.5	29.6	24.9
RW	23.3	-	26.5	-	30.1	30.3	30.0
GT	24.6	-	40.4	40.0	42.9	43.0	74.8
JD	22.6	-	35.4	-	37.9	36.7	62.4
AH	25.5	33.6	38.7	39.0	39.3	39.9	56.5
Mean	24.4	29.7	33.5	37.9	36.2	36.0	46.1
SD	1.3	2.7	5.0	4.6	5.4	4.9	19.9

Subject	Colloid Osmotic Pressure (mmHg)						% change
	0 min	10 min	20 min	30 min	40 min	50 min	50 v 0 min
SR	25.0	-	39.5	-	44.2	45.4	81.6
KM	28.8	30.5	33.0	46.7	51.4	51.4	78.4
DN	26.2	-	40.5	-	47.7	47.7	82.0
SW	26.3	28.5	30.5	35.4	40.5	45.1	71.5
JS	23.8	27.4	40.3	45.0	48.0	49.1	106.3
BB	24.7	29.3	36.0	40.6	43.9	51.8	109.7
JG	22.9	-	34.8	-	44.9	46.4	102.6
JL	23.4	29.3	37.8	42.0	46.5	47.6	103.4
GR	27.7	29.6	40.4	46.8	50.3	53.8	94.2
TL	25.6	26.3	29.0	35.5	40.3	46.3	80.9
Mean	25.4	28.7	36.2	41.7	45.8	48.5	91.1
SD	1.8	1.4	4.2	4.8	3.7	3.0	13.7
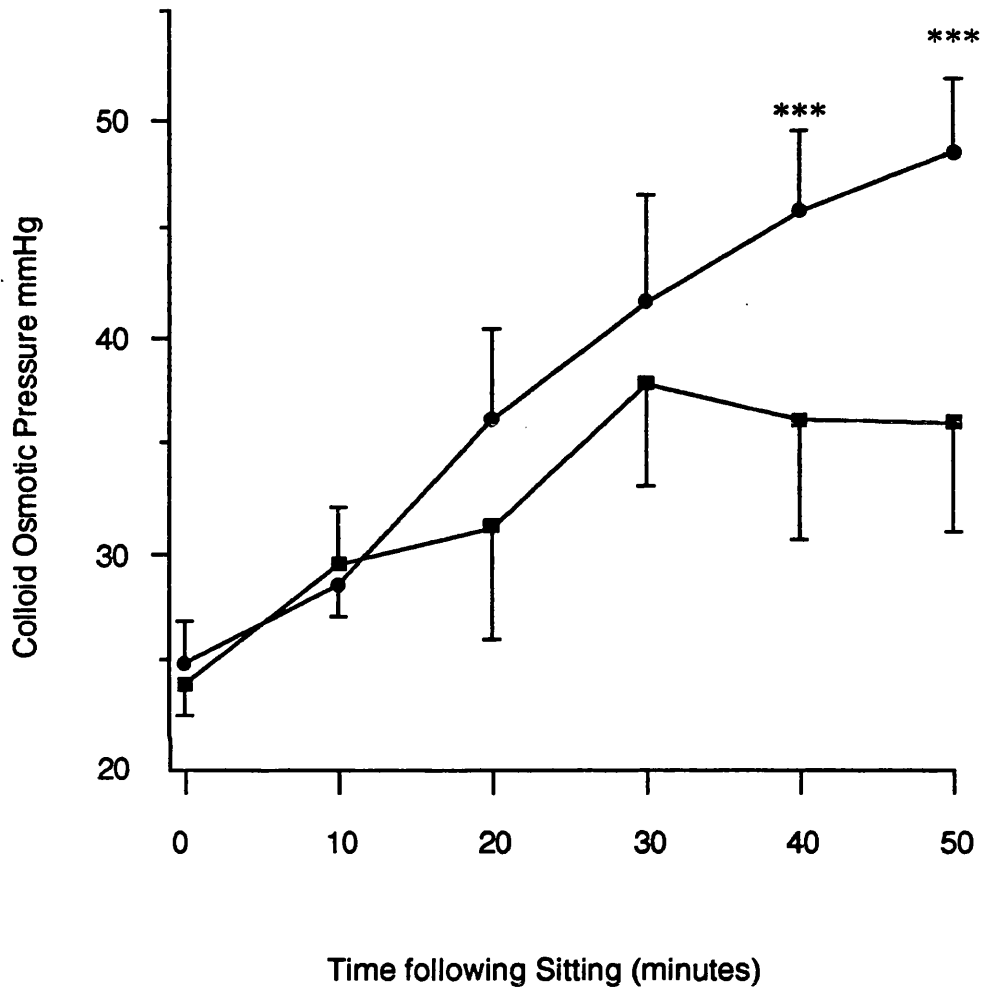
Figure 6.5

Changes in colloid osmotic pressure in diabetic (squares) and control
(circles) groups (points indicate means and bars indicate SD).

3.7 mmHg, range 40.3 to 51.4 mmHg: p<0.002) and 50 minutes (diabetics- 36.0 ± 4.9 mmHg, range 28.6 to 43.0 mmHg; normals- 48.5 ± 3.0 mmHg, range 45.1 to 53.8 mmHg: p<0.002) of dependency. The percentage change in colloid osmotic pressure in the control group (91.1 ± 13.7%, range 71.5 to 109.7%) was significantly greater than that in the diabetic group (46.1 ± 19.9%, range 7.8 to 74.8%; p<0.002).

As would be expected similar changes were observed in the haematocrit. Figure 6.6 shows a series of spun haematocrit tubes from a diabetic and control subject to illustrate the visible differences in haematocrits. Table 6.6 shows the change in haematocrit in individual subjects in the two groups. Statistical differences between the diabetic and control gropus were observed at 20 (p<0.005), 30 (p<0.01), 40 (p<0.002) and 50 minutes (p<0.002). The percent change in haematocrit in the control group (40.8 ± 7.3%, range 29.8 to 55.4%) was significantly greater than that in the diabetic group (19.9 ± 11.1%, range 4.5 to 41.3%; p<0.002).

The colloid osmotic pressure was inversely correlated with toe blood flow, Table 6.7 and Figure 6.7.

6.4.5 Foot Swelling Rate

Swelling rates for both groups are shown in Table 6.8 and graphically in Figure 6.8. The diabetic group had a significantly higher mean swelling rate (0.099 ± 0.025 ml. min^{-1}. 100 ml^{-1}) than that of the control group (0.069 ± 0.022 ml. min^{-1}. 100 ml^{-1}), p<0.02. Swelling rate did not correlate with either toe blood flow or colloid osmotic pressure when the groups were analysed separately. This is perhaps not suprising considering the techniques used and the relatively small number of subjects studied. When the groups are pooled there was a significant relationship between toe blood flow (r_S = 0.80; p<0.001, Table 6.7) and a negative relationship with colloid osmotic pressure (r_S = -0.67; p<0.002) Table 6.7 and Figure 6.9.
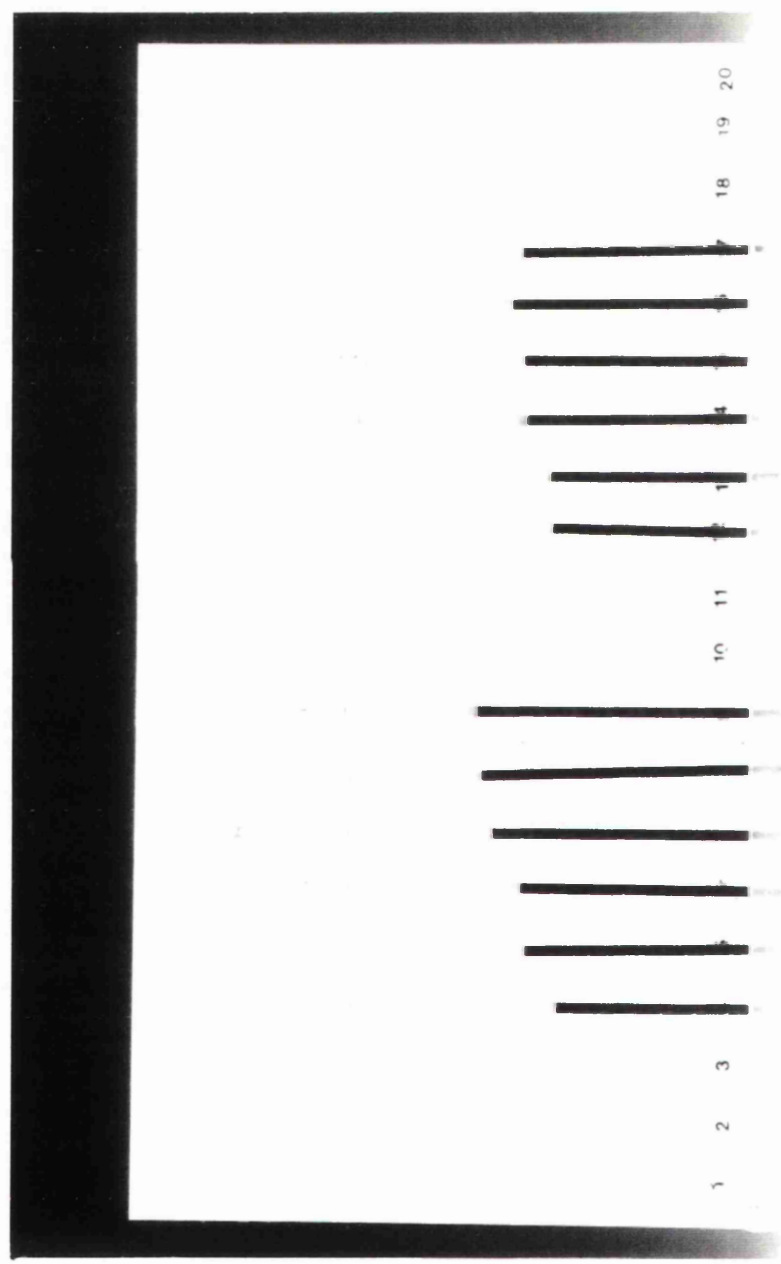
Figure 6.6: **Photograph** demonstrating the difference in haemoconcentration between a non-diabetic and diabetic subject. The group of spun haematocrit tubes on the left are from the non-diabetic subject and those on the right from the diabetic subject. In each group, the tubes going from left to right represent spun venous blood samples taken at 0, 10, 20, 30, 40, and 50 minutes of sitting.

TABLE 6.6

Haematocrit and Percentage change in Haematocrit in
Diabetic (upper panel) and Control Subjects (lower panel)

Subject	Haematocrit (%)						% change
	0 min	10 min	20 min	30 min	40 min	50 min	50 v 0 min
TW	44.0	-	46.0	-	48.0	49.0	11.4
DH	42.0	47.0	50.5	52.0	52.0	52.0	23.8
RP	44.0	46.0	52.0	52.0	51.5	52.0	18.2
TH	44.5	46.5	49.0	49.0	46.0	46.5	4.5
PT	42.5	48.5	53.0	54.0	53.0	52.5	23.5
CM	43.5	49.0	46.5	51.0	51.5	50.0	14.9
WS	42.0	44.0	47.5	50.0	51.5	52.0	23.8
NM	46.0	-	50.0	-	50.0	50.0	8.7
RW	44.0	-	46.5	-	49.5	49.0	11.4
GT	37.5	-	51.0	-	52.0	53.0	41.3
JD	43.0	-	51.0	-	54.0	49.0	37.2
AH	43.5	49.5	52.0	52.0	51.5	52.0	19.5
Mean	43.0	47.2	49.6	51.4	50.9	50.6	19.9
SD	2.1	1.9	2.4	1.6	2.2	2.0	11.1

Subject	Haematocrit (%)						% change
	0 min	10 min	20 min	30 min	40 min	50 min	50 v 0 min
SR	42.5	-	58.5	-	59.5	59.0	38.8
KM	41.5	52.5	62.5	61.0	64.0	64.5	55.4
DN	40.0	-	55.5	-	55.5	56.5	41.3
SW	38.0	43.0	48.0	52.0	54.0	57.0	50.0
JS	38.5	46.0	52.0	54.0	57.0	55.0	42.3
BB	44.0	49.0	54.5	56.0	57.5	59.5	35.2
JG	42.5	-	52.5	-	58.0	59.5	40.0
JL	41.0	49.0	53.5	55.0	57.5	56.0	36.6
GR	47.0	51.0	56.0	59.0	62.0	61.0	29.8
TL	41.0	44.0	51.0	55.0	56.0	57.0	39.0
Mean	41.6	47.8	54.4	56.0	58.0	58.5	40.8
SD	2.6	3.6	4.1	3.1	3.0	2.8	7.3

Table 6.7

Spearman rank correlation coefficients between the various parameters

	Swelling Rate	BloodFlow at 10 min.	Blood flow at 50 min.	COP at 20 min.	COP at 50 min.	Capillary Pressure
Swelling Rate		$r_s = 0.53$ $p < 0.015$	$r_s = 0.80$ $p < 0.001$	$r_s = -0.51$ $p < 0.02$	$r_s = -0.67$ $p < 0.002$	$r_s = 0.37$ $p < 0.23$
BloodFlow at 10 min.	$r_s = 0.53$ $p < 0.015$		$r_s = 0.85$ $p < 0.001$	$r_s = -0.40$ $p < 0.04$	$r_s = -0.88$ $p < 0.001$	$r_s = 0.61$ $p < 0.04$
Blood flow at 50 min.	$r_s = 0.80$ $p < 0.001$	$r_s = 0.85$ $p < 0.001$		$r_s = -0.57$ $p < 0.01$	$r_s = -0.85$ $p < 0.001$	$r_s = 0.51$ $p < 0.10$
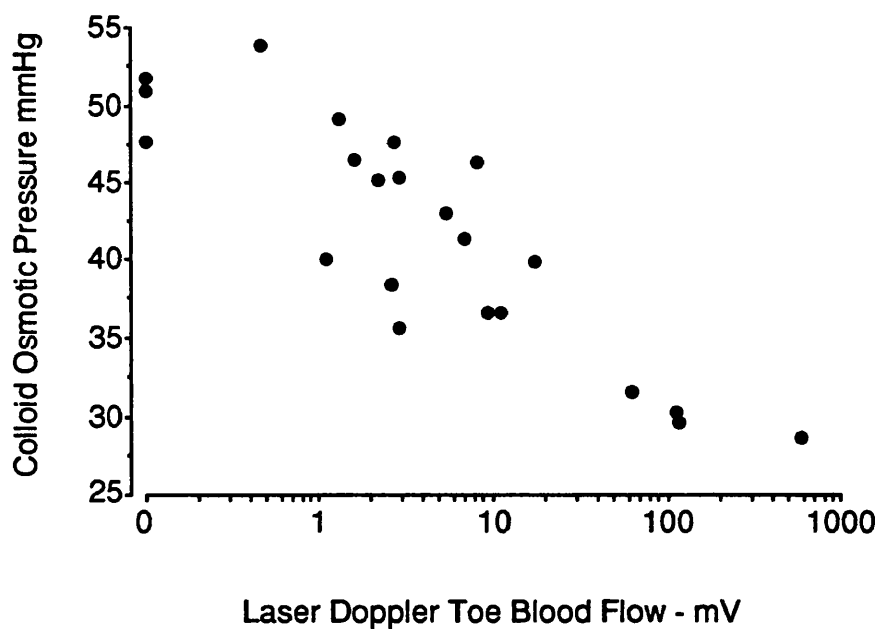
Figure 6.7 - Relationship between colloid omotic pressure at 50 minutes and average toe blood flow at between 25 and 50 minutes. Laser Doppler blood flow is shown on a log scale.

TABLE 6.8

Foot Swelling Rates in Diabetic and Control Subjects

Diabetic Subjects	Swelling Rate ml/100ml/min	Control Subjects	Swelling Rate ml/100ml/min
TW	0.100	SR	0.055
DH	0.090	KM	0.050
RP	0.100	DN	0.060
TH	0.110	SW	0.040
PT	0.035	JS	0.060
CM	0.090	BB	0.080
WS	0.100	JG	0.090
NM	0.110	JL	0.100
RW	0.145	GR	0.050
GT	0.110	TL	0.100
JD	0.110		
AH	0.090		
Mean	0.099		0.069
SD	0.025		0.022
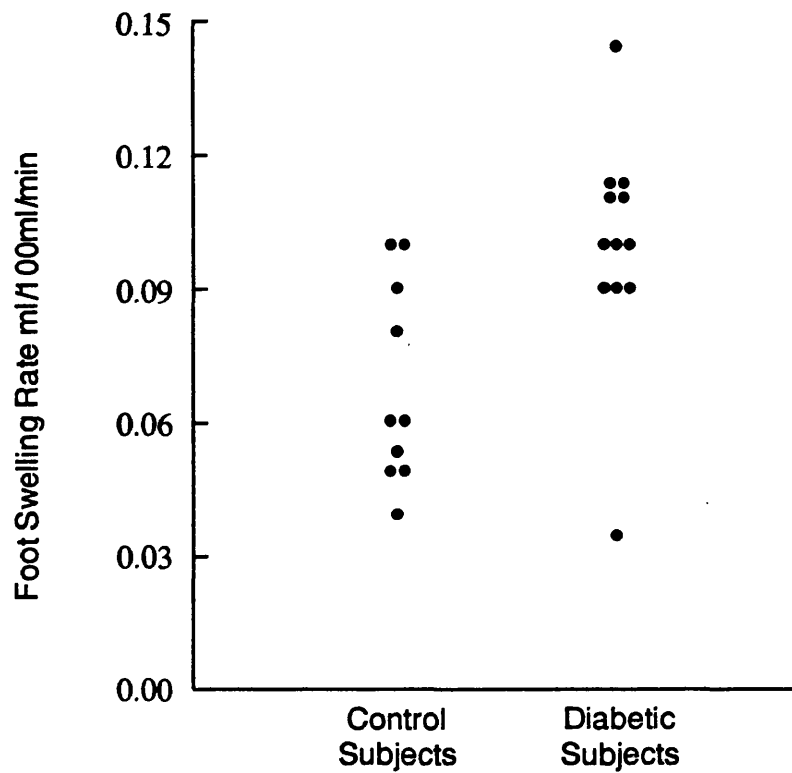
Figure 6.8: Foot swelling rates in diabetic and control subjects.
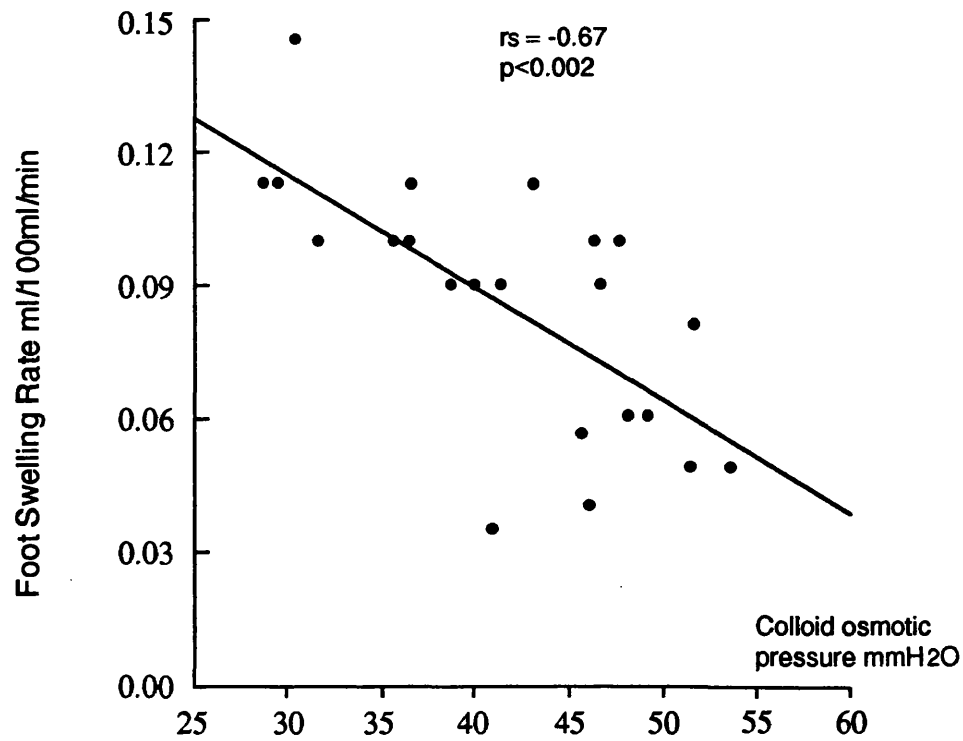
Figure 6.9: Relationship between swelling rate and osmotic pressure at 50 minutes.

6.4.6 Capillary Pressure

Because of the difficulty of the technique it was only possible to measure capillary pressure in six diabetic and six normal subjects. Subject details, results, and the temporal relationship to the earlier part of the study are shown in Tables 6.9 and 6.10. Mean capillary pressure in the control group was 85.3 ± 1.7 cm H_2O. The mean for the diabetic group was significantly higher; 92.2 ± 4.6 cm H_2O (p< 0.007), Figure 6.10. Capillary pressure did not correlate with swelling rates and skin blood flow but was negatively correlated with colloid osmotic pressure at 50 minutes (r_s = -0.83; p<0.01), Figure 6.11. Capillary pressure did not correlate with prevailing blood glucose r_s = 0.13.

TABLE 6.9

Capillary Pressure, Skin Temperature and Blood Glucose in the Diabetic Subjects

Subject	Blood glucose (mmol/l)	Skin Temp. (°C)	Capillary pressure (mm H2O)	Mean Capillary Pressure (mm H2O)
CM	3.5	26.3	86.0, 88.0	87.0
PT	3.6	23.7	91.5	91.5
WS*	3.9	32.3	100.0	100.0
TH	21.9	31.8	96.0, 92.5	94.3
RP	3.4	24.9	89.0	89.0
AH	21.9	25.0	91.5, 91.0	91.3
Mean	9.7	27.3		92.2
SD	9.5	3.7		4.6

TABLE 6.10

Capillary Pressure and Skin Temperature in Control Subjects

Subject	Skin Temp. (°C)	Capillary pressure (mm H2O)	Mean Capillary Pressure (mm H2O)
JL	26.0	86.0, 82.5	84.3
SW	24.5	85.0	85.0
JS*	26.8	83.0	83.0
TL*	26.1	88.0, 88.0	88.0
GR	26.4	84.0, 87.5	85.7
KM	27.5	86.0	86.0
Mean	26.2		85.3
SD	1.0		1.7

* Denotes that studies performed on a different day to venous sampling study.
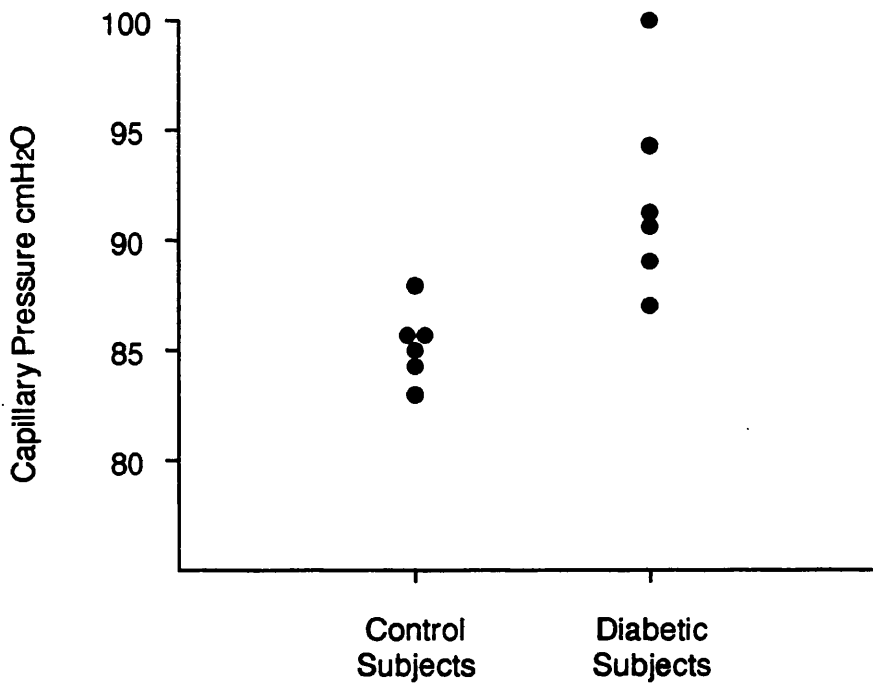
Figure 6.10- capillary pressure in control and diabetic subjects
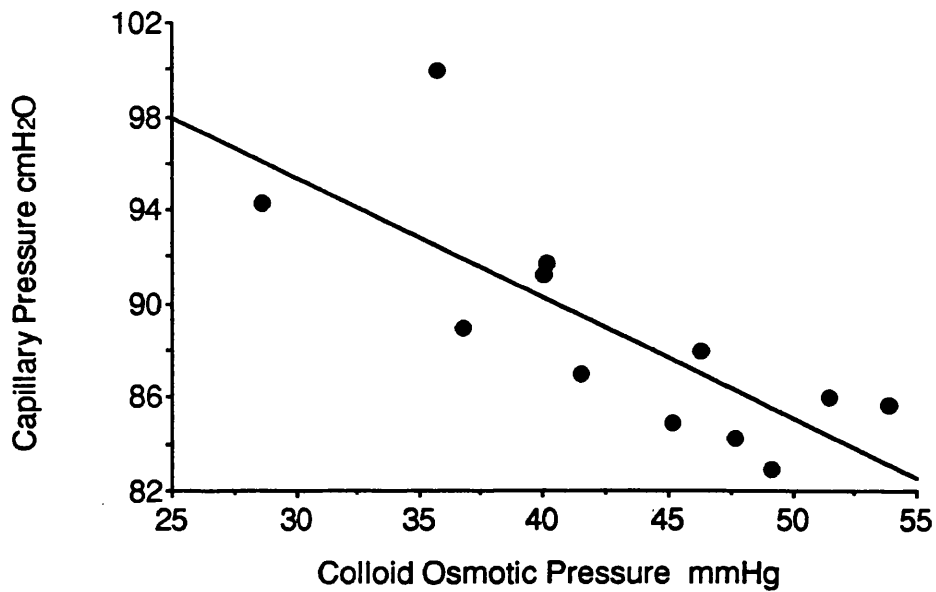
Figure 6.11- The relationship between capillary pressure and colloid osmotic pressure (r = - 0.83; p<0.01).

6.5 DISCUSSION

The principle findings of this study were of increased foot swelling rates, reduced haemoconcentration, increased capillary pressure and increased toe (shunt) blood flow in the diabetics subjects.

At the start of the sitting period, toe skin temperature and pulp blood flow were very much lower in the diabetic and control groups than had been found in similar groups described in Chapters 5. This is may be explained by the lower environmental temperature and the additional procedures undertaken during the equilibration period. Thus, postural vasoconstriction elicited by lowering the foot for cannulation and reflex vasoconstriction resulting from the discomfort of foot cannulation are likely to have been contributory in reducing the starting skin blood flow and temperature. Although there was no significant difference in starting toe blood flow and temperature between the groups, in several of the diabetic subjects (TW, TH, NM, RW) these were considerably elevated in comparison with the control group.

On sitting toe blood flow was also very much lower than had been found in the dependent foot in the studies described in Chapter 5. The difference is accounted for by lower starting blood flow and the much greater hydrostatic pressure to which the vessels were subjected; in the previous study the foot was lowered only 50 centimetres whereas in this study it was lowered one meter. Toe blood flow and skin temperature during dependency were significantly elevated in the diabetic group but this was principally due to the four diabetic subjects previously mentioned (TW, TH, NM, RW). The finding of a sub-population of diabetics subjects apparently free from neuropathy but with reduced vasoconstrictor responses to low environmental temperatures and unable to reduce shunt blood flow on dependency to the same extent as that in non-diabetic subjects is similar

to the observations made in Chapter 5 and suggests a reduction in sympathetic tone in these subjects.

In contrast to the findings in the toe, in both diabetic and control subjects, blood flow from the dorsum of the foot fell during sitting to extremely low levels; in several subjects it was indistinguishable from the biological zero value. These findings agree with the demonstration by Flynn (1989) that the postural regulation of capillary blood flow assessed by video-microscopy is not impaired in the diabetic foot.

In the normal subjects the increase in colloid osmotic pressure (23 mmHg) was similar to that (20 mmHg) described by Moyses (1984) but very much greater than that (13 mmHg) described by Noddeland (1981). The smaller increase in the latter study can be explained by the differences in experimental protocols; venous pressure was increased to only 49 mmHg in subjects in Noddeland's study whereas in the present study it was increased to 74 mmHg.

Venous blood from the dorsum of the foot is a mixture of blood which has left the capillaries and that which by-passes the nutritive vessels through the arteriovenous anastamoses. In normal subjects, Noddeland (1981) found that during orthostasis in warm feet, plasma colloid osmotic pressure rose by only a few mm Hg, whereas when cold the increase was approximately 13 mmHg. The lack of haemoconcentration of the venous blood in the warm feet may be due to either high capillary blood flow or continuing shunt flow diluting of the haemoconcentrated blood that has left the capillary bed. In the present study, the high shunt flow observed in the diabetic group would explain the relative lack of haemoconcentration of the venous blood, indeed the plasma osmotic pressure and percentage change in plasma osmotic pressure correlated with toe blood flow. Furthermore the rise in colloid osmotic pressure was least in the four diabetic subjects in whom toe blood flow in the dependent position was greatest. The alternative possibility of higher dependent capillary blood flow does not appear to

explain the lack of haemoconcentration as there was no significant difference in the laser Doppler measurements from the dorsum of the foot. However, this cannot be totally discounted as dorsal blood flow in half of the diabetic group was higher than the highest flow in the control group, and at very low blood flow rates the laser Doppler method may not be able to distinguish very small differences which may nevertheless result in differences in haemoconcentration. Furthermore, the changes in capillary osmotic pressure reflect changes occurring in a relatively large volume of tissue whereas the laser Doppler only samples a small volume, and as the blood flow abnormalities may be patchy this could give rise to misleading results in relationship to the blood flow changes in the capillary circulation of the whole foot. Such spatial sampling problems could explain why some diabetic subjects appeared to have reduced shunt blood flow to the same extent as in the normal subjects yet failed to increase colloid osmotic pressure to the same extent.

In the control group mean capillary pressure was 85.3 ± 1.7 cm H_2O. The calculated venous pressure is approximately 81.4 cm [93 cm, the vertical distance between the foot and the jugular notch, minus 9 cm, the distance between the jugular notch and tricuspid valve (Amoroso 1989), minus 2.6 cm, the allowance made for the negative pressure recorded at this site in the seated position (Grassi 1984). That capillary pressure is close to venous pressure suggest almost complete pre-capillary vasoconstriction and virtual absence of shunt flow. Although mean capillary pressure was found to be significantly higher in the diabetic group, only a small numbers of subjects could be studied, so the results need to be interpreted with caution, nevertheless, the findings are supported by a recent study in which capillary pressure in the finger was found to be elevated in diabetic subjects in the absence of hypertension (Sandeman 1990). Higher capillary pressures would be compatible with either reduced pre-capillary vasoconstriction and/or open arteriovenous shunts; the latter being in continuum with the venous end

of the capillary. Thus the finding of persistently high shunt flow in the diabetic group would be in keeping with the finding of elevated capillary pressure, however there was no correlation between the two, nor was there a correlation between capillary pressure and dorsal blood flow, or swelling rate. This is not surprising as the subjects had walked across to the measurement area, activating the calf muscle pump, only a relatively small number of subjects could be studied (in only 1 of the 4 diabetic subjects with the most elevated blood flows was it possible to obtain measurements), and in several subjects the studies could not be performed on the same day as the other measurements.

Regarding swelling rates, in a similar study, with the foot dependent one meter, Moyses (1984) found a swelling rate of 0.105 ml. min^{-1}.100 ml^{-1} in normal subjects, somewhat greater than that found in the present study. Compression of the tissue by the strain gauges would not account for this difference as extremely light tensioned Periflow mercury-in-silastic gauges which exert a force of only 0.2 gm for a 1% elongation (Janssen Scientific Instruments, Beerse, Belgium) were used, and these were never stretched more than 10% of their original length. The differences may be explained by the very low skin temperatures achieved in this study. Landis (1933) and Hyman (1968) have shown that capillary filtration is significantly lower at lower skin temperatures when presumably blood flow is lower. This has been confirmed by Hassan (1988 b) who found that the foot swelling rate increased from approximately 0.04 ml. min^{-1}. 100 ml^{-1} at a foot skin temperature of 26.0°C to 0.08 ml. min^{-1}. 100 ml^{-1} when the foot skin temperature was increased to 36.0°C.

The increased mean foot swelling rate in the diabetic group could result from increased capillary permeability, failure of pre-capillary vasoconstriction (resulting in high capillary pressures, high capillary blood flow and lack of haemconcentration) and/or elevated flow through arterio-venous anastamoses which would increase pressure in the venous limb of the

capillary. Since many studies have demonstrated increased capillary permeability of the diabetic microcirculation to small and large molecular weight substances it would be reasonable to suggest that the increased swelling rate results from an increase in the permeability to water. One difficulty in interpreting many of these studies is that they fail to control for the effect on filtration of an increase in capillary pressure and/or blood flow. Thus for example, although the transcapillary rate to albumin has been shown to be increased in diabetic subjects (Parving 1976) this may simply be the result of increased capillary perfusion pressure, in the same way that transcapillary rate to albumin is increased in hypertension (Parving 1973). In support of this is the demonstration that an acute reduction of blood pressure in diabetic subjects with nephropathy resulted in a reduction of the elevated transcapillary rate to albumin (Parving 1985). Similarly, Poulsen (1976) concluded that capillary filtration coefficient was increased in the subcutaneous tissue of the forearm of diabetic subjects since forearm swelling following venous occlusion was increased. Venous occlusion will elicit the veno-arteriolar reflex. Poulsen failed to consider that the apparent increased capillary filtration coefficient may simply reflect a relatively higher capillary perfusion due to relative failure of this reflex in the diabetic subjects.

In this study the increased swelling rate in the feet of diabetic subjects may be explained by a failure to elicit the postural reflex and thus regulate shunt blood flow on dependency. Thus, dependent toe blood flow was elevated in the diabetic group relative to the control subjects and when all the subjects are considered together toe blood flow correlated with swelling rate. Furthermore, there was an inverse relationship between with swelling rate and venous colloid osmotic pressure, the latter as previously suggested reflecting dilution of the capillary blood by blood shunted through the arteriovenous anastomosis. This relationship was stronger for blood samples obtained later rather than at the time swelling rate was measured. This may

be explained by the time taken for the haemoconcentrated blood in capillary to reach the sampling vein in subjects in whom shunt and capillary flow were almost completely arrested.

In conclusion relatively young diabetic subjects with only minimal complications have evidence of impaired shunt flow regulation on extreme dependency of the foot. This is associated with and perhaps the cause of elevated capillary pressure, reduced haemoconcentration and an increased foot swelling rate. The mechanism underlying this disturbance of blood flow regulation is unclear. Unlike the previous study none of these subjects had clinically detectable neuropathy and only minimal complications. Subtle sympathetic neuropathy not detected by central cardiac autonomic tests may provide an explanation. Alternatively, peripheral vasodilation and increased shunt flow, due to increased core temperature, consequence upon an increased metabolic rate, may have resulted in the postural reflex being over-ridden. Similarly, peripheral vasodilation may result form hyperinsulinaemia (Tooke 1985) and hypoglycaemia (Hilsted 1984), and again it is possible that either of these may over-ride the control of shunt blood flow on dependency. Hypoglycaemia would not appear to be involved as none of the subjects was clinically hypoglycaemic and no subject had a glucose level less than 3.8 mmol/l, furthermore there appeared to be no relationship between plasma glucose and blood flow. The studies were performed after the patients breakfast insulin had been given, so hyperinsulinaemia is a possibility, but as insulin levels were not measured it is not possible to establish the relationship between this and any of the various determinants of filtration examined.

Despite having elevated swelling rates none of the diabetic subjects had a history of ankle oedema. This not surprising as the conditions of the study, maintaining the foot perfectly still in the dependent position for this period of time, are unusual. Under normal circumstances small movements of the leg would activate the calf muscle pump, which would lower venous

pressure and increase lymphatic flow, thereby counteracting oedema formation. The importance of calf muscle pump on venous pressure was demonstrated by Pollack (1949) who found that following a few steps venous pressure at the ankle decreased from 85 to 30 mmHg. In one of the present studies it was found that simply squeezing the calf muscle resulted in venous pressure (measured by a pressure transducer) falling from 74 mmHg to 35 mmHg (data not shown). In the elderly and relatively immobile diabetic patient it is easy to envisage the development of peripheral oedema due to infrequent activation of the calf muscle pump and failure of the veno-arteriolar reflex. Although clinically obvious oedema was not present in the patients studied, several studies have confirmed that in diabetic patients extracellular fluid volume is increased (Fauchald 1985, Sandeman 1989) and interstitial colloid osmotic pressure (Fauchald 1985) or interstitial albumin concentrations (Poulsen 1973) are reduced in patients free from cardiac failure and nephrotic syndrome. These findings suggest increased capillary filtration and are in keeping with the findings of increased swelling rate and capillary pressure described in this study.

CONCLUSION

The aim of this thesis was to investigate the skin microvasculature in diabetic subjects using a number of techniques, including the laser Doppler method in order to gain a greater understanding of the diabetic microcirculation and in particular its response to changes in posture and tissue injury. Before defining abnormalities it was necessary to determine the range of responses in non-diabetic subjects since at the time the studies were undertaken the laser Doppler technique was relatively new and had not been previously used to study the vascular responses described.

Initially it was considered that the laser Doppler would measure predominantly nutritive blood flow; however, in agreement with the findings of others, in preliminary studies it soon became obvious that when used in the pulp of the toe the measurement largely reflected arteriovenous blood flow. This proved an advantage in that it allowed both shunt and non-shunt blood flow to be assessed independently depending on the area of skin examined. The method has been validated by other workers against a large number of other techniques but in particular the correlation with capillary blood flow in the human skin has been found to be extremely good (Hassan 1988b). In the studies described in Chapter 2, it was found that point to point variability, epidermal thickness and capillary density all affected the measurement, hence various methods were devised either to reduce their influence or to establish their importance. Thus for example, a multiple sampling probe combined with a more reliable heating probe than that provided by the manufacturer was developed, thereby reducing the coefficient of variation for the measurement of the thermal injury response to very acceptable levels; epidermal thickness and capillary densities were determined and shown not to account for the differences in the laser Doppler measurements between diabetic and non-diabetic subjects; and in the postural studies, despite measuring blood flow at a single point,

differences between the groups were sufficiently large to negate the influence of point to point variation, and in these studies the percentage change in blood flow proved to be a reasonably reproducible parameter. Thus, with defined limitations the laser Doppler proved extremely useful for investigating the skin microcirculation in diabetic subjects. It was simple to use, non-invasive, and gave continuous measurements of blood flow so allowing dynamic changes to be followed; it was useful in following blood flow changes during change in posture, and because the measurements could be made in minute areas of skin it was found to be ideal for studying the microvascular responses to injury in diabetic skin where tissue trauma needs to be kept to a minimum.

As discussed in the introductory chapter, in many of the previous studies a reduction in the maximum blood flow in the microcirculation of the diabetic foot could only be inferred since the methods used were indirect measures of microvascular blood flow. Furthermore, doubt has been expressed regarding the presence of significant structural abnormalities in the microvessels of the skin (LoGrefo 1984). The studies described in Chapter 3 and 4 provide conclusive evidence of both structural and functional abnormalities. Skin microvascular responses to various forms of injury were reduced, basement membrane thickness was increased, and the capillaries were found to be smaller in diameter. Furthermore, the abnormalities in microvascular hyperaemic responses correlated with the structural abnormality. Although it is not possible to prove conclusively that the abnormality in microvascular response following injury is involved in the pathogenesis or the impaired healing of diabetic foot ulceration, the finding that the patients at greatest risk of ulceration, the subjects with severe microvascular complications, had the most severely impaired responses is supportive of this suggestion. Even if not primarily involved in the development of skin ulceration, in the presence of large vessel disease

this deficiency in microvascular perfusion following injury is very likely to play a critical role in the subsequent repair of damaged skin.

From a clinical stand point, an important question is whether the abnormality in microvascular blood flow can be rectified. The apparent link between the hyperaemic response and basement membrane thickness suggests the abnormality is irreversible, at least in the short term. The studies in Chapter 3 suggest that this is the case at least with regard to an acute alteration in diabetic control and to the local administration of insulin. The question remains as to whether changes in diabetic control over a longer period can affect the hyperaemic responses in individual subjects. Although structural factors dominate in determining microvascular flow in the fully vasodilated microvascular bed, the rheological properties of the blood may critically affect hyperaemic blood flow when there is structural narrowing. Both whole blood and plasma viscosity are increased in diabetic patients (Barnes 1977, Isogai 1976) and there is evidence of decreased red cell and leucocyte flexibility (McMillan 1978, Ernst 1986). Furthermore, there is evidence that some of these abnormalities may be reversed by long term improvements in diabetic control (MacRury 1990). The effect on hyperaemic blood flow of changes in blood rheology following alterations in metabolic control or by the use of drugs such as oxpentifylline (Hoechst Pharmaceuticals, Hounslow, UK.) warrant further investigations.

An important finding was that the hyperaemic response was impaired, although to a lesser degree, in patients free from clinical complications and with diabetes of relatively short duration, findings recently confirmed in diabetic children by Shore (1989). This group of patients did not have an increase in basement membrane width but capillary diameter was significantly reduced when compared with that of the non-diabetics subjects. It is possible that unlike the situation in subjects with complications these patients may have a reversible abnormality, perhaps related to alterations in the production, release or response to endothelium derived vasoactive

mediators such as EDRF, endothelin and prostacyclin, or alterations in the production, release or response to vasoactive neuropeptides such as substance P, and CGRP. Further studies in newly diagnosed diabetic subjects involving administration or inhibition of these vasoactive mediators may provide clues as to possible reversible mechanism in these patients.

The studies described in Chapters 4 confirm previous studies which have demonstrated increased total skin blood flow. The novel finding was of increased shunt blood flow on dependency particularly in patients with neuropathy. This abnormality may be one of the mechanisms involved in the formation of neuropathic oedema. Edmonds (1983) demonstrated that ephedrine reduced oedema formation in these patients. Studies of postural changes in microvascular blood flow in patients with neuropathic oedema before and during ephedrine treatment may help to confirm or refute the involvement of this mechanism in this interesting complication of diabetic neuropathy.

The demonstration of elevated capillary pressure in the dependent foot and elevated shunt blood flow with the foot at heat level and dependent, supports the haemodynamic hypothesis for the development of diabetic microvascular complications. Although the blood flow abnormalities were more common in patients with peripheral neuropathy they were also found in some patients without neuropathy and in relatively young and uncomplicated patients exposed to the more severe stress of almost full limb extension. These findings are compatible with the hypothesis advanced in the introductory chapter to explain why at any one time only a proportion of patients without complications are found to have elevated blood flow; namely that early in the disease, interspersed between periods of normal blood flow are, periods when the regulation of blood flow is lost, either as the result of sub-optimal control, elevated metabolic rate or transient peripheral sympathetic dysfunction. Repeated episodes of high blood flow and capillary pressure may then result in minor but progressive vascular

damage; this may further impair vascular responsiveness, with the process becoming self-escalating.

In final conclusion, the series of studies described in this thesis demonstrate impaired blood flow regulation in the microvasculature of the skin of the diabetic foot as shown by increased resting and dependent arteriovenous blood flow and increased capillary pressure. Furthermore, the maximum hyperaemic vascular response to injury is reduced and this is associated with an increase in basement membrane thickness and reduced capillary diameter. These findings are compatible with the haemodynamic hypothesis for the development of microvascular complications. Furthermore, the demonstration that significant structural microvascular disease exists in the diabetic skin, and that this is associated with a reduced hyperaemic microvascular response to injury and that both these abnormalities are more severe and almost invariably present in those at risk of foot complications supports the suggestion that diabetic microangiopathy is involved in the pathogenesis and progression of foot ulceration. The finding of similar though less severe abnormalities in patients without clinical complications and relatively early after diagnosis suggests that intervention at this stage to reverse these abnormalities may be possible and warrants further study.

TABLE A 2.1

Coefficient of variation of thermal injury responses measured using the Periflux PF2b, calculated a) from single site measurements and b) from the mean of multiple site measurements in two separate studies.

Subjects	Blood Flow (AU Volts)			
	Study 1		Study 2	
	mean of 9 sites	CV of 9 sites	mean of 9 sites	CV of 9 sites
	Volts	%	Volts	%
1	2.45	16.7	2.39	20.9
2	2.18	30.3	2.07	18.8
3	1.50	22.7	1.51	28.5
4	0.88	15.9	0.82	19.5
5	1.74	25.9	1.47	21.8
6	1.00	29.0	0.99	34.3
7	2.14	18.2	1.79	26.3
8	1.57	40.1	1.65	24.2
9	1.35	21.5	1.27	28.3
a) Mean within subject CV	24.5			24.7

b) CV based on mean of 9 sites-
calculated from paired studies ie study 1   8.2
and study 2.

TABLE A 2.2

Coefficient of variation of thermal injury responses measured using the Periflux PFI, calculated a) from single site measurements and b) from the mean of multiple site measurements in two separate studies.

| Subjects | Blood Flow (AU Volts) | | | |
| | Study 1 | | Study 2 | |
	mean of 9 sites Volts	CV of 9 sites %	mean of 9 sites Volts	CV of 9 sites %
1	0.80	26.0	0.88	20.2
2	0.62	14.9	0.65	13.5
3	0.63	17.2	0.62	13.4
4	0.68	13.6	0.71	22.0
5	0.65	12.0	0.76	21.2
6	0.74	20.0	0.66	17.3
7	0.62	18.5	0.64	18.5
8	0.64	15.8	0.78	13.4
9	0.71	15.4	0.65	10.3

a) Mean within subject CV	17.0		16.6	

b) CV based on mean of 9 sites-
calculated from paired studies ie study 1   7.6
and study 2.

TABLE A 2.3

Coefficient of variation of the method for determining swelling rates
calculated from paired studies in six individuals.

Subjects	Swelling Rates ml/100gm/min	
	Study 1	Study 2
1	0.095	0.100
2	0.088	0.085
3	0.060	0.060
4	0.060	0.065
5	0.050	0.060
6	0.043	0.050
Mean	0.066	0.070
Coefficient of variation of study 1 and study 2	5.3%	

TABLE A 3.1

Data from which the coefficent of variation for the mean peak blood flow response to injection trauma was calculated. Nine subjects were studied on two different days (study 1 and study 2). The mean peak flow response for each of the studies was based on the mean of the peak responses from two injection sites. The coefficient of variation based on the mean from 2 sites was 8.6 %.

Subjects	Blood Flow (AU Volts)	
	Study 1 mean peak response	Study 2 mean peak response
	Volts	Volts
1	0.48	0.52
2	0.40	0.38
3	0.29	0.30
4	0.40	0.42
5	0.52	0.50
6	0.47	0.52
7	0.33	0.32
8	0.39	0.52
9	0.36	0.39

Within subject CV based
on the mean of 2 measurement sites   8.6%

TABLE A 3.2

Blood flow at various time intervals following injection trauma in diabetic subjects. Each result represents the mean from the two abdominal sites

Subjects	Blood Flow (AU Volts)					
	2min	5min	10min	15min	30min	60min
DM	0.21	0.31	0.27	0.28	0.15	0.09
RG	0.10	0.12	0.13	0.10	0.09	0.07
CC	0.19	0.23	0.30	0.28	0.28	0.15
RC	0.15	0.18	0.18	0.18	0.19	0.14
GR	0.25	0.35	0.29	0.23	0.10	0.03
DA	0.27	0.43	0.42	0.40	0.32	0.15
RH	0.14	0.27	0.25	0.26	0.27	0.25
JB	0.21	0.30	0.28	0.25	0.23	-
GS	0.24	0.30	0.34	0.32	0.29	0.18
WS	0.16	0.20	0.23	0.22	0.19	0.12
RP	0.18	0.23	0.20	0.24	0.10	0.17
DS	0.15	0.18	0.16	0.14	0.08	0.18
KH	0.13	0.15	0.17	0.16	0.16	-
CM	0.09	0.14	0.12	0.13	0.10	0.13
FR	0.15	0.20	0.20	0.20	0.15	0.09
RC	0.30	0.40	0.38	-	0.18	0.16
JT	0.21	0.32	0.34	0.27	0.16	0.11
BH	0.13	0.16	0.15	0.14	0.14	0.12
KH	0.23	0.34	0.29	0.20	0.10	0.10
JD	0.15	0.21	0.18	0.16	0.16	0.12
JF	0.20	0.29	0.29	0.28	0.28	-
RD	0.28	0.35	0.31	0.31	0.24	0.19
DD	0.42	0.42	0.43	0.37	0.37	0.23
Mean	0.20	0.26	0.26	0.23	0.19	0.14
SD	0.07	0.19	0.09	0.08	0.08	0.05

TABLE A 3.3

Blood flow at various time intervals following injection trauma in control subjects. Each result represents the mean from the two abdominal sites.

Subjects	Blood Flow (AU Volts)					
	2min	5min	10min	15min	30min	60min
BM	0.28	0.37	0.35	0.36	0.34	0.29
RR	0.39	0.48	0.49	0.43	0.21	0.17
SWa	0.18	0.35	0.42	0.38	0.34	0.24
TC	0.21	0.28	0.28	0.28	0.21	0.18
MH	0.39	0.52	0.53	0.44	0.14	0.10
JJ	0.23	0.36	0.32	0.24	0.17	0.12
JN	0.18	0.29	0.31	0.28	0.24	0.18
GR	0.36	0.40	0.38	0.38	0.40	0.39
AR	0.20	0.42	0.46	0.49	0.48	0.31
SW	0.18	0.25	0.29	0.29	0.30	0.26
NG	0.48	0.53	0.59	0.58	0.56	0.21
NW	0.18	0.42	0.30	0.31	0.16	0.14
SR	0.25	0.34	0.35	0.32	0.32	0.30
JL	0.27	0.39	0.33	0.26	0.20	0.18
JS	0.29	0.37	0.18	0.14	0.06	0.15
JW	0.33	0.50	0.49	0.48	0.48	0.47
MO	0.37	0.47	0.52	0.49	0.39	0.32
GJ	0.26	0.29	0.33	0.29	0.18	0.11
EE	0.34	0.39	0.44	0.38	0.33	0.32
JR	0.33	0.31	0.38	0.36	0.29	0.26
MP	0.27	0.28	0.29	0.23	0.20	0.25
Mean	0.28	0.38	0.38	0.35	0.29	0.24
SD	0.08	0.08	0.10	0.11	0.13	0.10

TABLE A 4.1

Blood Glucose Concentrations in Group I, Group II and

Group III Diabetic Subjects

Group I		Group II		Group III	
Subject	Blood Glucose mmol/l	Subject	Blood Glucose mmol/l	Subject	Blood Glucose mmol/l
GC	11.3	MB	17.9	JM-S	21.1
PC	12.0	LA	8.9	GS	14.9
LP	19.5	MG	7.9	PB	19.0
RS	5.1	GC	11.9	ET	11.8
JG	8.1	GD	23.5	CB	14.8
MS	3.2	BC	9.8	DS	8.4
ME	6.7			JM	8.2
DK	5.8			HH	12.4
DP	7.1			TB	10.4
RC	3.2			KS	10.4
GS	14.3				
AR	11.8				
Mean	9.0		13.3		13.1
SD	4.9		6.1		4.3

TABLE A 4.2

Skin temperature in Control Subjects, Group I, Group II, and Group III
Diabetic Subjects

Control Subjects		Group I		Group II		Group III	
Subject	Skin Temp.°C	Subject	Skin Temp °C	Subject	Skin Temp °C	Subject	Skin Temp °C
RM	33.6	GC	29.4	MB	30.2	JM-S	29.8
GR	30.5	PC	29.5	LA	29.7	GS	28.5
AB	27.5	LP	31.8	MG	29.9	PB	32.8
McL	33.6	RS	30.8	GC	32.4	ET	34.0
KF	32.2	JG	31.2	GD	34.2	CB	31.3
PJ	28.8	MS	30.5	BC	29.0	DS	29.4
YvT	31.0	ME	29.8			JM	34.2
BB	29.4	DK	34.0			HH	33.2
PB	29.9	DP	-			TB	34.5
JMt	29.0	RC	33.8			KS	35.0
TG	-	GS	29.9				
PL	31.0	AR	32.5				
AL	29.7						
MMc	30.6						
RB	29.7						
MR	28.9						
GD	31.2						
Mean	30.4		31.2		30.9		32.3
SD	1.7		1.6		2.0		2.4

TABLE A 4.3

The effect of local anaesthesia on maximal thermal injury response.
The table shows mean blood flow meaurements from skin that has and
has not been anaesthetised

Study	No anaesthetic	Anaesthetic
	Mean maximum blood flow (V)	
1.	1.79 ± 0.37	1.62 ± 0.35
2.	1.61 ± 0.38	1.78 ± 0.52
3.	1.69 ± 0.34	1.57 ± 0.33
4.	1.46 ± 0.38	1.83 ± 0.43
5.	1.63 ± 0.36	1.37 ± 0.23
6.	1.80 ± 0.37	1.73 ± 0.60
7.	1.69 ± 0.39	1.72 ± 0.39
8.	1.57 ± 0.26	1.60 ± 0.43
Mean	1.66 ± 0.11	1.65 ± 0.15

TABLE A 4.4

Capillary density in Control Subjects and Diabetic Groups

Control Subjects		Group I		Group II		Group III	
Subject	Capillary Density Cap/mm^2	Subject	Capillary Density Cap/mm^2	Subject	Capillary Density Cap/mm^2	Subject	Capillary Density Cap/mm^2
RM	86.3	GC	44.8	MB	65.9	JM-S	100.2
GR	99.8	PC	62.1	LA	66.2	GS	65.4
AB	38.2	LP	54.6	MG	54.2	PB	97.1
McL	93.8	RS	47.0	GC	71.9	ET	70.1
KF	89.8	JG	64.9	GD	53.5	CB	51.8
PJ	70.1	MS	59.2	BC	29.4	DS	46.3
YvT	72.0	ME	118.9			JM	30.9
BB	70.7	DK	62.6			HH	103.1
PB	59.5	DP	45.4			TB	85.1
JMt	31.1	RC	52.8			KS	69.2
TG	39.7	GS	90.3				
PL	44.8	AR	47.3				
AL	26.7						
MMc	315						
RB	45.0						
MR	52.3						
GD	51.8						
Mean	59.0		62.5		56.8		71.9
SD	23.6		21.7		15.3		22.4

TABLE A 4.5

Endothelial cell profile number, endothelial cell nuclear no., pericyte nuclear no. and endothelial/pericyte ratio in control subjects

Subject	Endothelial Cell Profile No.	Endothelial Cell Nuclear No.	Pericyte Nuclear No.	E/P ratio
R M	6.38	1.38	1.25	1.10
G R	4.36	1.82	1.27	1.43
A B	6.71	3.00	1.57	1.91
McL	4.51	1.43	1.29	1.13
K F	5.25	1.25	1.25	1.00
P J	4.88	1.50	0.75	2.00
Yv T	5.38	1.38	1.13	1.22
B B	6.83	1.50	1.50	1.00
P B	5.17	1.33	1.00	1.33
J Mt	3.80	1.50	1.10	1.36
T G	4.60	1.4	0.40	3.50
P L	4.50	1.10	0.63	1.75
A L	4.50	2.00	1.5	1.33
M Mc	5.22	1.11	0.66	1.68
R B	4.67	1.33	0.78	1.71
M R	5.33	1.67	0.67	2.49
G D	5.63	1.25	0.50	2.50
Mean	5.18	1.53	1.02	1.67
SD	0.86	0.44	0.37	0.66

TABLE A 4.6

Endothelial cell profile number, endothelial cell nuclear no., pericyte
nuclear no. and endothelial/pericyte ratio
in Group I subjects

Subject	Endothelial Cell Profile No.	Endothelial Cell Nuclear No.	Pericyte Nuclear No.	E/P ratio
GC	5.00	1.50	1.00	1.50
PC	5.17	1.50	1.17	1.28
LP	4.83	1.50	1.17	1.28
RS	4.27	1.45	1.18	1.23
JG	4.60	1.40	1.00	1.40
MS	4.71	1.33	1.28	1.04
ME	4.78	1.56	1.10	1.42
DK	4.83	0.83	0.83	1.00
DP	5.43	1.43	1.14	1.25
RC	4.00	1.00	0.88	1.14
GS	4.30	1.10	1.20	0.92
AR	5.77	1.30	0.75	1.73
Mean	4.81	1.33	1.06	1.27
SD	0.50	0.23	0.17	0.23

TABLE A 4.7

Endothelial cell profile no., endothelial cell nuclear no., pericyte
nuclear no. and endothelial/pericyte ratio in Group II subjects

Subject	Endothelial Cell Profile No.	Endothelial Cell Nuclear No.	Pericyte Nuclear No.	E/P ratio
M B	5.33	0.70	1.00	0.70
L A	3.86	1.00	0.86	1.16
M G	5.00	1.67	1.00	1.67
G C	5.00	1.44	0.67	2.15
G D	5.14	1.71	1.14	1.50
B C	6.75	2.00	1.00	2.00
Mean	5.18	1.42	0.95	1.53
SD	0.93	0.49	0.16	0.54

TABLE A 4.8

Endothelial cell profile no., endothelial cell nuclear no., pericyte nuclear
no. and endothelial/pericyte ratio in Group III subjects

Subject	Endothelial Cell Profile No.	Endothelial Cell Nuclear No.	Pericyte Nuclear No.	E/P ratio
JM-S	4.67	1.33	1.00	1.33
GS	4.36	1.10	0.90	1.22
PB	4.63	1.38	0.75	1.84
ET	5.71	2.43	1.14	2.13
CB	5.29	1.85	2.00	0.93
DS	5.8	1.40	1.60	0.88
JM	4.50	1.40	0.70	2.00
HH	4.33	1.17	1.42	0.82
TB	4.5	1.00	0.77	1.29
KS	4.00	0.83	0.50	1.66
Mean	4.78	1.39	1.08	1.41
SD	0.61	0.46	0.47	0.48

TABLE A 4.9

Capillary wall thickness in Control Subjects, Group I, Group II,

and Group III diabetic subjects.

Control Subjects		Group I		Group II		Group III	
Subject	Capillary Wall Thickness μm	Subject	Capillary Wall Thickness μm	Subject	Capillary Wall Thickness μm	Subject	Capillary Wall Thickness μm
RM	6.60	GC	5.31	MB	4.84	JM-S	6.60
GR	5.29	PC	6.64	LA	4.73	GS	5.67
AB	6.11	LP	6.10	MG	6.20	PB	4.94
McL	4.68	RS	5.69	GC	5.61	ET	7.18
KF	5.89	JG	3.23	GD	4.75	CB	8.34
PJ	3.82	MS	5.30	BC	7.05	DS	6.83
YvT	4.55	ME	3.30			JM	5.94
BB	5.43	DK	5.16			HH	5.63
PB	5.78	DP	4.79			TB	5.73
JMt	4.74	RC	4.03			KS	5.47
TG	5.84	GS	4.99				
PL	4.94	AR	6.32				
AL	6.02						
MMc	3.47						
RB	5.09						
MR	6.28						
GD	5.68						
Mean	5.31		5.07		5.53		6.23
SD	0.86		1.10		0.95		1.00

TABLE A 4.10

Endothelial cell thickness in Control subjects, Group I, Group II,

and Group III diabetic subjects.

Control Subjects		Group I		Group II		Group III	
Subject	Endo. Cell Thickness μm	Subject	Endo.Cell Thickness μm	Subject	Endo.Cell Thickness μm	Subject	Endo.Cell Thickness μm
RM	2.29	GC	2.35	MB	1.23	JM-S	1.42
GR	2.12	PC	1.93	LA	1.55	GS	1.99
AB	2.01	LP	1.97	MG	2.10	PB	1.86
McL	2.16	RS	2.15	GC	2.10	ET	2.42
KF	2.12	JG	1.91	GD	1.84	CB	2.74
PJ	1.45	MS	2.06	BC	2.45	DS	2.11
YvT	1.63	ME	1.14			JM	1.92
BB	1.88	DK	2.16			HH	1.68
PB	2.18	DP	1.78			TB	2.20
JMt	2.56	RC	1.33			KS	1.76
TG	2.55	GS	1.46				
PL	2.33	AR	1.77				
AL	3.88						
MMc	2.05						
RB	1.90						
MR	2.61						
GD	2.44						
Mean	2.25		1.83		1.88		2.01
SD	0.53		0.36		0.44		0.38

TABLE A 5.1

Reproducibility data for the percentage change in blood flow during the 4th minute of dependency with the foot lowered 50 cm below the heart level. Six subjects have been studied on 2 separate occasions. Data for horizontal and dependent flow in individual subjects is shown. Between subjects CV for horizontal, dependent and percentage change in flow has been calculated, as has the within subject CV for the percentage change.

	Study 1			Study 2		
	Horizontal Flow (V)	Dependent Flow (V)	Percent change	Horizontal Flow (V)	Dependent Flow (V)	Percent change
1	69	20.0	71.0	92	18.0	80.4
2	87	7.0	92.0	98	6.5	93.4
3	118	9.8	91.7	164	45.2	72.4
4	117	7.5	93.6	120	10.0	91.7
5	169	36.0	78.7	183	29.5	83.9
6	300	60.0	80.0	196	52.5	73.2
Mean	143.3	23.4	84.5	142.2	27.0	82.5
SD	83.9	21.1	9.2	44.7	18.9	8.9
Between subject CV %	58.5	111.0	10.9	31.4	70.0	10.8

Within subject CV for the precentage change in blood flow on dependency based on repeat studies = 10.4 %

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CLINICAL RESEARCH

Impaired microvascular hyperaemic response to minor skin trauma in type I diabetes

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Abstract

The microvascular response of foot skin to minor thermal injury and the skin of the anterior abdominal wall to injury from a needle was assessed by laser Doppler flowmetry in 23 patients with type I diabetes and 21 healthy control subjects. After minor thermal injury mean (SD) maximum skin blood flow was significantly lower in the diabetic group than the control group (0·53 (0·11) v 0·72 (0·10) V, in arbitrary units of flow, respectively, p<0·001) and was negatively correlated with the duration of diabetes (r= −0·60; p<0·01). After needle injury a similar pattern of impairment was seen, the peak flow value recorded being significantly lower in the diabetic group than the control group (0·28 (0·10) v 0·41 (0·09) V, respectively; p<0·001) and also negatively correlated with the duration of diabetes (r= −0·61; p<0·01). There was a significant relation between the response obtained at the two sites of injury in the diabetic group (r= +0·72, p<0·001) but not in the control group. The impairment in response was not related to diabetic control and was not explicable in terms of a reduction in superficial skin capillary density.

The inability of the diabetic skin microvasculature to respond normally to injury may be an important factor in the development of foot ulceration that often follows minor trauma.

Introduction

The disabling foot complications of diabetes are often precipitated by minor mechanical or thermal injury, suggesting disturbance of the healing process. An important component of the normal reaction to injury is hyperaemia, and, although measurement of blood flow after hyperaemic stimuli, such as release of arterial occlusion and exercise, suggests a limitation of maximum perfusion in diabetics,[1-3] no study has specifically examined the skin microvascular hyperaemic response to injury. The development of the laser Doppler flowmeter allows repeated non-invasive study of superficial microvascular flow in small areas of the skin and thus may be used to investigate the hyperaemic response to minor injury.

In this study laser Doppler flowmetry was applied to determine whether diabetics without large vessel disease showed evidence of limited microvascular perfusion in response to two distinct noxious stimuli in different areas of the skin. In addition, as the flowmeter measures volumetric flow (quantity and velocity of moving red blood cells)[4,5] the number of superficial skin capillary loops/mm² was estimated by television microscopy to find out whether any observed differences in flow were related to the density of the capillaries.

Patients and methods

A total of 23 patients (13 men, 10 women) with type I diabetes and 21 healthy control subjects (11 men, 10 women) were studied. The diabetic patients were volunteers randomly selected from the Charing Cross diabetic clinic. Controls comprised academic and technical staff from the medical school. Mean (SD) duration of diabetes was 13·3 (8·8) years (range 1-29 years), and mean age of the diabetic group was 30·9 (6·4) years (18-45) compared with 29·7 (5·2) years (20-40) for the control group. Smokers and hypertensive subjects were excluded from the study, and none of the subjects had a history of complications of the foot. All subjects had normal ankle reflexes. Vibration sensory thresholds at the big toes, measured with a biothesiometer (Biomedical Instrument Company, Ohio), were within the normal centiles for age assessed with centile charts.[6] Severe large vessel disease was excluded by normal ankle to arm systolic blood pressure ratios.[7] Seven patients had independent ophthalmological evidence of minimal background retinopathy, but no patient had persistent proteinuria. Patients with a history of ketosis in the past six months were not selected for study.

Diabetic control was assessed by measuring blood glucose concentration at the time of the study and glycosylated haemoglobin concentration. To examine the short term effect of change in diabetic control in a given subject 10 diabetics who had glucose concentrations <10 mmol/l (<180 mg/100 ml) when first studied were restudied after omitting to take their insulin before breakfast (mean (SD) blood glucose at the time of study 17·6 (3·6) mmol/l (317 (65) mg/100 ml)).

All studies were conducted after a minimum acclimatisation period of 30 minutes in a temperature controlled environment (mean (SD) 22 (1)°C) in quiet surroundings. Subjects were rested on a specially constructed couch that allowed passive lowering of either leg.

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MEASUREMENT OF BLOOD FLOW

Blood flow was measured with a laser Doppler flowmeter (Periflux model PFIC, Mk II, Perimed Limited, Sweden). Red laser light produced by the instrument is conducted to the skin surface by a single fibre optic light guide, held in place on the skin by a plastic probe holder. The backscattered light, some of which is altered in frequency by red cells moving in the microvasculature, is returned to the instrument by fibre optic light guides contained within the same probe as the incident light guide. Dual efferent light guides are used to reduce "noise" resulting from variations in the light signal. The frequency shift of the backscattered light is detected by the instrument, which produces a voltage signal directly proportional to the quantity of blood flow (velocity and number of red cells) in the microvasculature of superficial skin.[4][5] In practice, the pencil like probe is placed in the probe holder affixed to the surface of the skin with double sided sticky tape, and blood flow is recorded on a chart recorder in arbitrary units of volts.

INJECTION TRAUMA

Blood flow measurements were made from the skin of the upper abdomen just below the costal margin, 8-10 cm lateral to the midline (about T9 dermatome). Flow was recorded from both right and left sides and results given as the mean of the two sites. After the resting flow had been recorded for five minutes the laser probe was removed from its holder and injection trauma produced by inserting a 25 gauge needle into the skin in the exact centre of the probe holder to a depth of 0·5 cm, previously set by a needle guard.
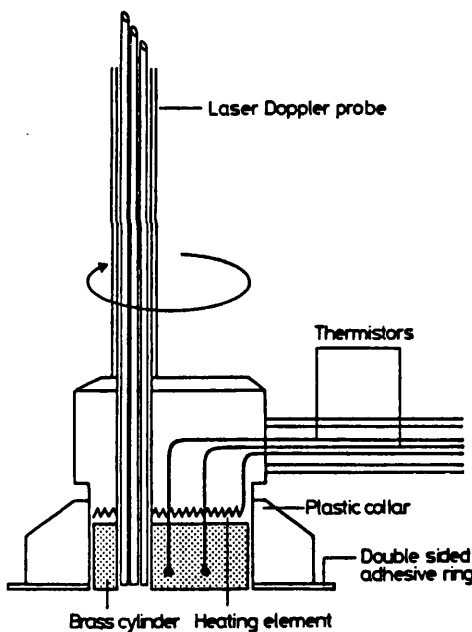
FIG 1—Cross sectional diagram of heating probe with modified laser Doppler probe in place. Rotation of heating probe within plastic collar is indicated by arrow.

Preliminary studies showed that a predictable peak in laser Doppler flow that in normal subjects was roughly 20 times the resting flow value developed within 15 minutes of the injection. Thereafter flow values rapidly fell to five times rest flow values over the first two hours, gradually returning to normal over the next two or three days. Thus to derive peak flow after needle injection the following measurement procedure was followed: the Doppler probe was immediately replaced and flow recorded continuously for the first 15 minutes and thereafter for five minute periods at 30 and 60 minutes. Fifteen minutes after the first needle prick had been given the manoeuvre was repeated on the opposite side of the abdomen. If any bleeding occurred the results were considered to be invalid, and a new site was chosen. The technique was reproducible with a coefficient of variation of 8·6% determined from nine duplicate pairs.

THERMAL INJURY

Mild thermal injury was produced by heating the skin on the dorsum of the foot to 44°C for 30 minutes. The heating probe supplied by the

manufacturers was inadequate as it did not contain a feedback thermostat, the heat distribution was uneven, and the central area over which blood flow was to be measured was not directly heated; thus a heated probe holder was specially constructed. This consisted of a cylindrical brass core heated by a thermostatically controlled element (fig 1). The circular end face (diameter 0·9 cm) was held in direct contact with the area of the skin to be heated by being placed in a plastic collar previously affixed to the skin by a double sided adhesive ring. The heated probe holder had a small diameter (2·4 mm) eccentrically placed channel through which a modified fine bore laser Doppler probe tip could be inserted. Rotation of the heated probe holder about the plastic collar allowed blood flow to be measured from an area that had been directly heated immediately before rotation. In this way the mean flow for nine separate areas (40° rotations) could be determined, which was more reproducible than a single measurement (coefficient of variation was 7·6% when determined from duplicate experiments in nine subjects, compared with a mean coefficient of variation of 16·3% for repeat single point measurements).

Blood flow was recorded from the heated skin after the foot had been lowered passively 50 cm below the level of the heart. Local heating to 44°C removes postural regulation of blood flow, which normally results in a reduction of blood flow on dependency. By lowering the leg and thereby increasing hydrostatic pressure and passively dilating the blood vessels, we found that skin blood flow was maximised.
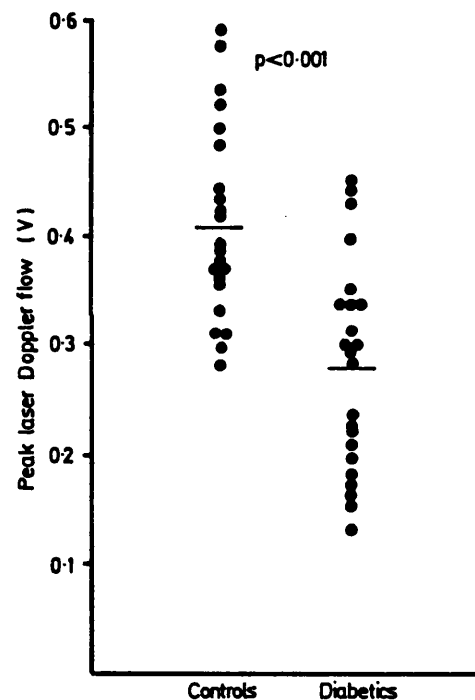

FIG 2—Injection trauma: peak laser Doppler blood flow response in diabetics and controls. Bars represent means.

NUMBER OF CAPILLARIES

The apexes of superficial dermal capillary loops were visualised with a television-microscope system as previously described.[1] Using this system, we recorded on video tape images from an area of skin on the dorsum of the foot, which were then analysed by two independent observers to derive the mean number of capillaries/mm² of the skin surface. The number of capillaries were counted in 16 of the diabetics and 14 control subjects from the original groups.

STATISTICAL METHODS

The results are expressed as mean (SD). Differences between the groups were analysed with the Mann-Whitney U test.

Results

Injection trauma—Mean (SD) resting blood flow was not significantly different in the two groups (diabetics 0·018 (0·006)V; controls 0·017 (0·006)

V). After needle injury in both groups blood flow showed an immediate and substantial increase, peaking 10-15 minutes later, followed by a fall towards the flow at rest, with a similar time course in the two groups. Mean peak blood flow was significantly lower in the diabetic group than the control group (0·41 (0·09) v 0·28 (0·10) V, respectively; p<0·001) (fig 2). Peak blood flow did not correlate with age in either group but was inversely related to the duration of diabetes (r= −0·61; p<0·01) (fig 3).
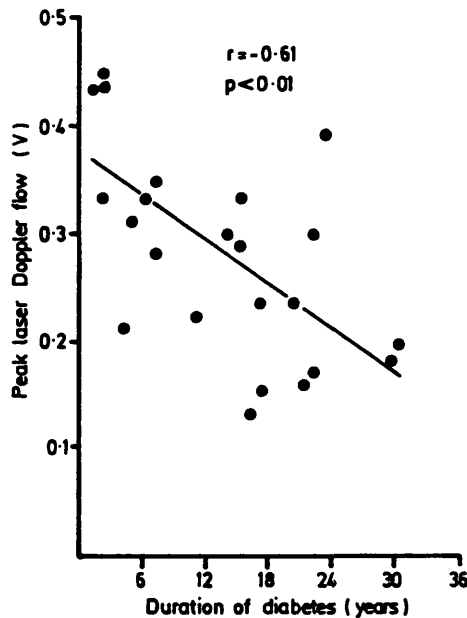

FIG 3—Relation between peak blood flow response to injection trauma and duration of diabetes. Regression line is indicated.
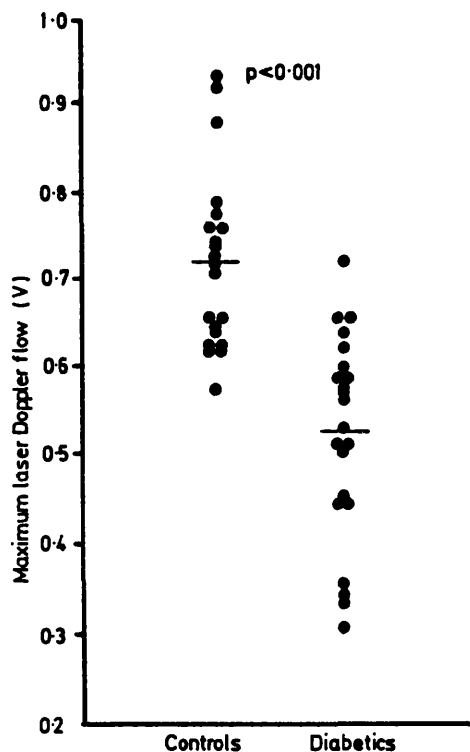
FIG 4—Mild thermal injury: maximum laser Doppler blood flow in diabetics and controls. Bars represent means.

Heat trauma—Mean maximum skin blood flow was significantly lower in the diabetic group than the control group (0·72 (0·10) v 0·53 (0·11) V, respectively; p<0·001) (fig 4). As with the response to injection trauma, maximum skin blood flow did not correlate with age in either group but was inversely related to the duration of diabetes (r= −0·60; p<0·01) (fig 5).

Maximum blood flow response to thermal injury showed a significant correlation with the peak blood flow after injection trauma in the diabetic group (r=0·72; p<0·001) (fig 6a) but not in the control group (fig 6b).

Capillary numbers—The mean number of skin capillaries was not significantly different in the two groups (diabetics 52·7 (8·7); controls 50·7 (5·7)). Maximum blood flow in the foot did not correlate with the number of capillaries in either group.

Influence of diabetic control—In the diabetic group skin blood flow response to both injection trauma and thermal injury did not correlate with blood glucose concentrations at the time of the study or glycosylated haemoglobin concentrations. Withdrawal of insulin and hyperglycaemia (mean glucose concentration 17·6 (3·6) mmol/l (317 (65) mg/100 ml)) had no significant effect on either the response to injection trauma (0·23 (0·07) controlled v 0·24 (0·07) uncontrolled; NS) or the response to thermal injury (0·48 (0·13) controlled v 0·47 (0·11) uncontrolled; NS).
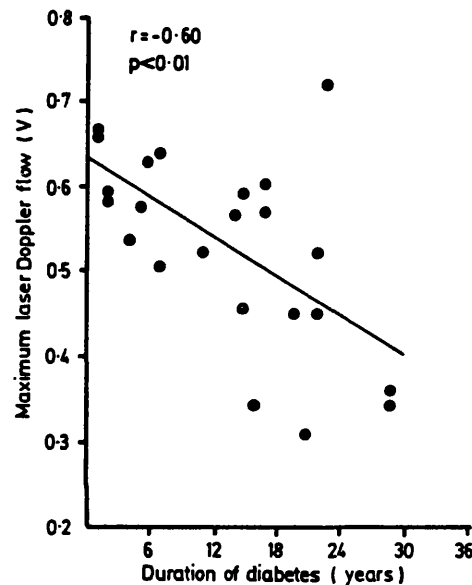

FIG 5—Relation between maximum blood flow values in response to thermal injury and duration of diabetes. Regression line is indicated.

Discussion

This is the first study to show an impaired skin microvascular vasodilator response to local trauma in patients with diabetes. Although impaired vasodilatation of the peripheral circulation in diabetes has been inferred from several previous studies, interpretation has been difficult owing to the methods used. There are theoretical problems in deriving blood flow values from xenon clearance studies,[9] and in most studies of diabetics in which this technique has been used muscle rather than skin blood flow has been examined. In some studies venous occlusion plethysmography has been used, which when applied to the calf measures predominantly muscle arterial inflow[10] and even when applied to the digit or foot measures total tissue perfusion rather than superficial skin blood flow.[11][12] In addition, the method for provoking hyperaemia in previous studies has been indirect—for example, body heating[10]— relied on exercise or vascular occlusion,[1-3] or used vasodilators,[10][12-14] none of which may be guaranteed to reflect the mechanisms that occur naturally in hyperaemia of injury. The only technique that uses direct thermal injury is that of transcutaneous oxygen tension measurement, but many factors other than limitation of microvascular perfusion influence this measurement.[15] Thus increased skin thickness, reduced oxygen diffusion, increased oxygen affinity of haemoglobin, or increased skin metabolism may explain the low values observed in diabetics.[16]

Laser Doppler flowmetry is a completely non-invasive method and may be used to measure skin microvascular blood flow at the point of injury. Synchronous assessment of skin microvascular
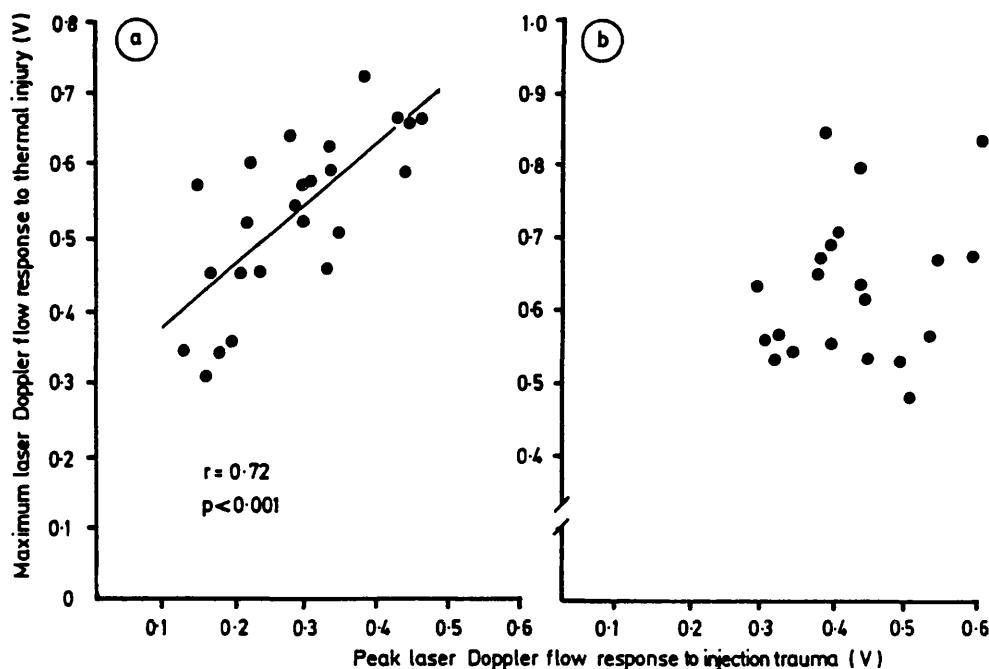
FIG 6—Relation between maximum laser Doppler blood flow response to heat trauma and peak laser Doppler blood flow response to injection trauma in the diabetic group (a) and the control group (b). Regression lines are indicated.

blood flow with laser Doppler flowmetry and direct capillary flow measurements with television microscopy suggests broad comparability between these two methods.[17]

The reduction in hyperaemic response to two different types of injury cannot be explained by a reduction in capillary numbers that is related to duration of diabetes, as mean density of capillaries in the two groups was similar and there was no correlation between the blood flow value and the number of capillaries on an individual basis. The finding of the correlation in impaired hyperaemic responses to injury in widely separated sites on the skin suggests that this microvascular abnormality may be a widespread feature in diabetics.

The impaired vasodilator response observed may represent a failure of release of local vasoactive mediators. This suggestion is supported by the observation that the response of the flare of histamine is reduced in diabetics[18] and also by the recent finding that substance P, a potent neurogenic vasodilator, is depleted in diabetic nerves.[19] An alternative explanation for the limited vasodilatation may be an impaired microvascular response to released vasoactive mediators, perhaps having a structural basis in the microvascular wall thickening and reduced distensibility that characterises long standing diabetes.[20]

The laser Doppler technique appears to be ideally suited for the study of the microvascular response to vasoactive mediators and their blockade. The clarification of the underlying mechanism may be important for improving the cutaneous microvascular response to the injury initiating many diabetic foot complications. The lack of relation of the response with retrospective and current diabetic control emphasises the complexity of the abnormality described and the need for further study.

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Blood flow in the skin of the foot related to posture in diabetes mellitus

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Abstract

Normal healthy subjects show a reflex rise in precapillary resistance in the skin of the foot when they rise from lying to standing. To investigate the integrity of this reflex in patients with diabetes mellitus blood flow in the plantar region of the big toe was measured, using a laser Doppler flowmeter. The responses of diabetic patients with and without peripheral sensory neuropathy and healthy control subjects matched for age and sex were studied, with the foot at heart level and the foot passively lowered to 50 cm below the heart.

In normal subjects mean blood flow recorded during the third to fourth minute of dependency fell to 18·1 (SD 11·9)% of the preceding resting flow determined with the foot at heart level. In the diabetic patients without neuropathy blood flow fell to 28·9 (18·6)% of the preceding resting flow. In the diabetic patients with neuropathy blood flow fell to 53·5 (23·7)% of the preceding resting flow, which was significantly different from the value achieved by the diabetics without neuropathy (p<0·02) and the healthy controls (p<0·002). Six normal subjects were indirectly heated to release sympathetic tone and achieve the same mean skin temperature of the foot as the diabetic patients with neuropathy, and blood flow fell to 38·7 (24·3)% of the preceding resting flow, a value not significantly different from the response seen in the patients with neuropathy.

These findings suggest that the postural control of blood flow in the foot is disturbed in patients with diabetic neuropathy, and this disturbance is compatible with a loss of sympathetic vascular tone. The resultant hyperperfusion on dependency may account for the oedema seen in some patients with neuropathy and may also act as a stimulus for the thickening of capillary basement membranes.

Introduction

The precapillary resistance in the skin of the foot rises on standing, thereby limiting the rise in capillary pressure resulting from the vertical column of blood between the heart and the foot.[1] Evidence suggests that this vasoconstriction is mediated by a sympathetic axon reflex.[2] We examined this reflex in patients with diabetes for
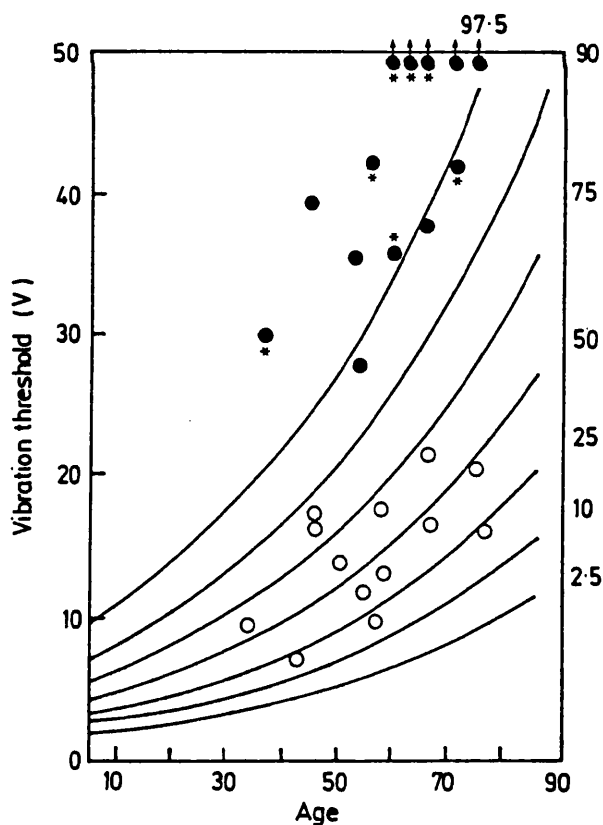

FIG 1—Centile chart of vibration thresholds in the big toe.[6] Open and closed circles represent values in diabetics in the present study without and with neuropathy, respectively. Asterisks indicate patients with autonomic neuropathy.

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two reasons: firstly, oedema may occur in patients with diabetic neuropathy,[3][4] which might represent failure to limit the rise in capillary pressure on dependency; secondly, thickening of basement membranes, the histological hallmark of disease of the small vessels in patients with diabetes, is promoted by raised capillary pressure.[5] The reflex change in microcirculatory flow in the skin of the foot on dependency may be studied with a new instrument, the laser Doppler flowmeter. This technique is non-invasive and, unlike existing techniques, does not entail local heating of skin,

breathing, and the Valsalva manoeuvre.[7] Diabetics without peripheral neuropathy had no evidence of autonomic dysfunction or history of ulceration of the foot. In all subjects foot pulses were palpable and ratios of ankle to arm systolic pressure (measured by Doppler ultrasound) were normal. The table gives details of the subjects.

Measurement of blood flow—Blood flow was measured using a laser Doppler flowmeter (Periflux, model PF1C, MkVII, Perimed Limited, Sweden). This instrument produces a voltage signal directly proportional to microvascular blood flow in superficial skin vessels.[8] Blood flow was measured in the plantar aspect of the left big toe, except in subjects who had

Details of subjects in the three groups. (Values are means (SD))

	Age (years)	M:F	Duration of diabetes (years)	Plasma glucose (mmol/l)	Haemoglobin A_1 (%)
Normal controls	59·5 (13·8)	7:6			
Diabetics without neuropathy	55·2 (12·5)	7:6	15·5 (8·1)	9·5 (4·2)	9·1 (1·5)*
Diabetics with neuropathy	59·5 (11·6)	9:4	12·8 (11·3)	9·2 (2·7)	8·9 (1·1)

*Normal range: 4·0-7·5%.

Conversion: SI to traditional units—Plasma glucose: 1 mmol/l ≈ 18 mg/100 ml.

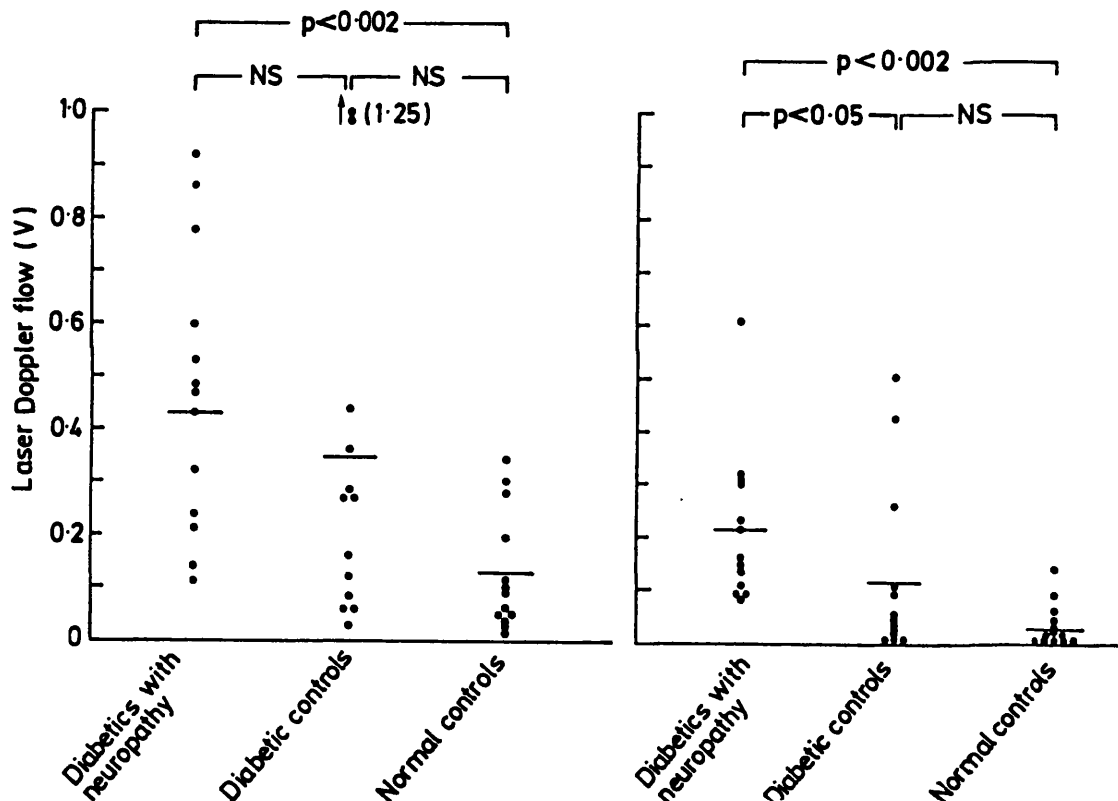FIG 2—Blood flow in the toe recorded by laser Doppler technique with toe at heart level (left) and 50 cm below heart level (right). (Horizontal bars indicate means.)

injection trauma, or venous occlusion, all of which may disturb local vasomotor reflexes.

Patients and methods

We studied three groups of subjects: diabetics with peripheral sensory neuropathy, diabetics without neuropathy, and healthy controls matched for age and sex. Patients were classified as having neuropathy if ankle jerks were absent and vibration sensory threshold, determined with a biothesiometer (Biomedical Instrument Co, Ohio), was above the 90th centile for age when compared with published centile charts for normal subjects of varying ages (fig 1).[6] Seven of the subjects with neuropathy showed evidence of cardiac autonomic dysfunction, eight had a history of ulceration of the foot, and four had mild, unexplained peripheral oedema. Autonomic neuropathy was assessed by measuring the response of the heart rate to standing, deep

ulceration of the foot or who had had the left foot amputated, when the other foot was used. Skin temperature was measured continuously using a Comark electronic thermometer type 1625 and copper-constantan thermocouple placed within 0·5 cm of the laser Doppler probe. Tests of blood flow were conducted in a room where the temperature was controlled (21-23°C). The subject lay recumbent on a specially constructed couch, which was hinged to permit passive lowering of either leg from the hip. Resting blood flow was recorded over two minutes after 30 minutes of acclimatisation. The leg was then lowered so that the toe was placed 50 cm below the midaxillary line, and flow was recorded between the third and fourth minute of dependency, when blood flow is stable.

Effect of skin temperature on blood flow during dependency—To examine the effect of different temperatures of the skin of the toes on the vascular response to dependency we studied a separate group of six normal subjects (mean (SD) age 49·7 (21·4) years) under the same conditions as described above, and again after indirect heating (a heat blanket pressed to the

abdominal wall) to release central sympathetic drive and raise the temperature of peripheral skin.

Statistical methods—The results are expressed as means (SD). Differences between the groups were analysed using the Mann-Whitney U test.

Results

Skin temperature—Skin temperatures measured after acclimatisation in the supine position were significantly higher in the patients with neuropathy (32·2 (2·0)°C) than in the patients without neuropathy (28·7 (3·1)°C, p<0·002) and the normal subjects (27·7 (3·3), p<0·002). There was no significant difference in values between the diabetics without neuropathy and the normal subjects.

Resting flow—Figure 2 shows resting flow values. Mean resting flow in the diabetics with neuropathy was significantly higher than that in the normal subjects (0·44 (0·23) v 0·13 (0·11) V (arbitrary units of flow), p<0·02). Mean resting flow in diabetics without neuropathy (0·34 (0·37) V) was not significantly different from that in either the control subjects or the diabetics with neuropathy.

Blood flow during dependency—Figure 2 shows measurements of flow during the third to fourth minute of dependency. Mean flow on dependency was significantly higher in diabetics with neuropathy (0·22 (0·15) V) than in the normal subjects (0·03 (0·04) V, p<0·002) or the diabetics without neuropathy (0·12 (0·17) V, p<0·05).

Percentage change in blood flow on dependency—In the normal subjects mean blood flow during the third to fourth minute of dependency fell to 18·1 (11·9)% of the original resting flow (fig 3). In the diabetic patients without
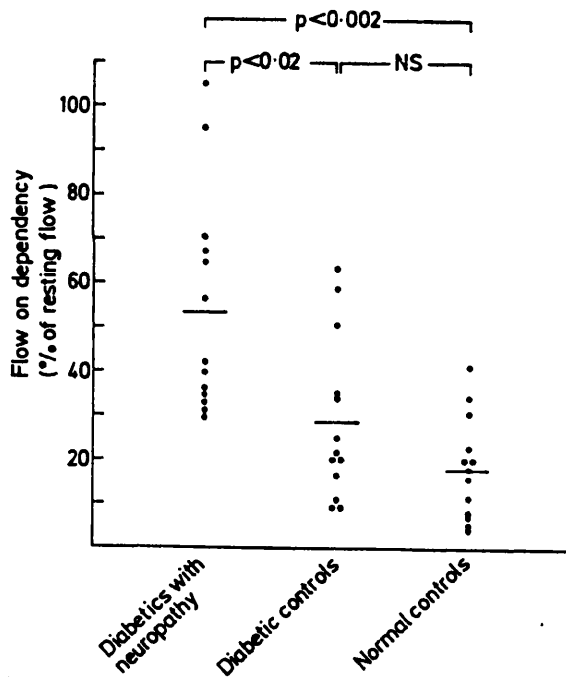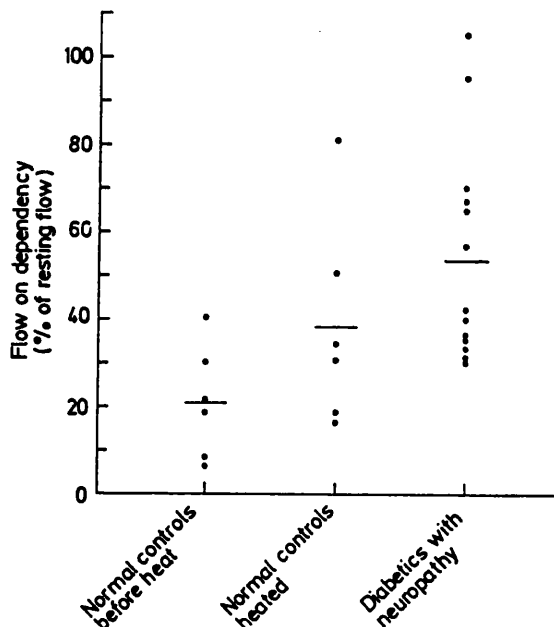
FIG 4—Vascular response to dependency in normal controls before and after skin temperature was raised by indirect body heating compared with response in group with neuropathy.

FIG 3—Flow on dependency expressed as percentage of preceding rest flow.

neuropathy blood flow fell to 28·9 (18·6)% of the original resting flow, but this was not significantly different from that achieved by normal controls. In the diabetic patients with neuropathy blood flow fell to 53·5 (23·7)% of the original resting flow, which was significantly different from the value achieved by the diabetics without neuropathy (p<0·02) and the healthy controls (p<0·002).

Effect of skin temperature on blood flow during dependency—Figure 4 summarises the percentage change in blood flow during the third to fourth minute of dependency in the six normal subjects before and after indirect heating. The values in the diabetics with neuropathy are plotted on the same graph. Before indirect heating blood flow fell during dependency to 20·9 (13·0)% of the original resting flow. After indirect heating the fall in blood flow was less in all six subjects, with a mean value of 38·7 (24·3)% of the original resting flow. The skin temperature of the normal subjects after indirect heating was not significantly different from that found in the diabetic patients with neuropathy (32·4 (2·4) v 32·2 (2·0)°C). Vascular

response to dependency in the normal subjects after heating was not significantly different from that observed in the diabetic patients with neuropathy (38·7 (24·3) v 53·5 (23·7)%).

Discussion

Most measurements of blood flow in the feet of diabetic patients have been taken with the foot at heart level. This is clearly unrepresentative of the conditions to which the peripheral circulation must adapt for most of the time. Lowering the foot below heart level elicits the venoarteriolar reflex, which results in an increase in precapillary resistance.[12] The non-invasive technique of laser Doppler flowmetry, though it does not provide an absolute measurement of volume flow, is well suited to determining such reflex changes in local flow as it does not damage the skin or depend on venous occlusion or local heating. Comparative studies with television microscopy, which measures flow velocity in single capillaries, suggest that the laser Doppler technique detects flow in subcapillary plexuses and shunts as well as the capillary loops, but the reflex responses determined by the two techniques are broadly comparable.[9]

The finding that skin temperature was raised in the group with neuropathy is in keeping with previous observations and presumably reflects the higher resting flow observed in this group.[10 11] This increased flow is exaggerated on dependency, the value attained being seven times that in healthy control subjects, compared with a fourfold increase when the foot was at heart level. This exaggeration indicates the failure of the venoarteriolar reflex. Although the mean fall in flow in the diabetics without neuropathy was less pronounced than that in the healthy controls, this value was distorted by three or four diabetics with particularly impaired responses. These patients might conceivably have had sympathetic neuropathy without detectable sensory and cardiac autonomic neuropathy, a hypothesis supported by the observation that the sympathetic nervous system may be more susceptible to early impairment in patients with diabetes.[12]

Indirect heating causes release of sympathetic tone and opens up peripheral arteriovenous anastomoses to promote the dissipation of heat. Our study showed that the response of blood flow to dependency in the skin of diabetic patients with neuropathy is similar to that seen in vasodilated normal skin, suggesting that the reflex failure observed may represent an abolition of sympathetic tone with persistence of arteriovenous shunting on dependency.

Considerable evidence exists to support the concept of increased shunting in the foot of diabetics,[10] but the exaggeration of this abnormality in the dependent position is a new observation.

Several inferences may be drawn from this study. Failure of precapillary vasoconstriction on dependency will result in the capillary bed being exposed to a greater hydrostatic load. This may account for the development of peripheral oedema seen in patients with neuropathy. Furthermore, chronic exposure to capillary hypertension may act as a localising stimulus to thickening of capillary basement membranes. Vracko described increasing thickness of capillary basement membrane from thigh to foot, which is particularly pronounced in patients with diabetes.[13] Both these inferences require direct experimentation to substantiate them. Preliminary results from our laboratory suggest that capillary pressure is indeed higher in the dependent foot of diabetics compared with normal subjects.

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