DOCTOR OF PHILOSOPHY

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THE DEVELOPMENT AND APPLICATION OF A PRESSURE DELIVERY PROCEDURE TO SIMULTANEOUSLY ASSESS PLANTAR PRESSURE AND ENDOTHELIAL FUNCTION IN THE DIABETIC FOOT

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
AB	aluminium bar
ABW	adjustable balance weight
ACh	acetylcholine
AF	atrial fibrillation
AGEs	advanced glycation end products
AGI	antioxidant vascular protectant
ANOVA	analysis of variance
ATPase	sodium-potassium adenosine triphosphatase
AU	arbitrary units
AVA	arterio-venous anastomoses
BH4	tetrahydrobiopterin
Ca	calcium
cAMP	cyclic adenosine mono-phosphate
CAV1	caveolin-1
cGMP	cyclic guanine mono-phosphate
CGRP	calcitonin gene-related peptide
CI	confidence interval
cm^2	square centimetre
COX	cyclooxygenase
DAG	diacylglycerol
d/dig	digit
DP2	Doppler probe
EDHF	endothelium derived hyperpolarising factor
EMLA	topical anaesthetic cream containing lidocaine and prilocaine
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
ET-1	endothelin - 1
F	F ratio
FAD	NOS activity assay ingredient
FAU system	junction associated actin filament system
FCP	flow cessation pressure
FLPI	full-field laser perfusion imaging
FMD	flow-mediated dilatation
FMN	flavin mononucleotide
g	grams
hsCRP	high sensitivity C- reactive protein
ICC	interclass correlation coefficient
IGT	impaired glucose tolerance
IL	interleukin
iNOS	inducible nitric oxide synthase
K	potassium
kDa	kilodalton (atomic mass unit)
kg/cm^2	kilograms per square centimetre
kPa	kilopascals
LDF	laser Doppler flowmetry
LDI	laser Doppler imaging

LDLs	low density lipoproteins
L-NMMA	Nitric oxide synthase inhibitor N^G-Monomethyl-L-Arginine
LSCI	laser speckle contrast imaging
µA	microamps
MEGJs	myoendothelial gap junctions
mm	millimetres
mmHg	millimetres of mercury
MPF	maximum peak blood flux
MPJ	metatarsophalangeal joint
MWU	Mann-Whitney U test
n	sample size
Na	sodium
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate reduced form
Ncm^2	Newtons per square centimetre
NO	nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal nitric oxide synthase
O_2	oxygen
p	probability, significance
PAI -1	plasminogen activator inhibitor 1
PAOD	peripheral arterial occlusive disease
PG12	prostacyclin
PIV	pressure induced vasodilation
PKC	protein kinase C
PORH	post occlusive reactive hyperaemia
PSH	plasma thiol
PU	perfusion units
r	Spearman's correlation coefficient
R^2	coefficient of determination
ROS	reactive oxygen species
SBF	skin blood cell flux
S.D	standard deviation
secs	seconds
SCI-DC	Scottish Care Information-Diabetes Collaboration
sICAM	soluble intercellular adhesion molecule
SKBF	skin blood flow
SNP	sodium nitroprusside
sVCAM	soluble vascular cell adhesion molecule
$TcPCO_2$	transcutaneous cardon dioxide tension
$TcPO_2$	transcutaneous oxygen tension
TNF-α	tumour necrosis factor-α
T2DM	Type 2 diabetes mellitus
vWF	von Willebrand factor

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DECLARATION

I declare that I am the author of this thesis and that all references cited have been consulted. This thesis is a record of the work undertaken by myself, and has not been previously submitted for a higher degree.

Lynne Flynn Date

I certify that the work reported in this thesis has been performed by Lynne Flynn and that during the period of study she has fulfilled the conditions of the ordinances and regulations governing the degree of PhD at the University of Dundee.

Prof. Rami Abboud Date

Dr Faisel Khan Date

ABSTRACT

Background: Diabetes is a metabolic disorder characterised by chronic hyperglycaemia, with Type 2 diabetes being the most common form. Over time, chronic lower limb complications such as peripheral neuropathy and arterial disease can develop. Approximately one in twenty people with diabetes will then go on to develop a foot ulcer every year, and more than one in ten ulcers will progress to leg or foot amputation. Investigating the impact of plantar pressure on peripheral blood flow, and in particular endothelial dysfunction is an important element in increasing our understanding of the mechanisms which contribute to foot ulcer formation. However, to date no studies have simultaneously investigated the interaction of elevated plantar pressure and endothelial dysfunction.

Aim of the study: To develop and validate a pressure delivery device and utilise this equipment to investigate the impact of plantar pressure on endothelial dysfunction of the superficial blood vessels supplying the plantar aspect of the forefoot. To devise and utilise protocols for measuring blood flow, both during post-occlusive reactive hyperaemia (PORH), and when the vessels are stimulated to dilate with an endothelium-dependent and an endothelium-independent vasodilator under 50% and 100% of simulated walking plantar pressure on the feet of subjects with diabetes mellitus.

Methods: A pressure delivery system was developed and tested for repeatability in normal healthy volunteers. The system consisted of a stainless steel spring housed in a metal casing to allow unimpeded movement of the spring. Iontophoresis of solutions

with different possible vehicles were investigated, and a suitable protocol for the delivery of an endothelium-dependent vasodilator, acetylcholine (ACh) and an endothelium-independent vasodilator, sodium nitroprusside (SNP) utilising a methylcellulose thickening agent was developed. Laser speckle contrast imaging (LSCI) in conjunction with PORH was tested for suitability of use on the plantar aspect of the foot and a comparison with laser Doppler flowmetry (LDF) carried out for the 3rd metatarsophalangeal joint (MPJ) area. Endothelial dysfunction was assessed with vasodilation via iontophoresis of ACh and SNP with the simultaneous delivery of 50% followed by 100% of simulated walking pressure applied by the developed pressure delivery system on the 3rd MPJ.

Results: The required equipment and protocols were successfully developed which would allow the simultaneous investigation of the interaction of plantar pressure and endothelial dysfunction. On utilisation of the equipment, the post occlusive peak flux was significantly higher in the subjects with Type 2 diabetes than in the control subjects in all areas under study except the 1st toe. A significant positive association was found between the utilisation of LSCI and LDF measurements obtained at the plantar aspect of the 3rd MPJ. With vasodilation of the superficial vessels on the plantar aspect of the 3rd MPJ using ACh, the main impact of reduction of blood flux occurred when only 50% of normal walking pressure was applied to the area. When delivering the vasodilator SNP with iontophoresis to the same area of the foot, increase in pressure was found to have a greater impact of reduction of blood flux on the group with Type 2 diabetes than the control group.

Conclusions: Investigation of the interaction between peak plantar pressure and endothelial dysfunction was achieved with the utilisation of the equipment and protocols developed. With PORH the subjects with Type 2 diabetes displayed a significantly higher post occlusive peak flux in comparison to the control group. A strong, significant positive association was found between the LDF for resting flux under the 3rd MPJ and LSCI, thus, LSCI was found to be suitable for assessing endothelial function in superficial vessels. Reduction in blood flux when pressure was exerted under vasodilation with ACh occurred with 50% of normal walking pressure. Increase in pressure with SNP delivery was found to have a greater impact on blood flux for the subjects with Type 2 diabetes than the control group i.e. the group displayed a greater reduction in blood flux with simulated normal walking pressure delivery.

Chapter One

Introduction and aims of the thesis

1 INTRODUCTION

1.1 BACKGROUND TO THE RESEARCH

Diabetes mellitus has been defined as a metabolic disorder with multiple aetiology characterised by chronic hyperglycaemia and manifests with carbohydrate, fat and protein disturbances resulting in insulin secretion and/or defects. Type 2 diabetes is the most common form of diabetes and is characterised by problems with insulin action and secretion (WHO 2011). A recent estimation of worldwide prevalence for 2011 indicated that there were 366 million cases of diabetes, with an expectation of this rising to 552 million people by 2030. In the UK there are currently 2.9 million people who have been diagnosed with diabetes, with an estimation of around 850,000 people undiagnosed. In Scotland in 2011, the prevalence was 4.3% which was 223,494 diagnosed adults, with 90% having Type 2 diabetes and of that group more were men than women, 55% being male and 45% female (Quality and outcomes framework (QOF) 2011).

Over time a large number of those presenting with Type 2 diabetes will develop chronic complications such as retinopathy, nephropathy, peripheral neuropathy and peripheral arterial disease. Lower limb and foot problems are a major cause for concern with diabetes, particularly when there is poor glycaemic control, and can ultimately lead to lower limb amputations (Abboud 1995; Stratton *et al*. 2000). Diabetes has been cited as the major cause accounting for just under half of all adult lower limb amputations (Amputee Statistical Database for the United Kingdom 2007). Around one in twenty people with diabetes will develop a foot ulcer each year and more than one in ten foot ulcers results in amputation of the foot or leg. It has also been found that up to 70% of people die within five years of having an amputation resulting from diabetes (Abbott *et al.* 1998). Many factors have been investigated as having a role in the development of foot ulceration in diabetes including peripheral neuropathy, environmental factors such

as accidental trauma, especially from ill-fitting footwear, muscle dysfunction, abnormal blood flow and endothelial dysfunction, as well as elevated plantar pressures. Overall opinion indicates that there is a combination of factors generally involved in the formation of foot ulcers (Abboud *et al.* 2000; Plank *et al.* 2000; Bowering 2001; Cobb & Claremont 2001; Newton *et al.* 2001; Khan & Newton 2003; Hahn *et al.* 2007; Petrofsky *et al.* 2009; Tong *et al.* 2011). The work presented in this thesis will concentrate on investigating a combination of two of the factors, namely elevated plantar pressures and endothelial dysfunction.

1.1.1 Elevated plantar pressures with diabetes

There are many factors that can play a role in the alteration and increase in plantar pressures found in association with diabetes, with the most commonly associated pathology being peripheral neuropathy (Abouaesha *et al.* 2001; Pataky *et al.* 2005; Bus *et al.* 2005; Acharya *et al.* 2011; Tong *et al.* 2011; Waldecker 2012). Sensory neuropathy has been found to lead to abnormal gait, which results in increased plantar pressure (Cavanagh *et al.* 1991; Masson 1992). Kersting & Rosenbaum (1992) noted that a loss of sensation could prohibit the feed-back mechanism when overloading of the plantar surface was experienced. Thus, the individual would not know that they required to alter the load, which would result in high pressures occurring in plantar areas. Motor neuropathy causes weakness and imbalance of the intrinsic muscles of the foot which in turn allows an imbalance to occur between the long flexor and extensor muscles leading to forefoot slap (Abboud *et al.* 2000; Bowering 2001). This imbalance creates a likelihood of the metatarsophalangeal joints bearing abnormally high pressures during gait (Bus *et al.* 2005; Tong *et al.* 2011). Involvement of the autonomic nervous system has been implicated in callus formation with a reduction in sweat leading to anhidrotic

skin (Boulton 1987), and to fissuring with a higher chance of bacterial infection occurring, as well as the loss of the peripheral sympathetic vascular tone (Bowering 2001). Another contributing factor is limited joint mobility (Veves *el al.* 1992; Masson 1992). Subjects with diabetes and limited sub-talar joint mobility exhibiting clinical signs of neuropathy were found to display the highest mean forefoot pressures and also had the highest prevalence of plantar ulceration in comparison with those without neuropathy and healthy control subjects (Fernando *et al.* 1991). Structural changes in keratin in the skin, muscle, cartilage, tendons, ligaments and bony alignment have also all been linked with an increase in plantar pressures (Abouaesha *et al.* 2001; Kogler & Shorten 2001; Bus *et al.* 2005).

1.1.2 Endothelial dysfunction with diabetes

When diabetes is present, the endothelium has been found to be more vulnerable to pathological changes with many mechanisms thought to possibly contribute to this (Kampoli *et al.* 2009). As expected due to the nature of the disorder, hyperglycaemia is implicated in endothelial dysfunction (De Vriese *et al.* 2000). Evidence relating to the role of oxidative stress in the pathogenesis of endothelial dysfunction is growing with Alp *et al.* (2003) finding in animal studies that reactive oxygen species (ROS) in the form of endothelial cell superoxide was higher in diabetes. Kampoli *et al.* (2009) reported that hyperglycaemia with elevated free fatty acids and insulin resistance acted together to target endothelial cells, and this was thought to lead to oxidative stress. Advanced glycation end products (AGEs) formation is a natural aspect of ageing, but is accelerated in diabetes. Sampathkumar *et al.* (2005) correlated serum levels of AGEs with diabetes and microangiopathy in association with retinopathy and neuropathy, and found the serum levels to be higher than in control subjects. There is increasing

evidence that protein kinase C (PKC) activation in diabetes leads to endothelial dysfunction by several mechanisms as indicated in Figure 1.1 (Khamaisi & Raz 2006).

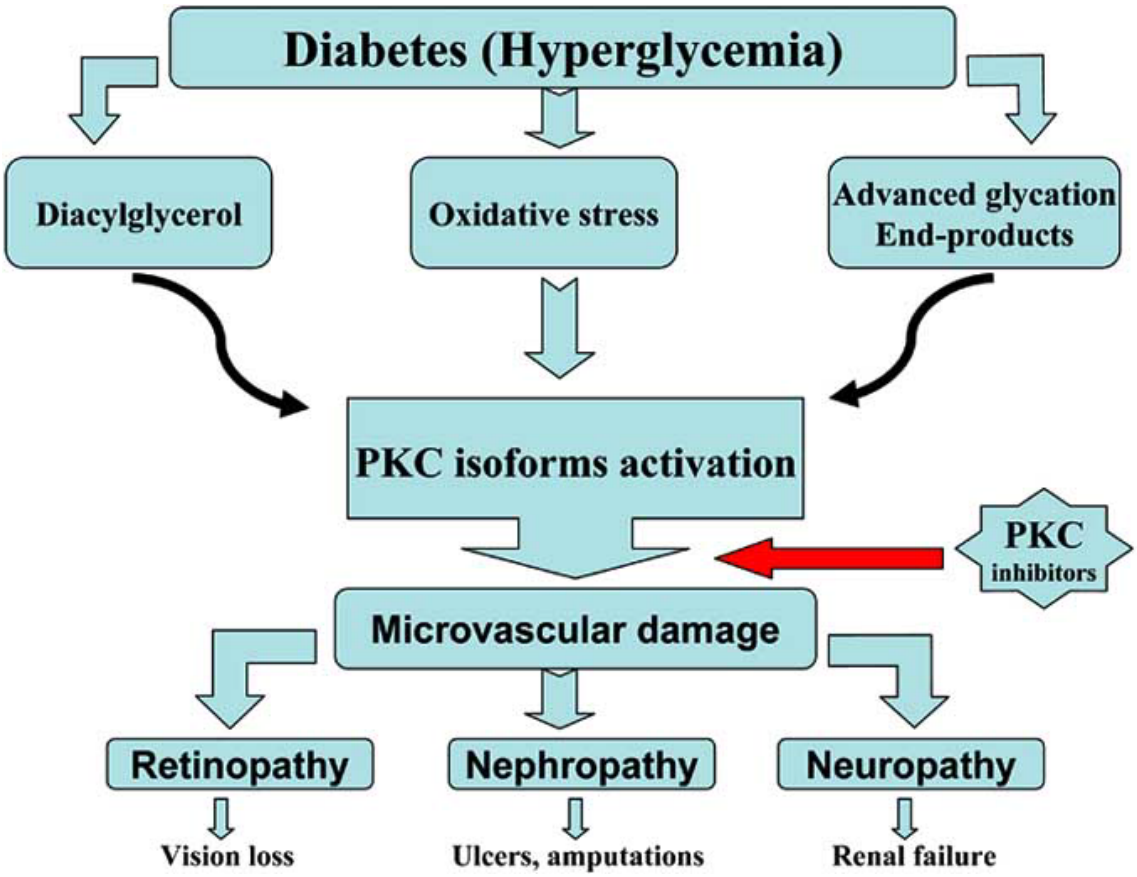

Figure 1.1The process from diabetes to micro-vascular dysfunction with the role of PKC activation (adapted from Khamaisi & Raz 2006).

Circulating biomarkers have also been implicated. Caballero *et al.* (1999) when investigating groups who were at risk of developing diabetes, found elevated levels of the cellular adhesion molecules in response to inflammation. Endothelin-1 (ET-1) levels were significantly higher in those with relatives who had diabetes, subjects with impaired glucose tolerance (IGT) and the group who had diabetes when compared to a group of control subjects. Von Willebrand factor (vWF) was found to be higher in the group with diabetes in comparison to those with relatives having diabetes and the

control group. Soluble intercellular adhesion molecule (sICAM) levels were found to be higher in the group with diabetes and the IGT group whereas the soluble vascular cell adhesion molecule (sVCAM) levels were higher in the group with diabetes and the group with relatives having diabetes than in the control group. It was noted that they were being used to aid the adhesion of circulating leukocytes to their surface and this was considered to be an early stage in transendothelial migration of leukocytes and the subsequent development of atherosclerosis. Von Willebrand factor has been reported as a response of the activated endothelium and has been found to be associated with the complications found with diabetes (Freestone *et al.* 2008). Brooks *et al.* (2008) compared blood flow responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP) which is a none endothelium-dependent vasodilator, with blood borne biomarkers of endothelial dysfunction including plasminogen activator inhibitor1(PAI-1), sICAM, sVCAM and high sensitivity C-reactive protein (hsCRP) in groups with Type 2 diabetes with and without microvascular complications and a control group. They found a significant correlation between the ACh and SNP responses with diabetes ($r = 0.6$, $p < 0.0001$) which was especially strong in those with microvascular complications. Both ACh and SNP responses fell progressively with the development of diabetes with microvascular complications. Higher hsCRP levels were found across the groups, although this trend was not found with the other biomarkers used in the study. They postulated that an impairment of the vessels to react to vasodilator stimuli must be present with diabetes.

The actual effect that insulin has on endothelial nitric oxide synthase (eNOS) and the generation of nitric oxide (NO) is under debate. Kashyap *et al.* (2005) found insulin-stimulation of muscle NOS activity to be impaired in well-controlled Type 2 diabetes suggesting that impaired NOS activity could be involved in insulin resistance in this group. Vehkavaara *et al.* (2000) found the use of insulin therapy improved endothelium-

dependent and independent vasodilation which suggests that there was some positive impact on vascular function, however their claims could imply that aspects other than improvement in the activity of NOS could be involved. For example, Sartori *et al.* (1999) found insulin to have actions on the vasculature by stimulating the release of NO and sympathetic neural outflow. However the mechanism by which this occurs is unclear and Clark *et al.* (2003) noted that there was evidence that when there was a lack of blood flow to muscles, insulin could act to switch flow from the non-nutritive to the nutritive route and suggested that insulin resistance of muscle impaired glucose uptake could be due to insulin-mediated capillary recruitment i.e. an increase in the number of perfused capillaries in the muscle in response to the stimulus of insulin.

1.1.3 Simultaneous assessment of plantar pressure and endothelial function

To date no studies have simultaneously investigated the effects of plantar pressure on endothelial function and therefore no commercially available equipment exists which is capable of carrying out this investigation. Experimental systems have been developed to investigate the impact of pressure and blood flow using laser Doppler flowmetry and a variety of pressure delivery methods including a weighbridge device with a cantilever mechanism around a central pivot (Abu-Own *et al.* 1995; Fromy *et al.* 2000). Other examples include work with pressure delivery via a piston to the heel, with the piston actually acting as a probe for blood flow assessment (Meinders *et al.* 1996). Finally, a shoe based upon an orthopaedic insole with cut out area under the first metatarsophalangeal joint was the first to attempt measurement of blood flow on the plantar aspect during actual loading when walking (Cobb & Claremont 2001). However, movement artefacts prevented actual measurement during the stance and loaded phase of gait, and data was only possible during the swing and unloaded phase of gait.

1.2 RATIONALE FOR THIS STUDY

Investigating the impact of plantar pressure on endothelial dysfunction in diabetes is vital to increase our understanding of the mechanisms that contribute to soft tissue breakdown and ulceration of the foot. Currently, there is no commercial equipment available to investigate the interaction of pressure and endothelial dysfunction, thus, the purpose of this study was to develop and validate equipment that could simultaneously investigate the impact of pressure and endothelial dysfunction, and to analyse the possible differences in endothelial dysfunction in the skin blood vessels in a group of Type 2 diabetes when compared with a healthy control group. Endothelial function in this thesis was assessed using analysis of post occlusive reactive hyperaemia (PORH) with laser speckle contrast imaging (LSCI), which is a non-invasive, laser Doppler method for assessing microvascular perfusion, providing simultaneously high spatial and temporal resolution. This was followed with iontophoresis of two vasodilator substances, acetylcholine (ACh) an endothelium-dependent vasodilator and sodium nitroprusside (SNP) an endothelium-independent vasodilator which works via liberation of NO. Iontophoresis is the process where ionisable substances dissolved in solution create an electrolyte solution, which is capable of conducting an electric current. The substances can be readily delivered through the skin surface utilising the electric field created. External pressure was generated from a pressure device developed specifically to apply a given pressure to the local area whilst not damaging or interfering with the iontophoresis equipment or the laser Doppler probe used to measure the skin blood flow.

1.3 AIM, OBJECTIVES AND HYPOTHESES OF THE STUDY

The aim of the study described in this thesis is to develop and validate a pressure delivery device and utilise the equipment to investigate endothelial dysfunction of superficial skin blood vessels by measuring blood flow, both during post-occlusive skin reactive hyperaemia and when stimulated to dilate with an endothelium-dependent and an endothelium-independent vasodilator under different plantar pressure on the feet of subjects with diabetes mellitus.

The objectives are:

1. To develop and validate equipment which will allow the application of iontophoresis with ACh followed by SNP, blood flow assessment and plantar pressure exertion simultaneously upon the plantar aspect of the forefoot.

2. To investigate the possibility of utilising LSCI for PORH assessment in the lower limb, as it provides a global perfusion map which affords difference in the perfusion found in areas of the foot to be compared, as well as offering high spatial and temporal resolution, and to compare LSCI with the more established LDF.

3. To explore the relationship between endothelial function assessed by PORH of the lower limb and 50% and 100% of peak plantar pressure when exerted upon the 3rd metatarsophalangeal joint (MPJ) area of the plantar aspect of the foot in a non-weight bearing position in conjunction with vasodilation of the superficial blood vessels.

4. To compare this relationship in a group of subjects with diabetes with an age matched control group of subjects without diabetes.

The experimental hypotheses under test are:

1. There will be a significant difference in the maximum peak blood flux (MPF) response, the time taken to reach MPF and recovery as measured at 2 minutes and 5 minutes following MPF when measured using the LSCI system between subjects with diabetes and the control group of subjects without diabetes.

2. The LSCI and LDF systems will be comparable when used to measure blood flux on the plantar aspect of the foot under the chosen comparison site of the mid forefoot area i.e. the 3rd MPJ.

3. There will be a significant difference in blood flow when measuring endothelium-dependent vasodilation using ACh, and endothelium-independent vasodilation using SNP when delivering 50% of peak plantar pressure between subjects with diabetes and the control group of subjects without diabetes.

4. There will be a significant difference in blood flow when measuring endothelium-dependent vasodilation using ACh, and endothelium-independent vasodilation using SNP when delivering 100% peak plantar pressure between subjects with diabetes and the control group of subjects without diabetes.

1.4 THESIS STRUCTURE

1.4.1 Chapter 2 – Literature review

Chapter 2 provides a review of the literature focused around the topics of plantar pressure analysis and endothelial dysfunction. It initially explores the relationship between plantar pressure alterations and diabetes as well as plantar skin and pressure elevation in diabetes and the role that plantar pressure plays in ulcer formation. It outlines the methods for assessment of plantar pressures currently available bringing in both platform and in-shoe devices. Endothelial structure, function and physiology are discussed to provide the background for dysfunction of the endothelium. The endothelium-derived vasodilators and contracting factors are included prior to the discussion of endothelial dysfunction specifically related to diabetes. Methods of assessing endothelial dysfunction and blood flux are discussed with specific emphasis on LDF and iontophoresis, LSCI and PORH. Finally the relationship between pressure and blood flux is explored as well as the methods developed to assess pressure delivery and blood flow.

1.4.2 Chapter 3 – Design and development methods

Chapter 3 concentrates on the equipment design and development for the study. Initial development of the plantar pressure delivery system is discussed in conjunction with repeatability or test-retest reliability data. Development work around the LDF utilisation including calibration of the equipment, the choice and rationale for chamber size used in the study and the iontophoresis solutions and vehicle are discussed. An appropriate protocol for the delivery of iontophoresis and the vasodilators is outlined, as well as inter-day repeatability testing for LDF detailed. The LSCI inter-day repeatability is also

noted and a pilot for the initial use of LSCI in conjunction with PORH for the lower limb is detailed.

1.4.3 Chapter 4 - Application of the developed protocol

In Chapter 4 the choice of site for the study is discussed and ethical approval outlined. Details are provided for the volunteers with diabetes, their invitation to participate as well as the details for the control volunteers. The actual protocol for the assessment is included with details of the procedure provided. The statistical methods employed to analyse the data generated by the study are described and presented.

The results of the study are set out in relation to the order of completion during the data gathering process. The initial sections detail the results of the post occlusive reactive hyperaemia assessment utilising LSCI with the findings between the two groups taking the parameters individually. It then summarises the correlation between the areas under study in the forefoot with the LSCI system and reactive hyperaemia in the two groups under study, as well as the impact of pressure on baseline blood flux. The comparison of LDF and LSCI when utilised to measure blood flux on the foot in this study is then detailed. It then moves onto the results of the effect of the addition of 50% and 100% pressure delivery on endothelial function as measured with laser Doppler flowmetry (LDF), taking the group as a whole and each of the two groups individually, with an account of the differences found between the two groups initially with ACh and then followed by SNP.

1.4.4 Chapter 5-Discussion

A detailed discussion of the results of the study is carried out in Chapter 5. This includes debate around the findings of the study, and comparisons with other work in the area when possible. It follows the format of the results of the study in Chapter 4 by starting with the equipment developed specifically for this study, PORH with LSCI and possible mechanisms around the reaction and the implications for this study. It then follows with the discussion around the comparison of LDF and LSCI for measuring perfusion. The discussion then focuses on pressure and endothelial function, taking each of the vasodilators and discussing the results of this study in relation to other work in the area and the mechanisms for the vasodilation, as well as the evidence for endothelial dysfunction with diabetes, and the impact that pressure has on blood flux. Possible limitations of the study are also discussed.

1.4.5 Chapter 6-Conclusions

Chapter 6 brings the main findings of the study together and discusses recommendations for future relevant work accordingly.

Chapter Two

Literature Review

2 LITERATURE REVIEW

2.1 PLANTAR PRESSURE ANALYSIS

2.1.1 Introduction

Load bearing during bipedal locomotion in combination with adaptation to foot structural and functional changes are important functions of the human foot. Apart from the natural ageing process, disease dependent pathological changes which alter foot function and gait, such as those in conditions like diabetes, have an influence on foot loading (Bosch *et al.* 2009; Sawacha *et al.* 2012). One of the issues with the impact created by loading the foot during both normal and intensive activity is the repetitive nature of that loading itself, so any alterations of the biomechanical behaviour of the foot structure and/or soft tissue elements caused by a disorder have a greater impact with multiple repetitions (Natali *et al.* 2010). Thus, measuring pressure exerted on the plantar aspect during locomotion adds an important and dynamic element to evaluating foot function. However there are limitations to assessing plantar pressure as it only provides an indication of the vertical force acting on the foot ground interface, or with in-shoe systems the foot insole interface, but does not measure the shear stresses which are thought to impact upon the function of the foot during activity (Rouhani *et al.* 2010).

2.1.2 Association between diabetes and increased plantar pressure

The mechanisms for increased plantar pressure found in association with diabetes are multifactorial, however, generally the singly most commonly reported pathological disorder found with diabetes which has been associated with higher plantar pressures is lower limb neuropathy (Abouaesha *et al.* 2001; Lord *et al.* 2002; Pataky *et al.* 2005;

Bus *et al.* 2005; Acharya *et al.* 2011; Tong *et al.* 2011; Waldecker 2012). Caselli *et al.* (2002) found that the plantar pressure at both the forefoot and hindfoot was increased in the diabetic neuropathic foot. However, an investigation of plantar pressure distribution patterns in non-ulcerated and ulcerated patients with diabetes and neuropathy, found that the progression of neuropathy did not influence plantar pressure distribution as both groups presented with the same clinical signs and symptoms of neuropathy. Thus concluding that while a relationship existed between neuropathy and plantar pressure distribution, other predictive elements including vascular deficit, joint mobility and foot disorders should also be investigated (Bacarin *et al.* 2009). High plantar pressures and neuropathy were found to frequently coexist with diabetes; however, a direct causal relationship was thought to be speculative (Bevans 1992). Plantar pressure distribution in patients presenting with Type 2 diabetes without neuropathy was investigated and showed similar maximum peak pressures as a control group without diabetes leading to the conclusion that either motor or sensory neuropathy had to be present before plantar pressures became elevated (Tong *et al.* 2011). Abboud *et al.* (2000) found a delay in muscle contraction time with diabetes, with the anterior tibialis muscle reaching a significant delay. This delay was thought to alter the speed of forefoot contact during gait allowing the foot-flat stage to be achieved in a less orderly approach than in control subjects. The outcome of this was that the forefoot was subjected to higher plantar pressure. The impact of restricted ankle dorsiflexion on plantar pressure distribution was investigated by Malloy *et al.* (1999). They found that when the restriction of dorsiflexion was to a neutral or dorsiflexed position, there was no significant impact on plantar pressure. However when the limitation in available dorsiflexion was 5 to 10 degrees of plantarflexion, it produced a significant shift in the distribution of plantar pressure laterally and increased the pressure-time integral (impulse) at the forefoot. It should be noted that their study was carried out on healthy subjects with no lower limb

pathologies utilising ankle foot orthoses to limit mobility and the subject group consisted of only 5 male and 5 female volunteers. However, Fernando *et al.* (1991) found that patients with diabetes coupled with limited sub-talar joint mobility and exhibiting clinical signs of neuropathy displayed higher mean forefoot pressures and had more plantar ulcers when compared with diabetic subjects without neuropathy and healthy controls. It should be noted that clinical neuropathy was coupled with limited sub-talar joint mobility, so the differences noted between the groups could not solely be attributed to the presence of neuropathy.

In conclusion, while it has been commonly reported that peripheral neuropathy is associated with higher plantar pressures, other factors have also been linked such as vascular deficit, limitation in joint mobility, foot disorders such as digital deformity and delay in muscle contraction, and are commonly found concurrently with neuropathy. The evidence would indicate a strong link between neuropathy and elevated plantar pressures in association with diabetes, but not a causal relationship as such.

2.1.3 Alterations in plantar skin and pressure in diabetes

The evidence that connects elevation of plantar pressures with thickening of the epidermis and callus formation is contradictory. Abouaesha *et al.* (2001) studied 157 patients with diabetes and neuropathy but with no history of peripheral vascular disease or ulceration. Subjects who presented with forefoot callus displayed a reduced tissue thickness under all of the MPJs with the exception of the first, and an increase in peak pressure across all MPJs. Thus they found a strong inverse relationship between plantar tissue thickness and dynamic foot pressure measurements. Hashmi *et al.* (2006) attempted to quantify specific glycation products generated in the plantar epidermal

proteins in Type 2 diabetes. They found a greater pentosidine (a biomarker for AGEs) concentration in the subjects with Type 2 diabetes than in healthy controls. Utilising high frequency ultrasonography they measured the thickness of the epidermis and found it to be thicker in Type 2 diabetes than in healthy controls and to have decreased plasticity. Kogler & Shorten (2001) found that pathologies like diabetes resulted in atrophy of the heel and forefoot fat pads caused an increase in plantar pressures.

Callus was found to be located under high pressure areas and to further increase the loading under the feet (Boulton 1987); it was also thought to act as a foreign body elevating plantar pressures (Young *et al.* 1992) meaning that high plantar pressures are generally associated with evidence of stress-like callus formation (Abouaesha *et al.* 2001; Masson 1992). Peak plantar pressures fall following removal of callus (Pitei *et al.* 1999); however others have suggested that callus does not actually cause elevation in peak plantar pressure (Woodburn & Helliwell 1996; Potter & Potter 2000).

The skin pathology which has been widely reported in conjunction with high plantar pressures in diabetes is ulceration.

2.1.4 The role of plantar pressures in ulcer formation

Elevated plantar pressures have been cited as a risk factor involved in the pathogenesis of plantar ulceration in diabetes, however most authors would agree that the elevation in pressure is found in conjunction with other risk factors such as neuropathy, peripheral arterial disease, muscle dysfunction and foot deformities (Meinders *et al.* 1996; Mayrovitz & Smith 1998; Abboud *et al.* 2000; Lord & Hosein 2000; Plank *et al.* 2000; Bowering 2001; Cobb & Claremont 2001 and 2002; Khan & Newton 2003; Koitka *et al.* 2004a; Newton *et al.* 2005; Hahn *et al.* 2007; Tong *et al.* 2011). Patients with

diabetic neuropathy and a history of ulceration were found to display significantly higher pressure than those without ulceration (Plank *et al.* 2000). However, no difference was found in pressure at the site of ulceration and the contralateral foot. They concluded that while high plantar pressure was important in the development of ulceration, additional factors must be involved. Around 1 in 20 people with diabetes will develop a foot ulcer each year (Abbott *et al.* 1998), and Meinders *et al.* (1996) noted that while the occurrence of ulceration was common with diabetes, aetiological factors including neuropathy and micro-angiopathy as well as elevated plantar pressures had to be implicated. However the interaction of these factors was uncertain. Mayrovitz & Smith (1998) commented that the heel was subject to ulceration in conditions such as peripheral arterial disease and diabetes as it had a small surface area of contact with fairly high local pressure coupled with a limited basal blood flow. Reduction in blood flow and neuropathic factors when associated with the abnormal pressures found in diabetes have been proposed as the principal factors in the pathogenesis of ulceration, although the precise role of pressure is still unknown (Levin 1995; Jeffcoate & Harding 2003; Koitka *et al.* 2004a; Hahn *et al.* 2007).

Bacharin *et al.* (2009) found alterations in plantar pressure distribution in 2 neuropathic groups with and without ulceration. Although the subjects presenting with ulceration exhibited higher loads, both groups presented with similar clinical signs and symptoms of neuropathy, so they concluded that the progression of diabetic neuropathy could not be cited as influencing plantar pressure distribution. Thus, concluding that further work was required to investigate other predictive factors such as foot deformities, ankle joint mobility, autonomic and vascular deficits.

As well as a link between increased plantar pressures and plantar ulceration, there is also a risk with lower pressures, but experienced over an extended duration also adding

to that risk. Several studies have concluded that diabetes leads to alterations in gait, and one of the main findings has been a significant reduction in walking speed (Brach *et al.* 2008; Ko *et al.* 2011; Volpato *et al.* 2012; Raspovic 2013). Slower walking in subjects with Type 2 diabetes means that the contact time for the plantar aspect is generally increased in comparison to that found in subjects without diabetes. An investigation into gait patterns in older adults with Type 2 diabetes by Ko *et al.* (2011) also found that alterations in gait occurred prior to the onset of peripheral neuropathy, with a significantly shorter stride during fast walking occurring ($p = 0.033$). Again the impact of a shorter stride pattern would be longer contact and thus extended time when the plantar aspect of the foot would experience pressure. Baccarin *et al.* (2009) also noted a cautious walking pattern was present, with decreased speed and an increase time of double support, again leading to an increased contact time.

2.1.5 Summary

Plantar pressure alterations found in conjunction with diabetes have been investigated in numerous studies. While it has been firmly established that they play a key role in the development of foot problems encountered with diabetes like callus formation and importantly ulceration, the evidence for epidermal changes is contradictory and it would follow that other risk factors are undoubtedly involved in the pathogenesis of these conditions. The association and links between the several risk factors have not been fully investigated and in order to obtain a better understanding of the development of foot disorders, further research is required.

When investigating the role of elevated plantar pressures in the development of foot disorders like ulceration, it is essential that reliable methods of assessing plantar pressures are available. Several commercial systems have been developed to assess

barefoot plantar pressures and also those found when the foot is encased in footwear. Platform systems are useful for barefoot analysis assessing the foot to ground interface, whereas in-shoe insole based systems assess the foot to footwear interface. The following section outlines some of the more commonly used available systems.

2.2 METHODS FOR ASSESSMENT OF PLANTAR PRESSURES

2.2.1 Background

The foot during locomotion is normally the only part of the body in contact with the ground. Foot function is controlled by both intrinsic and extrinsic muscles which allow the foot to be both adaptive to deal with the changes in loading patterns during the contact phase of gait, and compliant so that walking over uneven ground is possible. When the foot makes contact with the ground an equal and opposite force is created caused by gravity and the velocity of the body, known as ground reaction force (Abboud *et al.* 1996). During locomotion, pressures are transmitted via the plantar surface to the deeper structures of the foot, and measuring plantar pressure provides an additional dynamic component to foot evaluation which is clinically important for the identification of foot deformities which in turn can impact upon the foot ground interface (Natali *et al.* 2010). In the late 1960's the majority of neuropathic foot ulcers were reported to occur under pressure bearing areas of the foot. This then lead to the development of systems to measure the pressures under the foot and the initial system utilising instrumented shoes to measure dynamic plantar pressure appeared in the 1970's (Alexander *et al.* 1990).

Currently there are several systems available to measure static and dynamic forces exerted on the foot-to-ground interface, foot-to-shoe and sole of the shoe to ground

interfaces. Most systems available measure vertical forces or pressure, but the important shear stresses are now being studied. Lord & Hosein (2000) investigated plantar shear stresses in a group of subjects with a history of diabetic neuropathic ulceration in comparison with asymptomatic adults. Shear was not found to be significantly different between the groups, but they did find a medial shift of stress across the forefoot in the group with neuropathic ulceration.

With the variety of systems available and thus types, number and size of sensors utilised inevitably leads to variation in measurements and units employed. Davis *et al.* (1996) noted that even when sensor size was standardised, different protocols utilised for gathering data could impact upon the results. Comparison of an in-shoe and a platform system found that the data could not be extrapolated from one method to the other; they recommended standardisation of collection of data but that the systems should be recognised as not interchangeable (Chevalier *et al.* 2010).

2.2.2 Platform systems

Platforms enable collection of data in a barefoot state i.e. without the influence of footwear which can mask high pressure areas. The whole foot/ground contact can be measured and subjects are able to walk naturally without wires or data boxes attached to them. They do require a walkway to ensure the plate is flush with the surface and targeting of the plate can be an issue (McPoil *et al.* 1995). Earlier systems tended to have few sensors and provided poor resolution but with improved technology higher resolution systems have been developed and are widely available on the market.

2.2.2.1 Musgrave plantar pressure platform system (Preston Communications)

One of the earlier plantar pressure platform systems was developed in Belfast at Musgrave Park Hospital, with the latest computerised system (5^{th} generation) consisting of two plates with an active area of 394 x 194mm^2 each containing 2048 5 x 5mm^2 force sensing resistors which are electronically scanned with a speed of 113,777 sensors/second and calibration is with a hydraulic probe (Preston Communications). Some issues had been found with higher levels of pressure and Young *et al.* (1993) found the system saturated above 15kg/cm^2 thus preventing use for conditions where high levels of pressure could be noted, such as diabetes. Musgrave system is not commonly used now and has been superseded by other more reliable systems.

2.2.2.2 Pedobarograph

This system follows a very early principle described in 1934 by Elftman utilising a light source and dots and squares on a glass plate and recorded on cine-camera, the size and density of the dots and squares indicating pressure levels. The system is now made up of a contact plate made of thin opaque plastic with a glass plate beneath. The glass plate is illuminated with strips of light. Alteration of internal reflections of light occur when the force is applied via the foot causing contact between the opaque plastic sheet and the glass, so when viewed from below, the light pattern is proportional to the pressure applied. A mirror held at an angle of 45^0 below reflects the light pattern and the data generated is connected to a computer, thus the pressure is recorded. The system can be calibrated with the application of known loads to the platform, but has the disadvantage of not being portable (Abboud *et al.* 1996; Urry 1999). Again, like the Musgrave system it has been superseded by more advanced systems.

2.2.2.3 Tekscan Matscan®and Walkway™systems (Tekscan Inc. Boston, Massachusetts)

Two resolutions of the systems, standard and high resolution are available in multiple sizes with associated increase in sensors ranging from 435.9mm x 368.8mm in area housing 2288 sensors to 50,688 sensors encased in a low profile mat of 2926.1 mm x 447.0 mm. Scanning speeds are 100Hz and 440 Hz. As the sensors are compact in size, the platform height at 5mm has a profile which allows utilisation of the system without the need to embed in a walkway (Tekscan, MA, USA 2013). Utilising the two-step gait protocol for measuring the parameters of dynamic pressure, 30 healthy asymptomatic adults were assessed on two occasions, one week apart. The system was found to offer moderate to good test-retest reliability (Zammit *et al.* 2010). Two Tekscan Matscan® systems were assessed for repeatability along with two Novel emed-x® systems with 22 healthy participants with 10 walking trials on each system during the same visit. The inter-system reliability was found to be good between the systems thus allowing repeatable data collection to occur between the systems (Hafer *et al.* 2013). Figure 2.1 displays the Tekscan Walkway™.

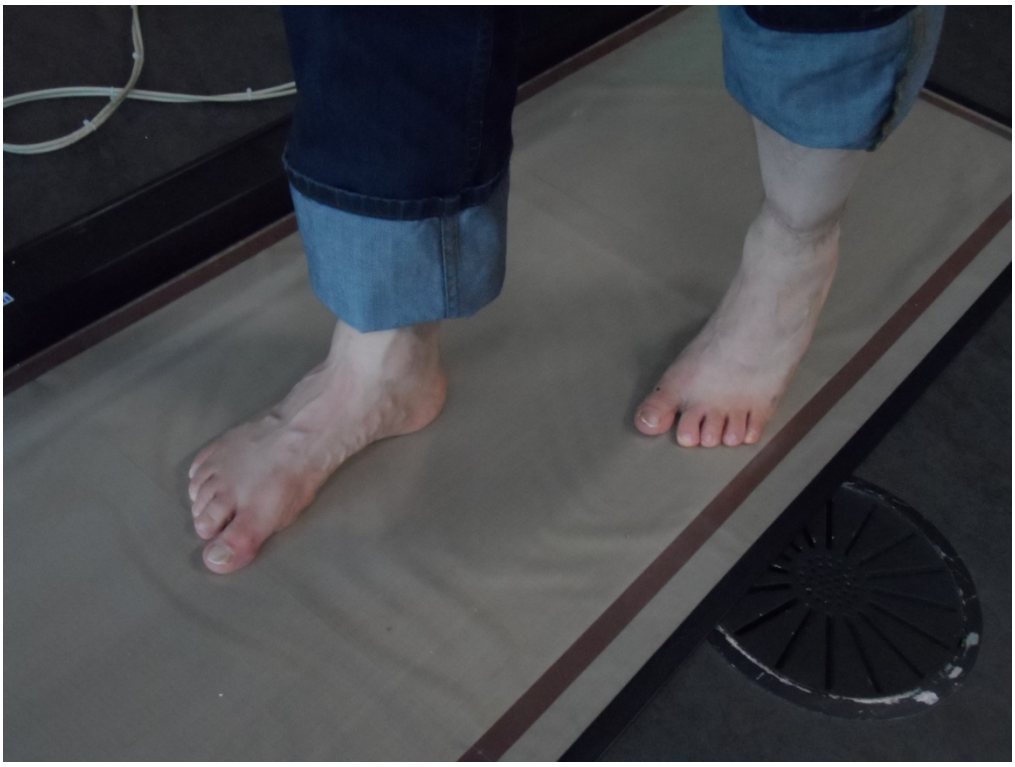

Figure 2.1 Tekscan Walkway™

2.2.2.4 The Emed® system (Novel gmbh Germany)

Novel currently produces 5 Emed® platform systems (Novel gmbh Germany) illustrated in Figure 2.2. They offer a range of size, number of sensors, sampling frequency, maximum total force and resolution as outlined in Table 2.1. All systems use calibrated capacitive sensors and connect to computers via the USB interface. Hughes *et al.* (1991) found the Emed®–f system, which was an earlier version to be reliable for most force or pressure measurements, and when the mean of three or more trials was calculated found reliability to be even better. Bryant *et al.* (2000) found their results utilising Emed®–sf also to correlate well with earlier studies. The Emed® ST4 was assessed for repeatability when mounted in the centre of a flat 10m walkway with 53 healthy volunteers. The mean number of days between repeating data collection for the same individual was 11.8 days. The coefficient of repeatability was less than 16.9% for the parameters considered which included peak pressure, contact area, contact time, the pressure-time

integral, force-time integral and instant of peak pressure, thus the system was found to be repeatable (Putti *et al.* 2008). The Emed® ST2 system was also tested for repeatability by Maetzler *et al.* (2010) and found to be comparable with the results of Putti *et al.* (2008) on the earlier system. This study utilised 23 healthy subjects who were tested and retested 7 days apart. The parameters were start and end of contact, contact time, peak pressure, instant of peak pressure, contact area and the pressure-time integral with the coefficient of repeatability being found to be less than 16% for all parameters, thus the updated system was also found to be repeatable.

Table 2.1 Current range of Emed® platform systems (adapted from novel gmbh Germany 2012).

Platform Type	Sensor Area (mms)	Number of Sensors	Resolution Sensors/ cm^2	Sampling Frequency	Max. force (Newtons)
emed a50	**389 x 226**	**1,760**	**2**	**50/60**	**110,000**
emed c50	**395 x 240**	**3,792**	**4**	**50/60**	**120,000**
emed n50	**475 x 320**	**6,080**	**4**	**50/60**	**193,000**
emed q100	**475 x 320**	**6,080**	**4**	**100**	**193,000**
emed x400	**475 x 320**	**6,080**	**1 or 4**	**100/400**	**193,000**

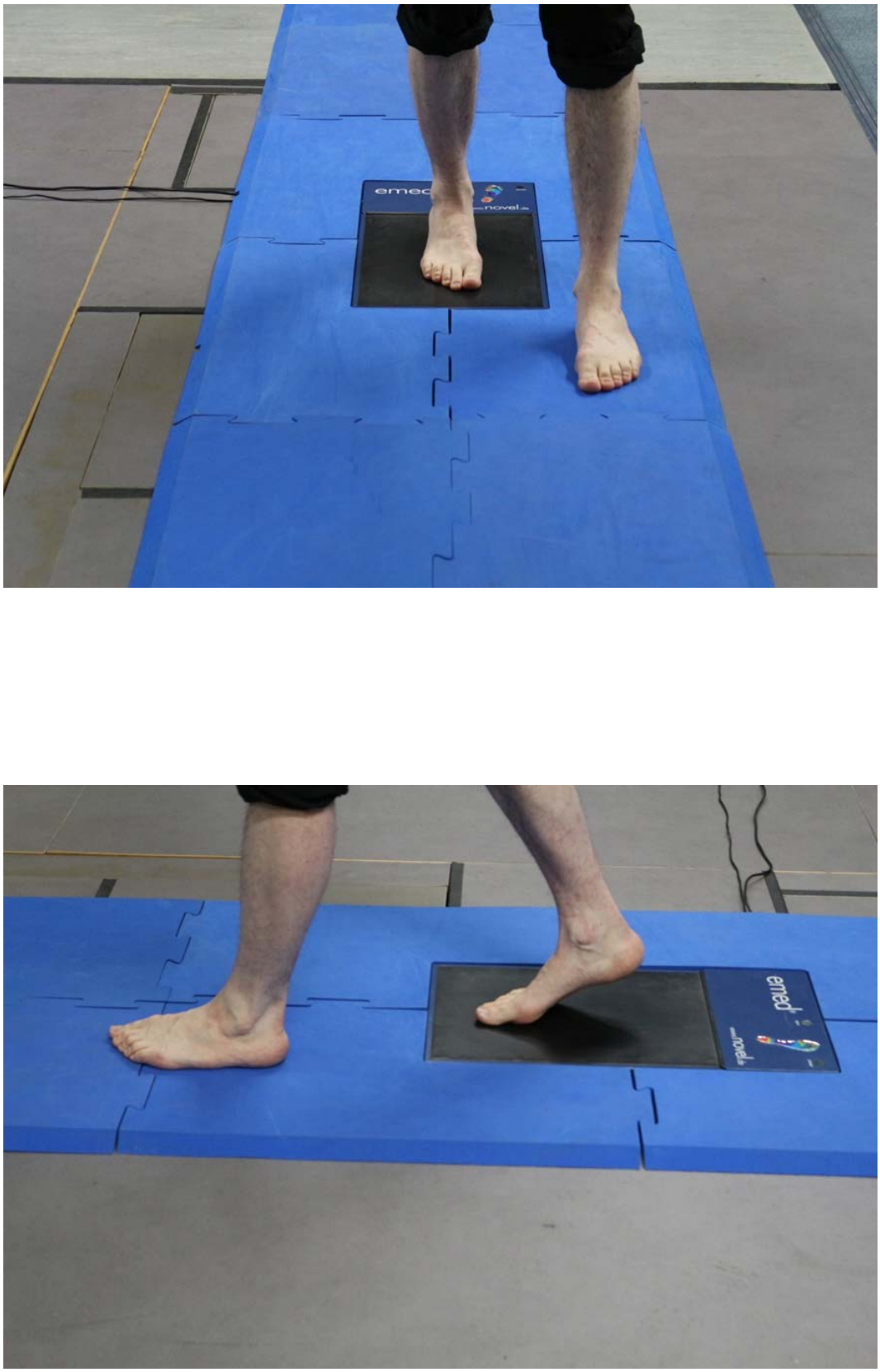

Figure 2.2 Emed® platform system in conjunction with portable walkway

2.2.2.5 Kistler® force plate (Kistler Instruments Ltd.)

The Kistler® force plate (Kistler Instruments Ltd.) utilises piezo-electric crystals which generate an electric charge along crystallographic axes, which can be measured via an amplifier (Figure 2.3). The transducer consists of four load cells, one located at each corner and measures applied forces and moments along three orthogonal directions. It is generally considered accurate and reliable; however it is sensitive to temperature and is not portable, requiring to be set in concrete (Lord 1981; Abboud *et al.* 1996).

Strain gauge sensors have also been used where one or more foils glued to a short beam bend on force being applied, causing a change in resistance of the material. Calibration of this change in resistance against known applied forces provide the magnitude of the force.

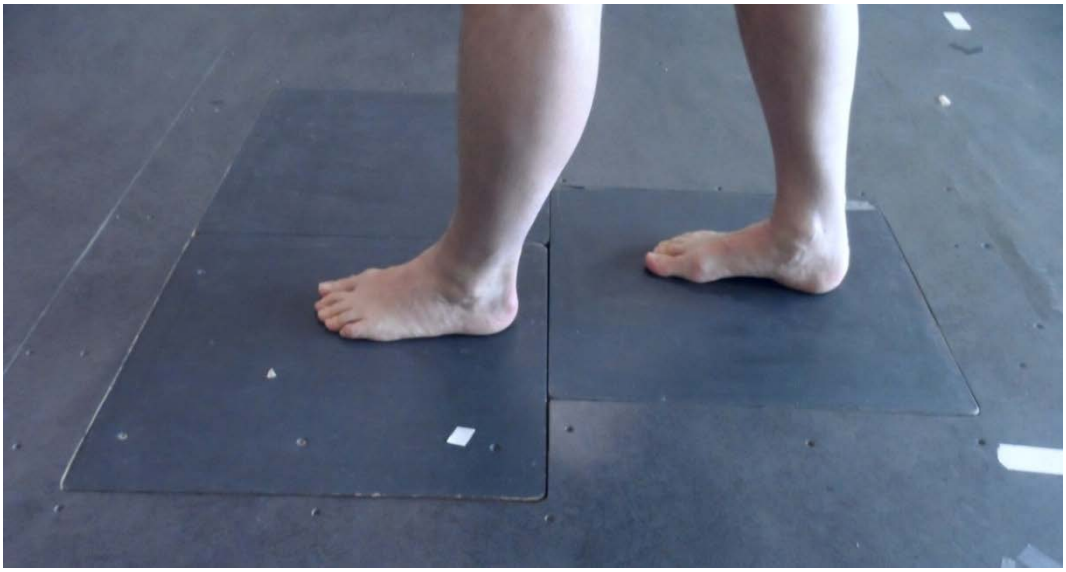

Figure 2.3 Kistler® force plates sunk into a permanent walkway

2.2.3 In-shoe systems

As technology has progressed, and sensors become smaller with higher sampling rates and faster computer systems became available, commercial in-shoe systems have been developed. In-shoe systems offer great advantages over platform systems in that they enable collection of data in the foot's normal functioning environment during activities. As no area has to be set into the surface to collect data, targeting is prevented and they can provide multiple footstep data from both feet during a single walk. However, the feet can miss some aspects of the sensing area with the influence of the shoes and unless the system is wireless, the positioning of the wires/data box can alter the subject's natural gait (Finch 1999). The material of the insole itself can alter the pressure e.g. a stiff insole and the depth of the insole with the sensors may increase pressures and make it uncomfortable for the subject (Rose *et al.* 1992*)*. If the material of the insole itself is slippery it may alter the natural gait and bending of the insole to fit the shoe which may in turn affect the sensors.

2.2.3.1 F Scan® system (Tekscan Inc. Boston, Massachusetts)

The system (Figure 2.4) uses an insole with a matrix of 960 force sensing resistors with a sensing range of 50.75 to 862 kPa, held in a micro-thin insole of 0.15mm. Insoles are available in sizes up to USA 14 shoe size, but can be trimmed to fit the individual shoe. While the depth of the insole is useful in that it does not take up space within the shoe, they are prone to wrinkling when inserted into the shoe, or when walking. The system does also suffer from calibration issues with the sensitivity of the insoles declining with use as much as 20.5% with multiple use (Abboud *et al.* 1996; Urry 1999). As Young *et al.* (1993) found issues with the Musgrave system which utilised force sensing resistors at higher pressure levels, McPoil *et al.* (1995) found that although the F Scan® system

displayed a linear correlation with known loads, the system was found to be less accurate when pressure levels exceeded 200kPa.

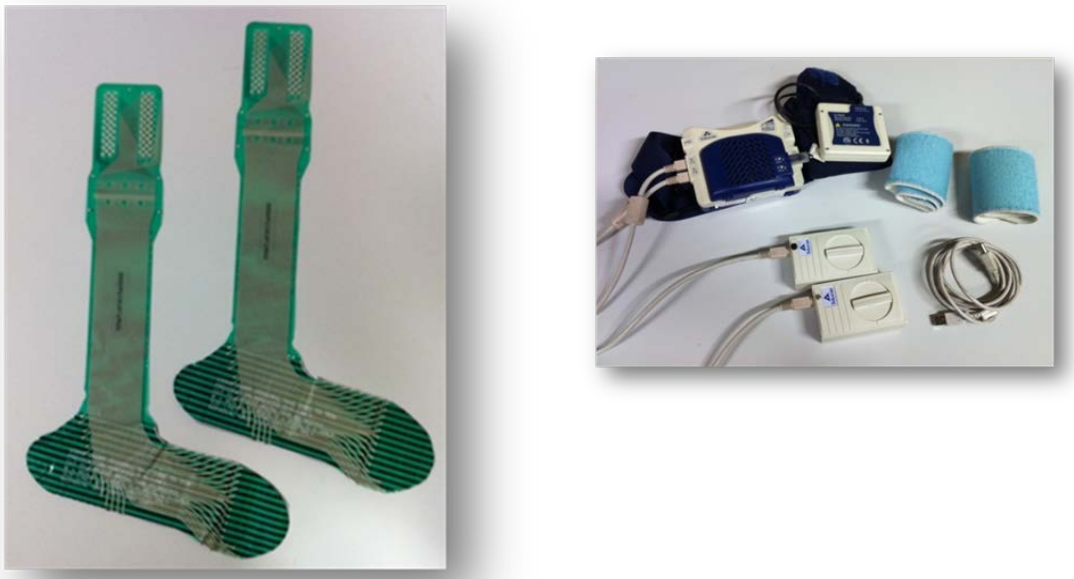

Figure 2.4 F Scan® system with insoles which can be cut to size

2.2.3.2 Pedar® plantar pressure measurement system (Novel gmbh Germany)

This in-shoe system (Figure 2.5) is one of the most commonly used in-shoe systems (Putti *et al.* 2007). It uses 2mm deep insoles to house 256 capacitive sensors with a frequency of 20,000 sensors/sec. The sensors measure change in capacity with a change in distance between two conducting wires which change with loading (Novel gmbh Germany). Similar to the equivalent platform system, calibration of the system was found to be straightforward to carry out and accurate, and McPoil *et al.* (1995) found less creep than exhibited with the F scan® insole when continuous and constant pressure was applied. The system is also available with Bluetooth technology which reduces the need for subjects to have wires and a data box attached to them thus preventing gait

alterations. A study utilising 53 healthy subjects who were clinically assessed to ensure no pathologies were present which might affect their gait, were assessed using the Pedar® system. The insoles were placed inside a specific model of shoe and 8 steps of data were collected per straight line for 8 walks per session. The same protocol was repeated during a second session. The Pedar® system was found to be repeatable for the pressure parameters tested (Putti *et al.* 2007).

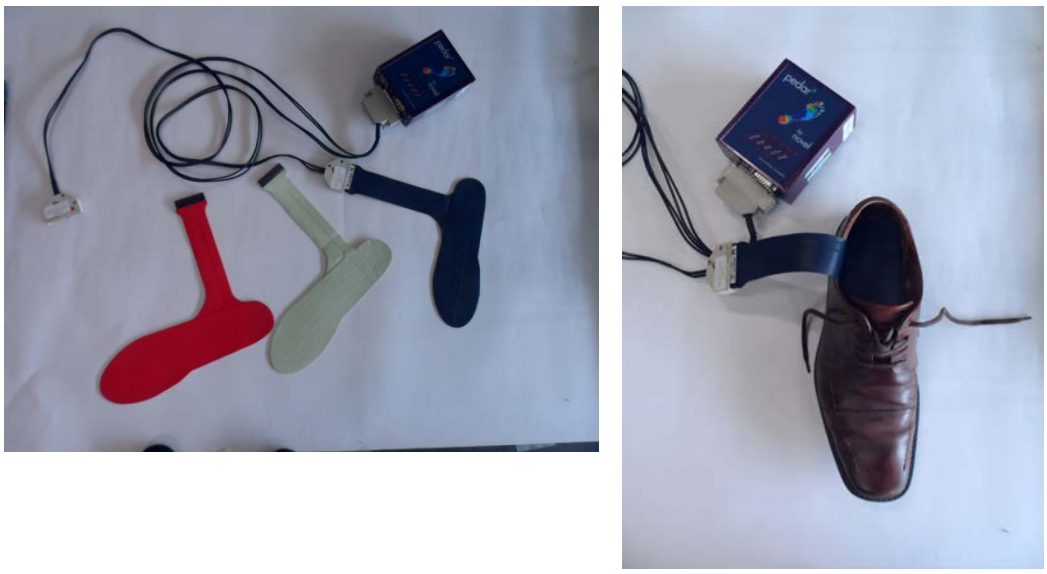

Figure 2.5 Pedar® in-shoe system insoles

2.2.4 Summary

The development of systems to measure plantar pressure has enabled evidence to be gathered about the function of the plantar aspect of the foot in relation to the shoe and external environment in health, and more importantly in disorders which impact upon the function of the foot such as diabetes. It has provided valuable data useful in the expansion of knowledge regarding the development of ulceration, and has provided a

method of investigating how useful offloading strategies are in the management of such problems. However, as plantar pressure alterations are only one of the risk factors in the development of ulceration, further work is required to investigate the relationship between the various risk factors. There have been great strides in the development of systems to measure plantar pressure, but there is no perfect system available, and there are advantages and disadvantages to both platform and in-shoe commercial systems. A major issue with any of the systems outlined here is the limitation to measuring vertical stresses only, and thus the inability to measure shear stress which also has an important role to play in the development of foot pathologies. In addition to plantar pressure, blood flow is a critical factor in determining the development of ulceration on the plantar aspect of the foot in diabetes. The circulation in the foot in areas of ground contact during ambulation was found to be reduced in subjects with diabetes, but it was only in the areas where elevated pressures were located such as the metatarsophalangeal joint areas of the forefoot that ulceration was found to develop. Thus reduction in blood flow and elevation of plantar pressure both were found to be associated with tissue breakdown (Proano *et al.* 1992). When heel skin microvascular perfusion was investigated in response to sustained pressure loading during surgery as well as in patients who were in long term residential care, it was found to be rapidly as well as significantly reduced with loading and to remain so with the duration of the loading period thus again leading to a risk of heel tissue breakdown (Mayrovitz & Smith 1998). With increase in demands made upon the microcirculation as a result of issues like injury, infection or prolonged pressure, the damaged microcirculation associated with diabetes may not be able to respond appropriately and resultant tissue breakdown and ulceration occurs (Flynn & Tooke 1992). Whilst a relationship between pressure and blood flow does exist in the pathogenesis of ulceration particularly in pathological states like diabetes, it appears to be a complex relationship and further evidence to provide a

better understanding of this relationship is undoubtedly required. This is perhaps further hindered by the fact that there are no commercial systems for measuring plantar pressure available which can readily be utilised in conjunction with blood flow measurement, to simultaneously assess both.

2.3 BLOOD FLOW ANALYSIS

There are many factors which are thought to play a part in the control of blood flow, of which endothelial function is one of the most important. Such elements as endothelium-derived dilating factors (nitric oxide (NO) and prostacyclin) and contracting factors such as endothelin, prostaglandins and reactive oxygen species (ROS) all play an important role. Over the past 20 years, great advances have been made for developing techniques for assessing endothelial function, and in this review some background to the more important factors is presented, and then some of the methods available for measuring blood flow are discussed. Each method has advantages and disadvantages, and I will provide information on some of the more commonly used methods.

2.3.1 Endothelial structure and function

The endothelium is made up of a monocellular layer covering all of the innermost surfaces of blood vessels (Triggle *et al.* 2012). Its cellular characteristics are basically the same as those in all human cells, with a nucleus surrounded by cytoplasm and organelles and held within a membrane. It has long been known as a lining, acting as a barrier to intravascular coagulation, but as far back as 1980, Furchgott and Zawadzki recognised the importance of the endothelium in the regulation of vascular function, and more recently multiple functions regulating blood flow and tissue homeostasis have been recognised (Daugherty *et al.* 1995; Furchgott 1996; Feletou & Vanhoutte 1999;

Mombouli & Vanhoutte 1999; Calles-Escandon & Cipolla 2001; Esper *et al.* 2006, Kampoli *et al.* 2009).

The cell membrane is a phospholipid structure which is double layered with water between the layers, and crossed by receptors or ion channels of complex proteins. There are many contractile proteins crossing the cytoplasm including the cortical web which is a structure responsible for shape and elasticity. This affords some sensitivity to changes in intravascular tension and stiffness when there is an increase in intravascular pressure. Passage of solutes and macromolecules across the membrane are regulated by the junction-associated actin filament system known as the FAU system. Cyclic adenosine mono-phosphate (cAMP) and cyclic guanine mono-phosphate (cGMP) generated by a Ca^{2+} nitric oxide guanylate-cyclase dependent pathway are second messengers which stabilise the FAU system. Stress fibres made of actin and myosin filaments forming bundles cross the cytoplasm in all directions. When blood flow increases the fibres flatten and align in the direction of flow (reacting to shear stress) and vice versa with reduced blood flow they increase in volume and lose alignment. The cell membrane is covered with caveolae, flask shaped invaginations and through them, the endothelium regulates fluid and macromolecular passage between the vascular and cellular compartments (Esper *et al.* 2006). Adjacent endothelial cells are connected by low resistance Myoendothelial gap junctions (MEGJs) which allow electrical continuity as well as hyperpolarization important for spreading vasodilation. Fenestrae (openings) found in the internal elastic lamina aid MEGJs in providing the electric coupling between the endothelium cells and vascular smooth muscle cells and the movement of small molecules between the cell layers (Triggle *et al.* 2012).

2.3.2 Endothelial physiology

Endothelial physiology is a huge field with many published papers and to carry out a full review of this area is out with the remit of this dissertation, however a brief overview will be detailed in this section. The endothelial cell senses physical or chemical stimuli inside the vessel (Figure 2.6) and can modify the shape of the vessel or release substances to counteract a stimulus and thus maintain homeostasis (Felton & Vanhoutte 1999). The endothelium has the capability of producing molecules as agonists or antagonists, creating a balance in both directions, these substances include vasodilators and constrictors, pro and anticoagulants, inflammatory and anti-inflammatory agents, fibrinolytics and antifibrinolytics, oxidising and antioxidising agents and many others (Daugherty *et al.* 1995; Esper *et al.* 2006).

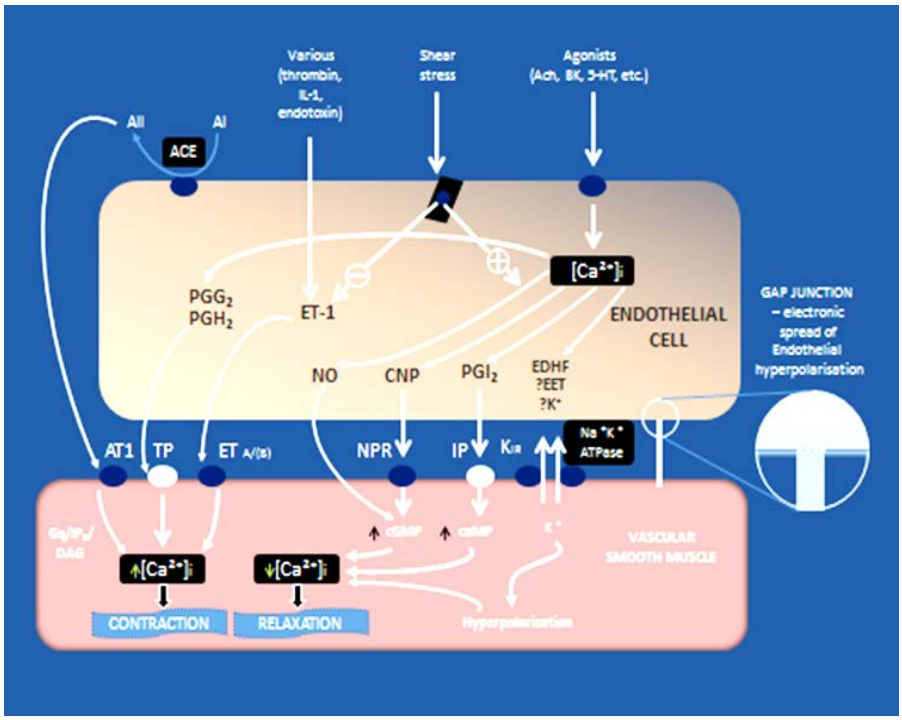

Figure 2.6 Vascular smooth muscle and endothelial cell communication- close communication between the endothelial cells and vascular smooth muscle regulates vascular tone and blood flow. Factors such as shear stress lead to endothelial dysfunction and platelet aggregation. The dysfunction caused by shear stress and other factors reduces the levels of EDHF (endothelium derived hyperpolarising factor), nitric oxide and PGI2 (prostacyclin). This combined with an increase in endothelium-derived constriction factors will lead to vascular smooth muscle cell depolarisation and results in constriction of the vessel. Contacts are made through the gap junctions which are closely associated with Na+/K+ ATPase (sodium-potassium adenosine triphosphatase, or sodium-potassium pump) on the vascular smooth muscle membrane (adapted from Rang & Dale's Pharmacology 2007).

2.3.3 Endothelium-derived vasodilators

2.3.3.1 Nitric oxide (NO)

Initially in 1980, Furchgott and Zawadzki demonstrated an endothelial-derived relaxation factor which caused vasodilation in the underlying vascular smooth muscle, and this was later found to be nitric oxide (Ignarro *et al.* 1987a; Ignarro *et al.* 1987b; Daugherty *et al.* 1995; Mombouli & Vanhoutte 1999). NO is present in just about all tissues and with a low molecular weight and lipophilic features it can cross cell membranes very easily by diffusion. It reaches the arterial smooth muscle wall by crossing the endothelial intima causing relaxation of the muscle and vasodilation. It also passes into the lumen and has been found to reduce platelet aggregation, tissue oxidation, inflammation, activation of thrombogenic factors, cell growth, proliferation and migration (Esper *et al.* 2006). It has been established that NO is involved in vasomotor tone, vascular homeostasis and neural and immunological functions (Calles-Escandon & Cipolla 2001), and a decreased production of NO is thought to play a role in vascular disease states such as atherosclerosis, hypertension and peripheral arterial disease (Daugherty *et al.* 1995).

2.3.3.2 Production of NO

Endogenous NO (Figure 2.7) is produced by the action of the enzyme NO-synthase (NOS) converting the amino acid L-arginine to L-citrulline and tetrahydrobiopterin (BH4) acts as an accelerator for the process (Moncada *et al.* 1991; Busse *et al.* 1993; Fleming & Busse 1993; Daugherty *et al.* 1995; Mombouli & Vanhoutte 1999; Calles-Escandon & Cipolla 2001; Alp *et al.* 2003; Esper *et al.* 2006). NOS in endothelial cells

is localised in the caveolae and is negatively regulated by caveolin, a 'scaffolding' protein encoded by the CAV1 gene (Mombouli & Vanhoutte 1999).

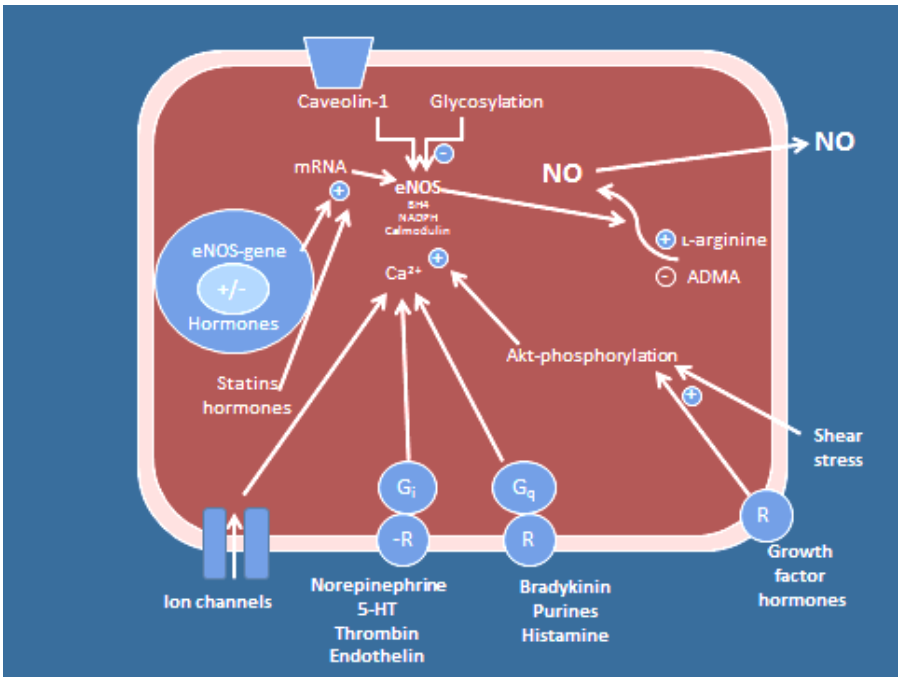

Figure 2.7 Production of nitric oxide – In the plasma membrane eNOS (endothelial nitric oxide synthase) is associated with caveolin-1. With an increase in free intracellular calcium and receptor mediated stimulation, eNOS dissociates from caveolae. The then free eNOS undergoes protein-kinase mediated phosphorylation and eventually the active eNOS catalyses the conversion of molecular oxygen to nitric oxide (NO) using L-arginine as the substrate and nitrogen donor.

Three isoforms of NOS have been isolated (Table 2.2), two constitutive and low production, and one inducible. NOS-1 is from neurological tissue, isolated from the brain and NOS-111 from endothelial cells; both are seen to respond to agonists which increase intracellular Ca^{2+}. The inducible NOS-111 is produced by macrophage and endothelial cells in response to pro-inflammatory cytokines. As a result it is said to participate in the defence of the host (Daugherty *et al.* 1995; Calles-Escandon & Cipolla 2001; Esper *et al.* 2006). The constitutive isoforms produce NO for short periods when induced by vasodilators like ACh or bradykinin. The inducible NOS synthesises NO for

longer periods, constantly when stimulated by cytokines e.g. tumour necrosis factor –α (TNF-α) (Fleming & Busse; 2003; Esper *et al.* 2006).

Table 2.2 Characteristics of the isoforms of NOS

Characteristics	Type I NOS (nNOS)	Type II NOS (iNOS)	Type III NOS (eNOS)
Mol. Wt. of monomer	160 kDa	130 kDa	133 kDa
Subcellular localization	Largely cytosolic (except skeletal muscle)	Cytosolic	Membrane bound
Regulation of expression	Constitutive Upregulated by sex hormones and after nerve injury	Not normally present Expression induced by cytokines/endotoxins	Constitutive Upregulated by sex hormones and shear stress
Substrates	Arginine O_2, NADPH	Arginine O_2, NADPH	Arginine, O_2, NADPH
Co-factors	FAD, FMN, BH_4	FAD, FMN, BH_4	FAD, FMN, BH_4
Prosthetic groups	Haem, calmodulin	Haem, calmodulin	Haem, calmodulin
Ca^{2+}-dependency of calmodulin binding	Yes Activated by $[Ca^{2+}]_i$ above normal resting concentration of cells	No Tightly bound – activity independent of $[Ca^{2+}]_i$	Yes Activated by $[Ca^{2+}]_i$ above normal resting concentration of cells
Levels of NO produced	pmoles	nmoles	pmoles
Major function	Neuronal messenger	Immunocytotoxicity	Relaxation of vascular smooth muscle

2.3.3.3 Stimulation for NO release

An increase in blood velocity creates a shear stress and this shear stress is the most important stimulation for NO release. It leads to vasodilation which is proportional to the amount of NO released by the endothelium, and this is known as endothelium-dependent vasodilation (Esper *et al.* 2006).

Specialist ion channels e.g. Ca^{2+} activated K^+ channels respond to shear stress and open up. This opening hyperpolarises the endothelial cell which generates NO by increasing

the force for Ca^{2+} entry, and activating NOS-111 (Busse *et al.* 1993; Calles-Escandon & Cipolla 2001).

2.3.4 Other endothelium-derived vasoactive factors

Like NO, prostacyclin (PG12) is a vasodilator which inhibits platelet aggregation through the activation of adenylyl cyclase rather than guanylyl cyclase (Daugherty *et al.* 1995). It is primarily produced by endothelial cells in the vascular wall; however, its vasodilator activity is determined by the expression of specific receptors located in vascular smooth muscle so if the arterial beds do not have these receptors prostacyclin cannot function as a vasodilator. PG12 facilitates the release of NO and in turn its own action is potentiated by NO (Mombouli & Vanhoutte 1999).

In studies investigating arteries such as the rat aorta in response to acetylcholine (Schini & Vanhoutte 1992), endothelium-dependent relaxations to acetylcholine or vasopressin were eliminated by inhibitors of L-arginine NO pathway, however, in vessels of smaller diameter this was not the finding (Vanhoutte 1993). Furthermore, shear stress activates a factor distinct from NO in perfused arteries, with the most likely substance being an endothelium-derived hyperpolarizing factor (EDHF). EDHF mediated endothelium-dependent vasodilation is insensitive to inhibition by a combination of NOS and cyclooxygenase (COX) inhibitors which results in hyperpolarisation of the vascular smooth muscle cells (Triggle *et al.* 2012). In vascular preparations acetylcholine and other endothelium-dependent agonists cause endothelium–dependent hyperpolarization which cannot be prevented by the inhibitors of NO formation. Nitric oxide synthase inhibitor NG-Monomethyl-L-Arginine (L-NMMA) is a known competitive inhibitor of NO synthase, and it also inhibits the release of NO from endothelial cells (Moncada *et*

al. 1991). In contrast to NO there is no basal release, but a stimulated release by substances like acetylcholine, bradykinin, adenine nucleotides, histamine, and thrombin.

2.3.5 Endothelium-derived contracting factors

2.3.5.1 Endothelin

Vascular tone is regulated through the action of vasoconstrictors and vasodilators which are locally produced and vascular tone is a result of the balance between these opposing factors. Nitric oxide is the most important of the dilators and endothelin is the most potent of the vasoconstrictors (Mather *et al.,* 2004). Endothelial cells also synthesise the prohormone big-endothelin and express endothelin-converting enzyme to produce the vasoconstrictor endothelin. Endothelin stimulates NO production so creating a limiting effect on its own activity, and in turn NO inhibits endothelin production, which provides a mechanism of action of nitrovasodilators (Masaki 1995).

2.3.5.2 Prostaglandins

In endothelial cells, metabolism by COX of arachidonic acid leads to prostaglandin H secretion, which is the precursor of thromboxane $A_{2.}$ Both act on receptors in the smooth muscle and cause vasoconstriction to occur. However under normal circumstances the small amount of vasoconstrictor released by the endothelial cells is masked by the production of prostacyclin (Mombouli & Vanhoutte 1999).

2.3.5.3 Reactive oxygen species (ROS)

Products of normal aerobic metabolism for example, superoxide anions, hydrogen peroxide, hydroxyl and lipid radicals, when they react with other molecules are referred to as ROS. Normally antioxidants neutralise their effects however, in pathological conditions ROS may reach excess known as 'oxidative stress.' De Vriese *et al.* (2000) defined oxidative stress as 'an increase in the steady state levels of reactive oxygen species and may occur as a result of increased free radical generation and/or decreased anti-oxidant defence mechanisms.' This may impact on cellular and tissue function and the excess ROS can oxidise NO forming peroxynitrite which can then lead to further oxidation (Mombouli & Vanhoutte 1999; Esper *et al.* 2006).

2.3.5.4 Inflammation

Endothelial cell activation can originate with inflammation, which in turn can lead to proliferation of the vascular smooth muscle cells and thus to vascular remodelling. This process is initiated by an increase in the vascular wall adhesion molecule (VCAM-1) and the intercellular adhesion molecule ICAM-1). The increase in the adhesion molecules on the endothelial cell membrane leads to a migration of, and accumulation of monocytes forming macrophages, and dendritic cells, as well as B and T lymphocytes which then cause an inflammatory response in the vascular wall to occur. The monocytes migrate to the intima (the inner layer of the arterial wall) and there they are transformed into macrophages. Foam cells are formed with the scavenger receptors binding oxidised low density lipoproteins (LDLs). The foam cells produce ROS and proinflammatory cytokines tumor necrosing factor and interleukin (TNF, IL-1β, IL-6) which then leads to the activation of more adhesion molecules thus cyclically producing more macrophages and B and T lymphocytes. This progressive accumulation of lipids

and foam cells then leads to fatty streak formation (Figure 2.8) (Esper *et al.* 2006; Khan *et al.* 2010; Savoia *et al.* 2011).

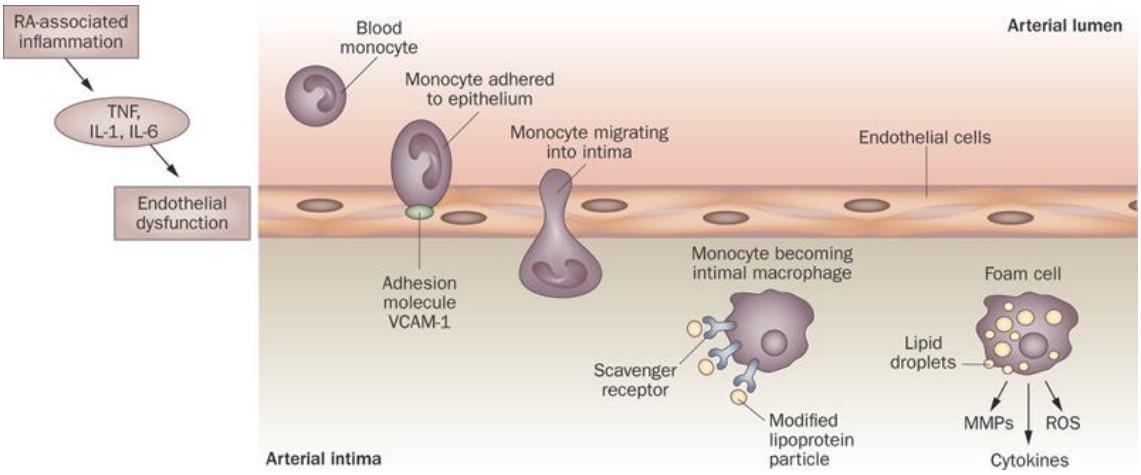

Figure 2.8 Inflammation and endothelial dysfunction leading to the formation of foam cells and fatty streaks (adapted from Khan *et al.* 2010)

2.3.6 Summary

An understanding of the structure of the endothelium is important when investigating possible functional disorders. It can be noted in Section 2.3 that there are numerous elements involved in the function of this structure for regulation of blood flow and tissue haemostasis, with NO being of prime importance. With such a variety of substances involved in the function, it is inevitable that there would be several possible mechanisms which can lead to dysfunction of the endothelium.

2.3.7 Endothelial dysfunction

As endothelial dysfunction can be either a decrease or increase in secretion of a vasodilator, an increased in sensitivity to vascular smooth muscle, and similarly with

vasoconstrictors, no single definition would cover the possible alteration of normal function; however what it does is to create abnormalities in the lumen of vessels (Mombouli & Vanhoutte 1999; Calles-Escandon & Cipolla 2001), and the mechanism by which endothelial dysfunction actually occurs remains obscure (Khamaisi & Raz 2006). Endothelial dysfunction has been given more emphasis in the pathogenesis of many disorders like atherosclerosis and hypertension and microvascular dysfunction with the complications of diabetes mellitus, PAD and kidney failure (Cracowski *et al.* 2006).

2.3.8 Endothelial dysfunction with diabetes mellitus

With both Type 1 and Type 2 diabetes, the endothelium has been found to be more vulnerable to developing pathological changes, and there has been a large variety of mechanisms thought to contribute to this (Kampoli *et al.* 2009). In studies involving children and adolescents changes in endothelial function have been identified prior to the manifestation of clinical indications of dysfunction (Elhadd *et al.* 1998; Khan *et al.* 2000). The pathogenesis of endothelial dysfunction in diabetes has been found to be multifactorial, and clinical studies have the added confounding element of the high prevalence of other cardiovascular risk factors also known to impact upon endothelial function (De Vriese *et al.* 2000; Khamaisi & Raz 2006). Hyperglycaemia is clearly implicated in the pathogenesis of complications associated with diabetes, and induces acute changes in intracellular metabolism such as activation of the polyol pathway, of protein kinase C and increasing oxidative stress as well as long term changes in macromolecular structure and function with the formation of AGEs (De Vriese *et al.* 2000).

2.3.8.1 Oxidative stress

There is an increasing amount of evidence implicating oxidative stress in the pathogenesis of diabetic endothelial dysfunction (De Vriese *et al.* 2000). The influence of puberty (a period noted for intense hormonal and metabolic changes) on endothelial dysfunction and oxidative stress was assessed in a group of 51 young patients with Type 1 diabetes of which 12 were prepubertal children, 16 were adolescents and 23 young adults, all of which had no clinical indications of angiopathy or microalbuminuria. Skin blood flow responses to ACh were found to be significantly higher in the adolescent group in comparison to the young adult group and the markers of oxidative stress used, such as soluble intercellular cell adhesion molecule-1(ICAM-1) and E-selectin were also significantly higher in the adolescent group while von Willebrand factor (vWF) although not significant was found to be higher in the adolescent group, however there was no difference found between the groups for plasma thiol (PSH). They concluded that factors which contribute to the impact of puberty on endothelial function were possibly complex and quite likely to be associated to metabolic alterations in diabetes, an increase in lipid peroxidation and oxidative stress (Elhadd *et al.* 1998). Post occlusive reactive hyperaemia (PORH) utilising laser Doppler flowmetry was studied in a group of 58 children with Type 1 diabetes in comparison to 58 ages and sex matched healthy controls (Schlager *et al.* 2012). Their findings indicated peak perfusion post occlusion was significantly higher in the group with diabetes, but other parameters were not significantly different such as baseline perfusion, time to peak perfusion and recovery time. This high peak perfusion found at such an early stage in the disease process, was thought to be possibly linked to an impaired vasoconstrictive ability of the precapillary arterioles. Factors which have been linked to alterations in macro and microvascular reactivity found in diabetes are an increase in oxidative stress as well as elevated proinflammatory cytokines serum levels. They also concluded that as

autonomic neuropathy has been found to occur at an early stage, structural changes associated with this such as thickening of vascular basement membranes might also be involved with endothelial dysfunction. In agreement, Joannides *et al*. (2006) also noted that the peak flow found with PORH was mainly dependent upon structural and metabolic factors. Yamamoto–Suganuma & Aso (2009) also investigated PORH with 104 subjects with Type 2 diabetes and 20 healthy controls, in agreement with Schlager *et al.* (2012) they found that an impaired PORH was associated with risk factors for micro and microvascular disease, however noted like Joannides *et al.* (2006) that maximum peak flow was dependent on structural and metabolic factors like adenosine and prostaglandins and thus not an index of endothelial function. This finding is somewhat puzzling as prostaglandins are known vasoconstrictor factors which can impact upon endothelial function.

Alp *et al.* (2003) found increased production of ROS, in the form of endothelial cell superoxide production was raised in diabetes. This coupled with a loss of endothelial NO bioactivity was thought to be a key feature of vascular disease found with diabetes mellitus in animal studies. In agreement, Pacher *et al.* (2002) also noted oxidative stress played a critical role in the early onset of diabetic cardiomyopathy in animal studies.

Kampoli *et al.* (2009) noted hyperglycaemia coupled with elevated free fatty acids and insulin resistance which characterise diabetes could act together to target endothelial cells. This combination was thought to lead to oxidative stress and in turn endothelial dysfunction. Elevated blood glucose levels was found to drive the production of ROS via multiple pathways which in turn resulted in uncoupling of the mitochondrial oxidative phosphorylation and activation of protein kinase C (PKC) in vascular cells (Khamaisi & Raz 2006).

2.3.8.2 Advanced glycation end products (AGEs)

AGE formation happens during the normal ageing process, but is accelerated during diabetes. They accumulate in the tissues with the formation of cross links and then generate oxygen-derived free radicals and cause a further increase in oxidative stress (De Vriese *et al.* 2000). Sampathkumar *et al.* (2005) evaluated a simple method of detecting serum levels of AGEs and correlated levels with diabetes and microangiopathy associated with retinopathy and nephropathy. The serum levels detected were higher with diabetes than in normal controls, and even higher with diabetes and known microvascular complications. They concluded that AGE could be a measure to predict microvascular complications in diabetes.

2.3.8.3 The role of protein kinase C (PKC)

As PKC is involved in a variety of cellular functions, the consequences of activation can be multiple (De Vriese *et al.* 2000). It is a family of multifunctional isoenzymes that phosphorylate protein substrates. Of the 10 known mammalian isoforms 8 are activated by diacylglycerol (DAG), and activation of PKC is through ligand activation of G-protein coupled receptors (Khamaisi & Raz 2006). Hyperglycaemia causes synthesis of DAG which then activates PKC and this pathway has been demonstrated in vascular tissues involved in diabetic complications like retinopathy and nephropathy, and there is increasing evidence that PKC activation in diabetes leads to endothelial dysfunction (Koya & King 1998; Khamaisi & Raz 2006).

2.3.8.4 Circulating biomarkers

Brooks *et al.* (2008) investigated microvascular function on 20 subjects with diabetes with complications, 20 subjects with diabetes without complications and 20 healthy controls utilising laser Doppler flowmetry and iontophoresis of ACh and SNP. They also compared their blood flow results with biomarkers of endothelial dysfunction, including plasminogen activator inhibitor-1 (PAI-1), soluble intercellular adhesion molecule (sICAM), soluble vascular cell adhesion molecule (sVCAM) and high sensitivity C-reactive protein (hsCRP). They noted that traditionally the increase in blood flow in response to the endothelium-derived vasodilator ACh is taken as an indicator of endothelial function. However, they argued that this could only be the case when the response to SNP, which is not an endothelium-derived vasodilator (but dependent on NO) was normal, and both substances responded in association with diabetes in this study. Thus, they concluded that there must be another associated impairment in conjunction with endothelial dysfunction and postulated that an impairment of the vessels to react to vasodilator stimuli must also be present with diabetes. The abnormalities found in association with diabetes and complications were comparable with the hsCRP marker for inflammation. This link was found to be much stronger than displayed with the other markers assessed in this study. This finding was not in agreement with other studies but possibly attributed to the lower levels of albumin/creatinine ratio which in this study was in fact only twice the upper limit of normal. Caballero *et al.* (1999) when investigating groups who were at risk of developing diabetes, noted that cellular adhesion molecules were found in response to inflammation (being utilised to help the adhesion of circulating leukocytes to their surface which is an early stage in transendothelial migration and development of atherosclerosis). They have been reported as being higher in a number of cardiovascular conditions and diabetes in some studies. Release of vWF was reported as another

response of the activated endothelium and associated with the development of long term complications of diabetes. Freestone *et al.* (2008) investigated endothelial dysfunction in atrial fibrillation (AF) using flow-mediated dilatation (FMD) and investigated the relationship to plasma vWF. They reported higher levels in the group with AF in comparison to healthy controls and mentioned this as a measure of reduced endothelial NO production.

2.3.8.5 Insulin

There is controversy regarding the actual effect of insulin on NOS activity and the generation of NO. Kashyap *et al.* (2005) compared the response to insulin in a group of 10 subjects with Type 2 diabetes with that of a group of 11 healthy controls. The base level of NOS found in the control group did not alter with the addition of insulin, whereas the NOS level of the diabetic subjects increased 2.5 fold with the addition of insulin. Their findings were that insulin stimulated muscle NOS activity was impaired in well controlled Type 2 diabetes and they concluded that impaired NOS activity could play a part in the insulin resistance in Type 2 diabetes. The use of insulin therapy in Type 2 diabetes was found to actually improve endothelium-dependent and independent vasodilation, suggesting that it had a beneficial effect on vascular function (Vehkavaara *et al.* 2000). However, their implication that insulin therapy provided some improvement for endothelium-independent vasodilation would in fact imply that other aspects than improvement in NOS activity could be involved.

Insulin has been found to exert cardiovascular actions by stimulating the release of NO and sympathetic neural outflow. The mechanism by which this occurs is not clear, and would appear to be either by stimulation of muscle blood flow and NO release by direct action on the vasculature, or by stimulation of neural mechanisms (Sartori *et al.* 1999).

Their findings, using sympathetically denervated limbs, would suggest that insulin had a direct vasodilator action in skeletal muscle in vivo. This action was directly mediated by stimulation of NO release and was masked by the sympathetic tone (vasoconstrictor) in innervated limbs. Sundell *et al.* (2002) looked at the effects of obesity on coronary flow response to insulin and found that young, obese, healthy men have reduced myocardial vasoreactivity. They noted that this could represent an early precursor of future arterial disease, however little evidence of this postulation could be drawn from their results. Laine *et al.* (1998) demonstrated that obesity was characterised by two defects in skeletal muscle: insulin resistance of cellular glucose extraction and an impaired endothelium-dependent vasodilation. They postulated that insulin resistance associated with obesity could not be overcome by normalizing muscle blood flow (a 75% increase in blood flow did not alter glucose uptake). In addition, Clark *et al.* (2003) when reviewing insulin action on blood flow and muscle metabolism noted that there was accumulating evidence to suggest that insulin resistance of muscle impaired glucose uptake could be partly due to impaired, insulin-mediated capillary recruitment.

2.3.9 Summary

As can been seen in Section 2.3.8, there are many areas of study and overlap of findings regarding the aetiological elements which contribute to glucose-induced damage to endothelial function. In several studies the intervention of strategies to correct one or more of the elements have provided a positive result, but this should not be taken as precluding other mechanisms from playing a role in endothelial dysfunction and in fact when for example, findings incorporate improvement in endothelium-independent vasodilation it must be presumed that other mechanisms are involved.

A wide variety of stimuli will invoke a response from an artery and these reactions can be utilised to evaluate endothelial function. Such stimuli include both physical and chemical elements, and measurements of biomarkers of endothelial activity, dysfunction or even damage have also been utilised to assess endothelial function. Both invasive and non-invasive methods of assessment have been developed for this purpose, however non-invasive methods have become the routine methods of choice (Steinmetz & Cole 1993).

2.3.10 Methods of assessing endothelial function

A non-invasive method of measuring the endothelial function of the peripheral vessels is flow-mediated vasodilatation (FMD) which is often referred to as the 'gold standard' technique. It utilises high resolution ultrasonography to measure diameter changes in an artery. Shear stress is thought to initiate stimulation of the endothelium to produce a NO-dependent response and FMD, by measuring the changes in the artery provides a direct indicator of the NO bioavailability. The technique does require accurate image analysis and as such operator training is necessary for utilisation (Beveridge & Khan 2012). Waveform analysis using Doppler ultrasound examines blood flow patterns. Laminar flow with the particles in the centre of the stream moving at maximum velocity and the peripheral particles stationary is the normal pattern of flow with undamaged, fully functioning vessels. With a stenosis, blood flow accelerates to form a jet and as the blood passes the actual area of stenosis, the flow pattern breaks down and produces areas of stagnation and often reverse flow. This disturbance is proportional to the degree of stenosis and also proportional to the range of recorded frequency shifts found within the pulsed Doppler sample volume. This alteration of flow velocities is known as spectral broadening (Steinmetz & Cole 1993).

Endothelium-derived biomarkers such as E-selectin, thrombomodulin and vWF can be utilised to identify possible cardiovascular risk. E-selectin plasma levels have been found to be indicative of endothelial cell activation as well as damage (Belch *et al.* 1997). Thrombomodulin, a thrombin receptor found on vascular endothelial cells is released into the circulation when endothelial damage occurs, and vWF plasma levels have an inverse correlation with endothelial function and as such can be utilised as a predictor for cardiovascular disease (Beveridge & Khan 2012).

Endothelial progenitor cells (EPCs) play a role in re-epithelialisation following injury to vessels and neovascularisation of ischaemic lesions. They are derived from bone marrow, and thought to have a role in the pathogenesis of atherosclerosis and cardiovascular disease (Werner *et al.* 2005). However, no precise definition of EPCs has been developed and no true standard of identification found and as such no protocol established for analysis of EPCs (Beveridge & Khan 2012).

Currently, there are several commonly used non-invasive methods of assessing microvascular perfusion available such as laser Doppler flowmetry, laser speckle contrast imaging, photoplethysmography, thermography, transcutaneous oxygen tension, ankle brachial pressure index and nailfold capillaroscopy all of the methods offer advantages and disadvantages which can be found in Table 2.3. Of the methods available, laser Doppler flowmetry with iontophoresis and laser speckle contrast imaging were found to be the optimum methods for utilisation in this current study and as such are discussed in detail in the following sections.

Table 2.3 Selection of the current available techniques for assessing microvascular perfusion with their advantages and disadvantages

Technique	Function	Advantages/Disadvantages
Laser Doppler Flowmetry	Uses laser light Velocity of this back scattered light is detected providing Doppler frequency information	**Advantages** -effective and reliable method for measurement of blood perfusion in the microvasculature -provides continuous, noninvasive and real-time measurement capabilities. -versatile and easy to use **Disadvantages** - motion artefact noise, lack of quantitative units for perfusion, lack of knowledge of depth of measurement
Laser Speckle Contrast Imaging	Interference when laser light has been reflected from different parts of an illuminated surface	**Advantages** -rapid skin blood flow measurements over wide areas coupled with good resolution. -full-field imaging of skin perfusion in near real time **Disadvantages** -relationship between speckle contrast and velocity is highly non-linear, thus a lack of quantitative units
Photoplethysmography	Optical reflection mode system measuring scattering and absorption characteristics of tissue associated with changes in blood flow, with near infrared spectroscopy	**Advantages** -non-invasive, Easy to use, cost effective and portable. **Disadvantages** -blood volume, movement of the vessel wall and orientation of erythrocytes may have an influence on the amount of light received. -cannot easily be calibrated.
Thermography	Thermal infrared imaging for the measurement of cutaneous circulation	**Advantages** -can be used over large areas -allows for real time recording **Disadvantages** -If temperatures are very close in range, infrared imaging camera may misread the information and individual objects can become indistinguishable - current technology in thermography only allows for imaging to be applied to surface temperatures
Transcutaneous Oxygen Tension	Transcutaneous oxygen and carbon dioxide sensors directly measure tissue gas tensions with a short sensor response times	**Advantages** -non-invasive and continuous **Disadvantages** Measurements do not equate directly to arterial blood flow due to skin perfusion
Ankle Brachial Pressure Index	Systolic pressure is measured at the ankle using either the posterior tibial artery or dorsalis pedis and the value in mmHg obtained here is divided by the brachial systolic pressure, utilising a pressure cuff and a hand held Doppler probe.A normal index value at rest is equal or higher than 1	**Advantages** Easy to perform Non invasive Equipment is cheap and readily available **Disadvantages** Not useful for small vessel disease or with calcification of vessels
Nailfold Capillaroscopy	Under 10x to 20x magnification has been used to assess digital microcirculation, and reproducibility of capillary width and number of loops/millimeter have been measured.	**Advantages** Simple to carry out and non-invasive, safe and cost effective Allows direct observation of nail fold capillaries**.**

2.3.11 Laser Doppler flowmetry (LDF)

2.3.11.1 Background

While many techniques such as photoplethsmography, thermography, transcutaneous oxygen tension, ankle brachial pressure index and nailfold capillaroscopy can provide an indirect indication of microvascular perfusion, laser Doppler flowmetry is one of the few that can assess the microcirculation directly. Changes in microvascular perfusion of the skin can be measured relatively easily utilising the non-invasive technique of laser Doppler flowmetry (Khan & Newton 2003; Rossi *et al.* 2006; Rossi *et al.* 2008; Fredriksson *et al.* 2010). The technique utilises the motion generated from a light source and captured by a detector, this is known as the Doppler effect. With LDF the moving erythrocytes, or in fact tissues, cause a scattering of the laser light, and the velocity of this back-scattered light is detected providing the Doppler frequency information (Wardell *et al.* 1993; Eun 1995; Khan & Newton 2003). The density and speed of the red blood cells when measured with a regular or constant haematocrit within the sample tissue, is known to be proportional to red blood cell flow, hence the utilisation of the term ‘flux’ as a unit of flow measurement.

2.3.11.2 Instrument and measurement techniques

Traditional LD flowmeters utilise a helium-neon laser light source of wavelengths varying between 630nm and 670nm with a fibre optic cable in contact with the skin surface (Figure 2.9). They provide an estimated perfusion depth of 1-1.5mm into the dermis and detect dermal blood flow without influence from the flow to the underlying skeletal muscle (Eun 1995; Fullerton *et al.* 2002; Khan & Newton 2003; Kvandal *et al.* 2006; Turner *et al.* 2008). An issue had been noted in that the optical fibres produce

low frequency noise artifacts; further developments have ensued to overcome this problem (Eun 1995).

Figure 2.9 Laser Doppler flowmeter utilised in conjunction with an iontophoresis controller

Semi-conductor laser diodes operating with near infrared light of 780nm to 820nm as a light source have been developed to replace the helium-neon lasers (Eun 1995; Khan & Newton 2003; Kvandal *et al.* 2006). The main advantages over traditional flowmeters being deeper penetration, and better reproducibility (Eun 1995; Khan & Newton 2003).

In contrast to single point measurements, laser Doppler perfusion imaging affords non-contact assessments (therefore desirable for wound assessments) with the laser beam position controlled by a system of mirrors. Currently, there are two main commercial laser Doppler imaging systems, produced by Lisca Development AB, Sweden and Moor Instruments Ltd UK. A digital image is produced, being developed as the combination

of many single-point recordings of a two dimensional matrix of the blood flow over an extended skin surface, and over a larger area, thus producing a detailed perfusion map.

Both LDF and LDPI have been found to be user-independent by utilising established protocols and maintaining external factors which can influence the readings, such as controlling environmental temperature, reducing movement as well as reflection artifacts where possible, removal of topical agents, as well as refraining from smoking and caffeine ingestion for a period of at least 2 hours prior to assessments (Fullerton *et al.* 2002; Khan & Newton 2003, Turner *et al.* 2008)

2.3.11.3 Utilisation of laser Doppler flowmetry

LDF has been utilised for many applications including dermatology, wound healing, diabetology and peripheral vascular aspects (Eun 1995; Fullerton *et al.* 2002). LDI and capillary microscopy have been used to assess subpapillary and nutritive microcirculation in 17 patients with arterial occlusive disease and chronic venous insufficiency ulcers in 4 regions, a non-granulating tissue area, a granulating tissue area, an area of skin adjacent to the ulcers (within a distance of 1-8mm), and a more distant area of 9-25mm (Ambrozy *et al.* 2009). Laser Doppler flux was significantly higher in granulating areas than in areas without granulation tissue and intact skin. In scar tissue flux was found to be higher than in both intact skin and in ulcers without granulation tissue being present. Thus, ulcers without granulation tissue had poor perfusion and did not display healing, whereas when granulation tissue was present, high perfusion was found. They also noted that the blood supply in the intact skin adjacent to the ulcers was affected by an altered microcirculation found in the ulcers. Furthermore, it was noted that LDI flux measurement could provide information to aid the optimum evaluation of microcirculation in ulcers of mixed aetiology. Krupa (2003) investigated the

relationship between plantar pressure and skin perfusion on the foot in subjects with Type 2 diabetes, peripheral neuropathy and a history of plantar ulceration. Although the heel area was found to display high pressures, in comparison to the forefoot areas in the study it displayed a higher skin perfusion, thus findings indicated that understanding of the skin vasculature response to plantar pressures could add valuable insight into re-ulceration issues.

Pressure ulcers were investigated as a complication of tissue ischaemia resulting from diabetes. When comparison was made between subjects with diabetes and controls, skin blood flow decreased markedly in subjects presenting with diabetes without neuropathy, as measured with single point LDF in response to initial locally applied increasing pressure. This inability of the microcirculation to respond to local pressure was noted as a possible factor in ulcer development in this group (Fromy *et al.* 2002). The utility of LDI versus transcutaneous gases ($TcPCO_2$ and $TcPO_2$) for the assessment of limb perfusion in cases with severe ischaemia was assessed by Figoni *et al.* (2006), in order to determine the degree and progression of the pathological process leading to possible amputation. They compared 31 patients who required amputation with 29 age–matched non ischaemic controls and found that LDI could detect a proximal to distal gradient of perfusion of the lower limb which was not discernible with $TcPO_2$. LDI was also found to discriminate between ischaemic and non ischaemic skin which was not possible with $TcPO_2$ thus overall LDI proved to perform better than $TcPO_2$. Skin perfusion in the hallux utilising LDI with 14 patients with peripheral arterial occlusive disease was compared with 11 healthy volunteers (Fischer *et al.* 1995). Significantly lower digital artery pressure and skin perfusion pressure was found in the patient group. They noted that LDI provided an easy and non-invasive method of simultaneously assessing digital artery and skin perfusion, enabling the severity of peripheral arterial occlusion to be assessed thus predicting possible healing of foot ulcerations.

One major limitation of laser Doppler flowmetry is that despite attempts, it is not possible to measure in absolute perfusion values and as such arbitrary units (PU or AU) are universally used. The arbitrary units are also referred to as blood flux rather than flow, and flux is the product of average speed and concentration of the moving erythrocytes in the tissue volume sample (Cracowski *et al.* 2006).

2.3.12 Laser Doppler flowmetry and iontophoresis

Iontophoresis is a useful technique where small doses of vasoactive substances are required to stimulate skin microvessels locally without systemic effects to gain a better understanding of mechanisms of vascular dysfunction. Utilising iontophoresis with LDF provides a non-invasive method using a small electric charge to aid migration of charged substances across the skin surface (Figure 2.9) (Newton *et al.* 2001; Ferrell *et al.* 2002; Khan & Newton 2003; Newton *et al.* 2005; Turner *et al.* 2008). The polarity of the active electrode used has to be the same charge as the substance itself (Pitei *et al.* 1997; Turner *et al.* 2008). During the process of iontophoresis, the electric dose, which is calculated from duration and current strength, is thought to correlate with the amount of substance delivered across the skin (Henricson *et al.* 2009). The most commonly utilised substances for this purpose are those for investigating endothelial function, namely ACh an endothelium-dependent agonist, and SNP an endothelium–independent vasodilator, although other substances have also been used such as bradykinin and substance P (Newton *et al.* 2001; Ferrell *et al.* 2002; Khan & Newton 2003; Kiotka *et al.* 2004b; Turner *et al.* 2008; Henricson *et al.* 2009). However, when using iontophoresis, several methodological issues need to be considered, which have been reviewed elsewhere (Turner *et al.* 2008).

2.3.12.1 Utilisation of iontophoresis

Iontophoresis in conjunction with LDF and LDI has been utilised to investigate many pathological conditions, in particular diabetes mellitus and peripheral arterial disease. Sensory neuropathy is recognised as a contributory factor to development of wounds. Kelly *et al*. (2001) studied endothelial responses in the diabetic foot with sensory neuropathy. Analysis of the ACh and SNP response revealed no significant differences between groups of subjects with diabetes and neuropathy, without neuropathy and controls without diabetes. However this study utilised a protocol with a higher current for a shorter duration of iontophoresis than found in most other protocols and with a small number of subjects, six in each of the groups with diabetes with and without neuropathy, as well as the healthy controls studied.

Children, adolescents and young adults with Type 1 diabetes were studied by Khan *et al.* (2000). They measured skin microvascular function using LDF with iontophoresis of ACh and SNP in the dorsum of the feet. Findings indicated that early reductions in the microvascular function in the form of endothelium-dependent and independent vascular responses, as well as a reduction in maximal vasodilator capacity, was present prior to any manifestation of clinical presentation.

Impaired pressure induced vasodilation (PIV) in young adults with Type 1 diabetes was investigated by Koitka *et al*. (2004a) who noted that vascular and neurological mechanisms were both likely to be involved in foot ulceration. They found an impaired vasodilation to ACh but not to SNP between the group with diabetes and the control group with twelve subjects in each. Although Sigaudo-Roussel *et al.* (2004) carried out their study on streptozotocin-induced diabetic mice and healthy control animals; they also found that endothelial impairment was enough to severely alter PIV. PIV

suppression was noted as possibly leading to complications such as foot ulceration in their study utilising ten subjects in both groups under investigation.

A pilot study to investigate the effects of local pressure on microvascular function in the diabetic foot, with 16 patients and 8 healthy controls was carried out (Newton *et al.* 2005). The subjects with diabetes displayed higher plantar pressures than the control group, but no significant difference was found in basal skin perfusion or in ACh response. Within the patient group, however, baseline flow was increased significantly ($p=0.041$) but the acetylcholine response reduced significantly ($p=0.03$) at the high-pressure compared with the low-pressure site; this was most apparent in those who were particularly at risk of ulceration due to high plantar pressures. The authors concluded that further work is required to determine whether, and under what conditions, this additional hyperaemia is protective or maladaptive.

2.3.13 Laser Doppler flowmetry and reactive hyperaemia

An additional tool for assessing the integrity of the microvasculature is to measure the reactive hyperaemic response, which is defined as a temporary increase in skin and muscle blood flow following an arterial occlusion (Humeau *et al.* 2002; Yamamoto-Suganuma & Aso 2009). Post occlusive reactive hyperaemia (PORH) has been specified as a useful, non-invasive and sensitive indicator for microvascular dysfunction, and has been associated with myogenic, neural and metabolic factors as well as endothelial function, with some debate around which of these elements are responsible for the peak as well as the recovery phase (Humeau *et al.* 2002; Cracowski *et al.* 2006; Rossi *et al.* 2006; Yamamato Suganuma & Aso 2009; Schlager *et al.* 2012).

The ability to continuously monitor perfusion has made LDF an important mode for study for post-occlusive reactive hyperaemia (PORH) (Humeau *et al.* 2002; Morales *et al.* 2005; Roustit *et al.* 2010). Useful parameters for reactive hyperaemia include time to peak flow and the value of peak flow. These parameters have been found to provide early detection of vascular alterations such as prior to the development of clinical symptoms in diabetes mellitus, and also to show the effects of smoking and peripheral vascular disease (Humeau *et al.* 2002; Morales *et al.* 2005; Rossi *et al.* 2005). PORH was compared in subjects with Fontaine classification of stage 11 PAOD displaying intermittent claudication, but with no pain on resting, with healthy controls (Rossi *et al.* 2005). In PAOD patient's skin perfusion was not impaired during baseline i.e. prior to occlusion, which was thought to be a result of compensatory mechanisms related to endothelial, myogenic and sympathetic activities. During hyperaemia however, patients displayed reduced vasoreactivity suggesting perhaps an exhaustion of compensatory mechanisms.

Although LDF is useful for monitoring the PORH response, until recently this has only really been possible with single point instruments (which have their inherent limitations as mentioned in a previous section) since these instruments are able to continuously monitor perfusion and capture the rapid changes in perfusion post occlusion. A recent development, however, has been the introduction of laser speckle contrast imaging which has the capacity to perfusion from larger areas of tissue in near real time.

2.3.14 Laser speckle contrast imaging (LSCI)

2.3.14.1 Background

In the1960s a ‘grainy’ pattern was discovered when laser light was viewed on a matt surface such as glass, paper or dull metal, and this speckle pattern lead to the term being adopted (Briers 2001). It has been described as an interference when light has been reflected from different parts of an illuminated surface (Briers 2001; Royl *et al.* 2006).

With movement of an object, changes are noted in the speckle pattern. When a solid object undergoes small movements the speckles appear to move with the object, however with large movements the pattern undergoes complete change and frequency of the fluctuations is dependent on the velocity of the motion. This phenomenon is known as ‘time-varying’ speckle; Stern in 1975 was the first to note the potential for speckle changes when assessing blood flow. Royl *et al*. (2006) noted that ‘during increased blood flow the intensity variations of the speckle pattern are more rapid’. The group found that in the exposure time the contrast of the pattern was reduced and quantification of the blurring effect was in fact possible with analysis of adjacent areas within the illuminated area. The speckle contrast K was then calculable thus; the mean velocity of moving elements could be calculated using this speckle contrast. It was noted that this offered a faster way of signal processing which allowed full-field imaging of skin perfusion in near real time (Briers 2001; Roustit *et al.* 2010; Tew *et al.* 2011).

2.3.14.2 Utilisation of LSCI in blood flow measurement

LSCI was reviewed as a method of assessing skin microvascular function and dysfunction and it was concluded to be a promising new method of assessment both

with and without pharmacological intervention in iontophoresis, with the main advantage being the reproducibility of the equipment (Figure 2.10) (Mahe *et al.* 2012). The reproducibility of laser speckle contrast imaging (LSCI) was compared with LDF as a method of assessing skin microvascular reactivity when utilising PORH (Roustit *et al.* 2010). It was found to offer both rapid skin blood flow measurements over wide areas coupled with good resolution. LSCI also displayed very good inter-day reproducibility when assessing forearm PORH and local thermal hyperaemia. Although the findings were positive, the study had some limitations such as low number of subjects and the authors concluded that more data would be required to clearly evaluate any linearity between LSCI and skin blood flow.

A comparison of inter-day reproducibility utilising PORH with single point LDF integrated probe LDF with LSCI was carried out (Tew *et al.* 2011). In agreement with Roustit *et al*. (2010) they found LSCI fared better than LDF due probably to the larger skin area under measurement, however they found that LDF was more sensitive to changes in skin blood flow at lower levels found on the finger pad area. It was also noted that the LSCI signal was more sensitive to changes in red blood cell velocity than concentration, and in agreement with Nakagami *et al*. (2010) they found that the speckle contrast was not linear to either velocity or perfusion. In fact, the relationship between speckle contrast and velocity was highly non-linear. It was concluded that this did not preclude the use of LSCI for quantitative analysis of perfusion, but that careful calibration and or interpretation would be required. However, the known percentage change in velocity did produce the same change in speckle contrast, thus LSCI was found to be more useful in measuring changes in perfusion rather than absolute measurements (Tew *et al.* 2011). The advantages over LDF are that it offers high spacial and temporal resolution and is relatively user friendly and cheap to use (Choi *et al.* 2004).

An investigation into the use of LSCI for the assessment of circulation in pressure ulcers was undertaken (Nakagami *et al.* 2010). Although this work was utilising animal studies and small numbers were used, nevertheless it was concluded that LSCI could have great potential for assessing the severity of tissue damage in deep tissue injury, although much more work would be required in the area.

Figure 2.10 LSCI-FLPI® system (Moor Instruments Ltd.)

2.3.15 Summary

LDF being non-invasive, has proven to be an easy to use method of assessing the microvasculature directly. It utilises a helium-neon light source of wavelength 630-670nm. Some issues with reproducibility have arisen, but generally by careful utilisation of protocols the artefacts which could arise have been controlled. LSI displays better reproducibility, but is limiting in that pressure cannot be directly applied

to the area when LSI is being utilised. It is not possible to utilise absolute units of measurement with laser Doppler equipment, and arbitrary units (AU) have been universally adopted, with blood flux being utilised which is the average speed and concentration of moving erythrocytes in the tissue volume under study. Iontophoresis is a non-invasive, local method of migrating substances across the skin surface using a small electric current which is safe and has little systemic impact. It is particularly useful when investigating blood flux in the locality of subjects with systemic disorders like diabetes, when little disruption to systemic blood flow is important. Reactive hyperaemia is a temporary increase in skin and muscle blood flow following a temporary arterial occlusion. It can be utilised as a non-invasive indicator for microvascular dysfunction as the peak flow and time to reach peak flow on release of the occlusion are related to NO activity and as such the method is a good indicator for endothelial function. LSCI was developed from an interference when light has been reflected from different parts of an illuminated surface. Movement of objects lead to changes in the speckle pattern. This method of assessment for blood flow offers rapid and real time capabilities as well as good inter-day reproducibility, however, little work to date has been carried out with this equipment on the lower limb and in particular to explore the relationship between pressure and perfusion.

2.4 THE RELATIONSHIP BETWEEN PRESSURE AND BLOOD FLOW

2.4.1 Background

Much work has been carried out in the past concentrating on the area of decubitus ulceration, as this form of tissue breakdown has long been associated with externally applied pressure leading to occlusion of local microvascular vessels with the outcome of ischaemic injury to the soft tissues (Schubert & Fagrell 1989; Xakellis *et al.* 1993; Abu-

Own *et al.* 1993 and 1995; Schubert & Heraud 1994; Colin & Saumet 1996; Mayrovitz *et al.* 1997; Mayrovitz & Smith 1998). Xakellis *et al.* (1993) showed that pressure induced tissue damage followed a time-pressure curve, i.e. the lower the applied pressure, the longer time required to cause tissue damage. They also stated that due to the exploratory nature of their study of two different age groups, definite conclusions could not be drawn, but their preliminary findings suggest that healthy adults, regardless of age, display an increase in dermal blood flow with time when low levels of compressive pressure are applied. This suggests a more complex association exists between blood flow and externally applied compressive pressure. Meinders *et al.* (1996) noted that when loads are high enough and the skin subjected to the loads for a prolonged period of time, ischaemia and necrosis can result. Their findings were that heel microcirculation is vulnerable to compression. However, they went on to note that the relatively high pressures exerted on the plantar aspect of the foot during normal standing and walking never resulted in necrosis. They reported that the capillary basement membrane is known to be at its thickest in the foot and is thought to be involved in resisting externally applied pressures which when coupled with the hyperaemic response following reduction of pressure could offer protection from tissue damage. The post occlusive hyperaemic response following heel loading was found to offer protection following rapid and significant reduction in heel perfusion when investigating the skin microvascular response of the plantar aspect of the foot to changes in externally applied pressure (Mayrovitz & Smith 1998).

A comparison of skin blood cell flux (SBF) on different sites, namely the sacrum and gluteus region was carried out by Schubert and Fagrell (1989). They found that the cause of greater numbers of pressure sores being sited at the sacrum could be related to the comparatively poorer regulation of microvascular flow recorded in this area. In a subsequent study it was noted that shear force could also play a role, but was much

more difficult to measure than compressional pressure, as well as vasomotion, i.e. spontaneous rhythmic changes in small vessel diameter produced by contraction and relaxation of their walls was noted as decreasing in frequency with age in animal studies (Schubert & Heraud 1994).

The effects of external pressure on transcutaneous oxygen tension ($tcPO_2$) and skin blood flow (SKBF) were investigated over the sacral region in healthy subjects with a significant decrease in $tcPO_2$ occurring when the external pressure was 40mmHg (5.3kPa) which continued to decrease up to 100mmHg (13.4kPa) when it stabilised. A significant decrease in SKBF was also found when the external pressure was at 20mmHg (2.7kPa) (Colin & Saumet 1996).

Sacks *et al.* (1985) used cylindrical indenters to assess skin blood flow and tissue deformations noted that the decrease in skin blood flow found in association with external loading was a result of 3 variables, the ratios of bone depth and diameter to indenter diameter, and the percent of tissue compression overlying the bone. In a subsequent study (Sacks *et al.*, 1988) the group observed problems with the sensitivity found with the laser Doppler signals when measuring skin blood flow under externally applied pressure. The problems were thought to be due to the sensitivity of the laser Doppler to red cell motion of any kind, including random motion associated with changes in vessel occlusion. This effect became particularly important when blood flow was compromised when the random aspect of the signal became significant in comparison to the diminished blood flow, so that signals were observed when the researchers would have reasonably expected the flow to have reached zero. This study questioned the accuracy of the use of laser Doppler flowmetry for assessing the microcirculation in conditions when the point is reached when the random red cell motion becomes significant.

2.4.2 Biological zero

The ‘biological zero’ or a critical closing pressure and whether it should be subtracted from laser Doppler values are controversial issues (Ashton 1975; Khan & Newton 2003). It implies an actual closure of the muscular blood vessels as the endothelial transmural pressure where the circular tension in the wall overcomes the pressure across it causing an abrupt shut down. At least two mechanisms have been cited as involved in this process, active closure of small arterioles when transmural pressure is lowered either by falls in intravascular pressure, or a rise in tissue pressure above intracapillary pressure (Ashton 1975). The impact of pressure on the microcirculation of the heel was investigated by Abu-Own *et al.* (1995) and they found that at an interface pressure of 50mmHg (6.67kPa) the LDF signal was reduced to a minimal value. They noted that the level of compression had reached the point likely to have caused occlusion as increased pressure caused no further alteration in the laser Doppler signal, which they felt could have indicated the ‘biological zero’. Meinders *et al.* (1996) using healthy volunteers applied static loads to the heel aspect of the foot with the subject in a supine position. Contact pressure and skin blood flow using laser Doppler were simultaneously measured. They found that pressure above 40 kPa stopped skin microvascular blood flow. Nielsen (1983) noted that blood flow could be stopped when the diastolic aspect of pressure was reduced to zero. External pressure required to stop blood cell flux was assessed and it was concluded that flow cessation pressure (FPC) at the ankle level was on average 28 mmHg (3.73 kPa) lower than the systolic ankle pressure. However, it was also noted that in those individuals with pronounced arterial occlusive disease FCP it could be equal or even higher than the ankle pressure (Svensson *et al.* 1990). Thus, there is some debate regarding the pressure at which biological zero occurs, and Khan & Newton (2003) noted that the value of biological zero was in fact insignificant in comparison to the blood flow measurements found in other studies.

2.4.3 External pressure and pressure induced vasodilation (PIV)

In a study of subjects with diabetes and severe diabetic neuropathy, nutritional blood flow to the plantar region of the foot was not found to be decreased, in comparison to control subjects (Proano *et al.* 1992). They found that the critical plantar foot pressure which caused nutritional blood flow to the skin to be arrested was 3 N/cm^2 (30 kPa) in both diabetic and control subjects, and below this value blood flow was independent of pressure. This finding indicated that large areas of the sole of the foot were generally subjected to circulatory arrest when in a standing position. This study was limited in that only 10 subjects and 8 controls were included, and fluorescein flowmetry does not measure rapidity of circulatory return as the laser Doppler would. However, it should be noted that although a limited study in terms of numbers of subjects, the neuropathic group did display severe neuropathy coupled with a known history of previous ulceration. The effects of applying an external uniform compression to the lower limb have also been investigated for any unwanted effects on the arterial flow to the lower limb. Fromy *et al.* (1997) reported that both arterial inflow to the lower limb and forefoot microcirculation were reduced even in healthy young subjects with a uniform pressure of 10 mmHg (1.33 kPa). Sabri *et al.* (1971) noted that compression produced by bandaging in horizontal supine subjects may be harmful unless carefully controlled in that external pressure of 15 mmHg (2.00 kPa) significantly reduced femoral arterial flow.

Fromy *et al.* (2002) used Doppler flowmetry over the internal anklebone in response to local pressure applied at 5.0 mmHg (0.67 kPa) per minute in three groups of subjects. One group displaying clinical and subclinical neuropathy, another without neuropathy, and the third was healthy matched control subjects all assessed at usual room temperature. Their findings were in contrast to Proano *et al.* (1992) in that even in

those subjects with diabetes but without neuropathy, blood flow response to locally applied pressure was impeded, and in agreement with the findings of Newrick *et al.* (1988) who also found peak flow to be reduced in neuropathic patients as well as noting a prolonged period for blood flow to return to baseline with neuropaths. In fact Fromy *et al.* (2002) noted that skin blood flow decreased significantly from baseline at much lower pressure in diabetic subjects, i.e. at 7.5 mmHg (1.00 kPa) in diabetic subjects and 48.8 mmHg (6.51 kPa) in controls which they noted could explain the high risk in diabetes of developing plantar and decubitus ulceration, although this extrapolation needs to be viewed with caution in light of the positioning of the study site not being on the plantar aspect of the foot. Fizanne *et al.* (2004) utilising rodents discovered that a transient increase in blood flow could be elicited in response to an innocuous local pressure application, and defined this as pressure-induced vasodilation (PIV). This response was dependent upon capsaicin-sensitive nerve fibres and calcitonin gene-related peptide (CGRP). A further study investigated the impact of diabetes on PIV utilising streptozotocin-induced diabetic mice, suggested that endothelial impairment in the mice was enough to severely alter the PIV response which appeared to be highly sensitive to endothelial NO levels (Sigaudo-Roussel *et al.* 2004). This was advanced to an investigation utilising human subjects by Koitka *et al.* (2004a) who found that PIV, which relied on unmyelinated afferent excitability, might be impaired in diabetic patients. They measured cutaneous blood flow using LDF on the head of the first metatarsus in response to applied pressure at 5.0 mmHg (0.67kPa) in warm conditions. Responses to iontophoresis of ACh (endothelium-dependent) and SNP (endothelium-independent) were measured in the forearm. Their findings suggested that PIV was impaired in the feet of diabetic subjects. The non-endothelial mediated response to SNP was preserved in diabetic subjects while the endothelium-mediated response to ACh was impaired. The study was limited in that Type 2 diabetic subjects were excluded

from the study and the subject numbers were 12 in each of the patient and control subject groups, and were derived from a young adult population. In a subsequent study PIV was found to be impaired by low skin temperatures (Koitka *et al.* 2004a). Local skin temperature variations have been noted to have an effect on postural skin blood flow (Hassan *et al.* 1986; Hassan and Tooke, 1988; Oberle *et al.,* 1988; Flynn *et al.* 1989). PIV reaction was also found to be age related, and was found to lacking in an older age group (Fromy *et al.* 2010), this finding was in agreement with Petrofsky *et al.* (2009), who also found PIV to diminish in an older group of subjects with diabetes when compared with a group of young subjects with diabetes and healthy controls.

2.4.4 Summary

Initial work regarding the impact of pressure on blood flow has been in the area of decubitus ulcers which are essentially soft tissue breakdown associated with externally applied pressure. The biological zero is a critical measurement which leads to closure of the vessels under pressure. It is a controversial subject and several different values have been proposed as the level of closure, however the values mooted are insignificant when compared with the actual blood flow measurement and as such the impact on measurements makes little impression. PIV has been found to be a transient increase in blood flow which can be elicited in response to an innocuous local application of pressure. It has been found to be age related, impaired at low temperatures and more importantly impaired with diabetes.

2.5 METHODS OF ASSESSING PRESSURE DELIVERY AND BLOOD FLOW

Abu-Own *et al.* (1995) set out to develop equipment to investigate the impact of compression of the lateral aspect of the heel on blood flow to the area. They utilised a weighbridge device with a cantilever mechanism around a central pivot which was loaded with weights at one end. The other end was placed in contact with the heel utilising an acrylic indenter attached to a pressure sensor. The pressure sensor was attached to the laser Doppler which then logged the data recorded on a computer. The weights used to apply forces were 50, 100, 200, 400, 600, 800, 1000, 1250 and 1500g. The interface pressure and LDF was measured using the pressure transducer. The study was carried out in a temperature controlled environment of 22^{o} C and 30% relative humidity following a period of 20 minutes acclimatisation. They noted some of the known limitations of LDF in that its reproducibility is good in a given population, but not in individual subjects, however no limitations were discussed around their pressure delivery system or any details given about calibration or repeatability with the system.

An experimental set up to allow the application of several levels of load on the plantar aspect of the foot with the subject in a relaxed supine position was constructed by Meinders *et al*. (1996) (Figure 2.11). The area under study was the central aspect of the heel area and in order to apply the load, the foot was placed in a shoe construction. The shoe was then attached to a rigid board placed at the end of the bed. The heel area of the shoe was cut out so that a piston with a $2.0cm^2$ flat contact area could be used to penetrate and apply the load. A spindle mechanism was placed behind the piston so that load could be released. The applied load was measured by a load cell situated underneath the piston and blood flow measured by LDF, with the LDF probe acting as the piston for the application of the load. The testing was carried out with constant temperatures and subjects were acclimatised to the environment for a period of 15

minutes and had refrained from alcohol for 24 hours as well as caffeine for 12 hours prior to commencement of the study. Experimental limitations were discussed, for example the small contact area of the piston could have induced pain when applied for 5 minutes. Pain is a stimulus for the sympathetic nervous system which also controls arterio-venous anastomoses (AVA) blood flow, so could have been responsible for some of the alterations in blood flux found. They also noted that as the study was carried out with the subjects in a supine position and not with the limbs in the dependent position, the rise in capillary pressure could have been limited by precapillary vasoconstriction. However when subjects are asked to stand the small inevitable movements which occur to maintain position could cause a disturbance of the LDF signal, thus the researchers made the decision carry out the study with the subjects in a supine position.

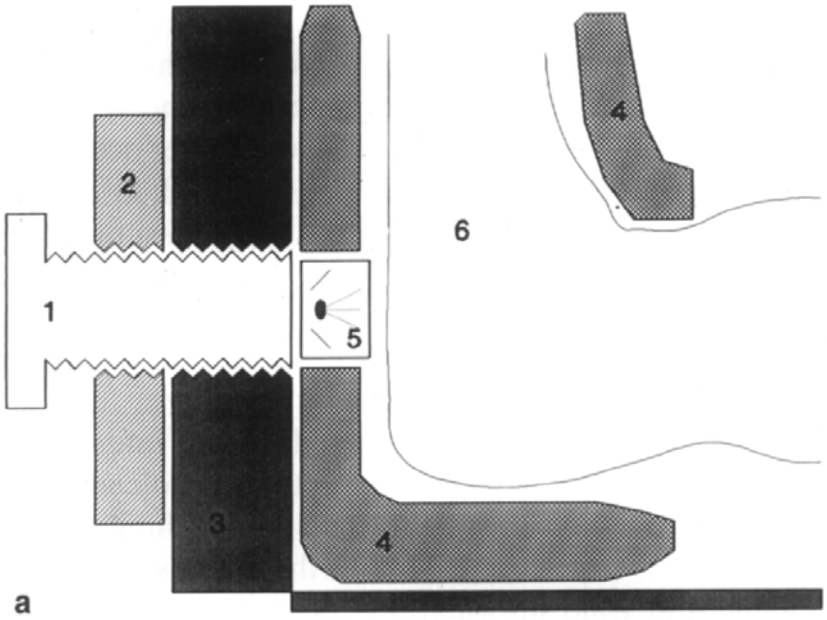

Figure 2.11 The experimental set-up: 1, spindle mechanism; 2, load cell; 3, board at the end of the bed; 4, shoe; 5, LDF probe with the laser source; and two photodiodes detecting the reflected light; 6, foot (adapted from Meinders *et al.* 1996).

A progressive calibrated pressure device to measure cutaneous blood flow changes to external pressure strain was developed by Fromy *et al.* (2000) as illustrated in Figure 2.12. This device consisted of a similar system to that of Abu-Own *et al.* (1995), with a mechanical aluminium bar (400mm long with a diameter of 8mm) used as the axis of the weighbridge. The homemade balance was designed to receive a cutaneous laser Doppler probe and a small circular plastic container to increase the pressure on the site of measurement at one end of the aluminium bar and an adjustable balance weight (15g) which could move along the bar to balance and maintain the bar in a horizontal position. A laser Doppler probe was connected to a laser Doppler flowmeter. The container was filled with water and the instrument was calibrated before each study with an electronic weighing system to find the linear relationship between the volume of water and the mass reading on the weighing system. This device was subsequently used for a series of studies measuring skin blood flow and locally applied pressure in the fingers of diabetic subjects (Fromy *et al.* 2002; Koitka *et al.*2004b; Fromy *et al.* 2010). No issues were recorded around the pressure device, although again the limitations of the LDF were detailed.

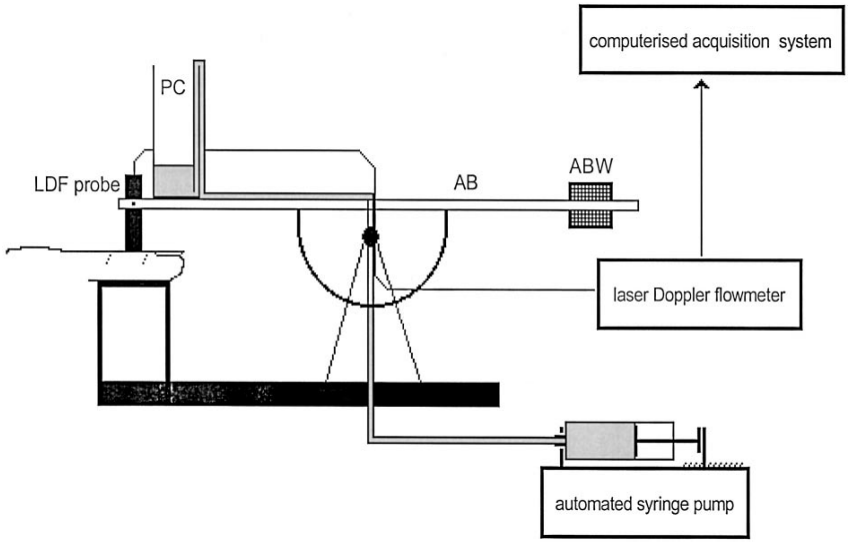

Figure 2.12 The mechanical device developed by Fromy *et al.* (2000).The device was used to apply a progressive calibrated pressure to a local area of skin and simultaneously measure cutaneous blood flow with a laser Doppler probe at the same site. PC: plastic container; AB: aluminium bar; ABW: adjustable balance weight (adapted from Fromy *et al.*(2000).

Cobb and Claremont (2001 and 2002) have investigated a method of measuring blood flux in plantar skin tissue under the first metatarsal head in standing and walking. They developed a custom made shoe for each test subject, using an orthopaedic insole made of plastazote foam, with a recess cut out at the first metatarsal head and a channel cut from the recess of the heel to house the sensor and electronic connection (Figure 2.13). A prototype sensor length 30mm and depth 12.5 mm made from double-sided copper clad board and acrylic sheet was developed. The device repeatability and device to device repeatability were recorded as better than 12.5%. The instrumentation recorder had an internal rechargeable battery to allow continuous recording for 50 mins. onto digital audiotape and was carried by the subject at waist level with a shoulder strap. Recorded data was then downloaded to a computer. As well as the limitations in possible subject numbers this posed, other problems like movement artefacts limited analysis to the swing phase of gait rather than capturing true foot to insole or foot to ground data. It should also be noted that having connections attached to the subject can lead to an alteration in normal gait pattern.

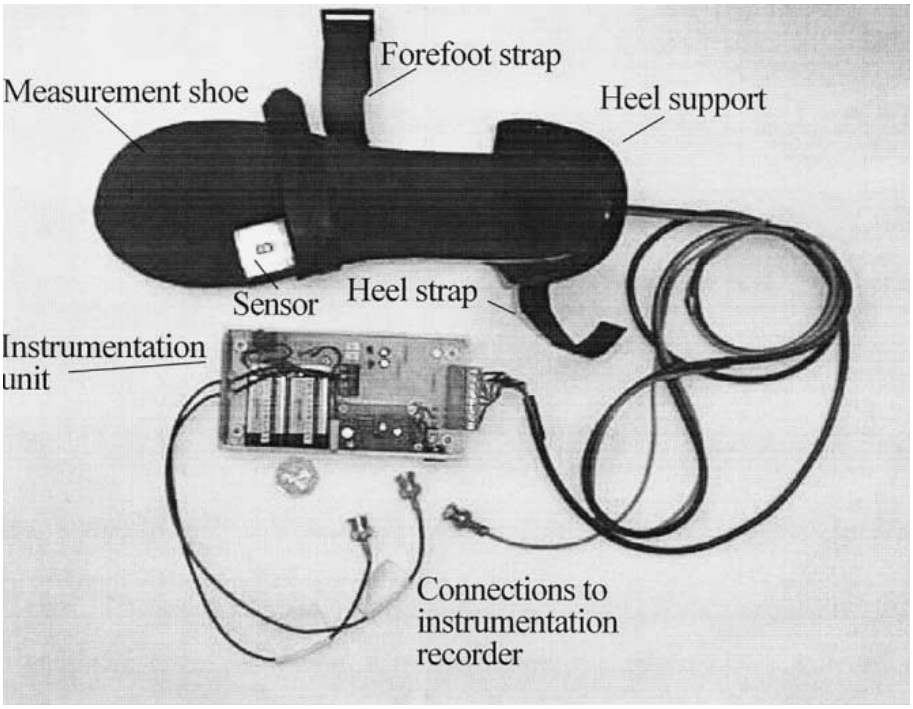

Figure 2.13 Measurement shoe and waist mounted instrumentation unit, with the sensor situated under first metatarsal head (adapted from Cobb & Claremont 2001).

Simultaneous measurement of plantar pressure and blood flow was taken a stage further with the development of a system which could measure both in a semi-weightbearing position. Santos *et al.* (2003) found a significant difference between the blood flow measurements under pressure in a supine position to that in a semi-weightbearing position (Figure 2.14). A significant difference was found between baseline laser Doppler flowmetry (LDF) in the supine and semi-weight bearing positions. Differences between both positions in the maximum hyperaemic response measured on release of the pressure and the time taken to actually reach the maximum hyperaemic response. This was a pilot study and only 4 subjects were included and as such it is difficult to see how meaningful statistical analysis could be achieved.

Figure 2.14 The device in a semi-weightbearing position (adapted from Santos *et al.* 2003)

Hahn *et al.* (2007) developed a lumped-parameter model to assess the impact of plantar pressure on the diameter of blood vessels in the big toe. The system utilised an energy system approach to develop the lumped-parameter differential equations which was based upon results of published data. It was hoped that this could then be expanded to

model the foot as a whole rather than just the hallux. Limitations included the fact that this was a lumped-model which resulted in averaging of blood flow for the region rather than providing actual values. However, further limitations could be the validity of the studies utilised to develop the model itself.

2.6 SUMMARY

Increased plantar pressures and impairment of the local microvasculature have both been cited as risk factors in the development of foot disorders, in particular ulceration found in association with conditions such as diabetes. The mechanisms for increased plantar pressures in association with diabetes are multifactorial, and contradictory evidence regarding the alterations in skin structure indicates that further work is required in that area. Although much development work has improved available measurement systems, there is still no perfect system available and there are pros and cons for both platform and in–shoe systems with further work required in the area of shear stress.

The endothelium is a complex structure with its function being regulated by the production of many dilatory and constricting substances. Recent work would indicate that impairment in nitric oxide production is associated with diabetes. When LDF is utilised with a strict protocol, artefacts can be limited to ensure reproducibility, and in association with iontophoresis it provides a sound method of investigating endothelial function. Nitric oxide activity can be measured non-invasively with reactive hyperaemia.

From the literature it would appear that further work is required to investigate endothelial function when under local pressure. Current commercial systems have not

been designed to measure both pressure and blood flow simultaneously, and methods developed to measure blood flow in conjunction with pressure to date have been at best experimental, with none of the systems utilised to investigate actual endothelial function in conjunction with pressure. This study aimed to develop equipment to measure both pressure and endothelial function simultaneously and to utilise reactive hyperaemia as a measure of endothelial function as well as to compare endothelial function under pressure in a group with diabetes when compared to healthy control subjects.

Chapter Three

Design and Development methods

3 DESIGN AND DEVELOPMENT METHODS

3.1 THE PRESSURE DELIVERY SYSTEM

3.1.1 Introduction

Studies of plantar foot pressure and blood flow to the lower limb are areas which have generated great interest, particularly when investigating pathologies such as diabetes mellitus. Much research has been carried out into the aetiological factors leading to issues with tissue viability, and amongst the factors, prominence has been given to both plantar pressure abnormalities and vascular disorders. Many advanced computerised systems have been developed and are currently available to measure plantar pressure both in healthy feet and in disease as noted in Section 2.2. Similarly, numerous systems are available to measure blood flow resulting in the capability of utilising non-invasive methods to assess both macro and microcirculation. The ability to measure plantar pressure and blood flow simultaneously still remains elusive due to a lack of commercially available equipment capable of measuring both. Thus, in order to investigate the impact of plantar pressure on blood flow to the plantar aspect of the foot, a pressure delivery system that could deliver known pressure had to be developed, capable of functioning in situ while the blood flow measurement was being carried out simultaneously.

3.1.2 Development of a plantar pressure delivery system

In order to enable pressure to be directly applied whilst simultaneous blood flux measurements were being undertaken, the decision to utilise the laser Doppler flowmeter DRT4® (Moor Instruments UK Ltd.) rather than e.g. laser Doppler perfusion

imaging was made as it was possible to have the probe measuring blood flux with LDF in skin contact. Around 10 months of exploratory work was undertaken to investigate and develop equipment which would provide the optimum method of accurately applying pressure to the plantar aspect of the foot while investigating endothelial function with iontophoresis.

Initial work considered having the subject in a weightbearing position, thus providing natural pressure delivery to the area. This proved to be difficult to achieve due to several factors, the bulk of equipment required to be in situ to undertake iontophoresis and blood flux measurements, the requirement to ensure that the probe and iontophoresis solution remained in contact with the skin, but the overriding factor was movement artefacts created by the natural body movement when an individual is maintaining a standing position prevented accurate blood flux measurements. Thus, the decision was made to place the subjects in the supine position where movement artefacts could be more easily controlled and the equipment would be visible and easily monitored. As ideally pressure delivery and blood flux measurements were to be carried out in the same position on the foot, investigations were then carried out regarding the possibility of a LDF probe acting as the final point of delivery for the pressure as well as measuring blood flux. During preliminary testing, the Doppler probe (DP2), which is a standard probe utilised with the DRT4® system suitable for use with an iontophoresis chamber, was found to have a strong, rigid plastic cylinder casing with a length of 30 mm and diameter of 6 mm. This outer durable shell was designed to house and protect the optic fibres. However the optic fibres themselves required to be free of pressure or angulation which could have resulted in damage as well as impacting upon the results. Thus a metal housing or ‘cuff’ was developed which could firmly hold the DP2 cylinder allowing it to act as the end point of the pressure delivery system, and to allow freedom for the optic fibres to function as normal which can be seen in Figures 3.1, 3.2 and 3.3.

The method of pressure delivery had to be accurate, light and easy to manoeuvre as well as delivering a pre-measured, sustainable pressure, without altering the function of the DRT4® equipment. After much thought and discussion, it was decided that the pressure could be delivered via a spring. The spring used consisted of a roll of high yield Type 301 stainless steel finished with zinc plate. The spring had 11.5 coils with an outside diameter of 4.775 mm, free length of 22.224 mm with a load at solid length of 47.282 Newtons/mm^2 (47282 kPa) (Lee Springs). The spring was housed within a metal tube for protection and to allow unimpeded movement. A measuring gauge was attached to the spring indicating the pressure delivered, and the pressure could be set and adjusted via a control dial as illustrated in Figure 3.4.

The delivery system also had to remain in situ and the limb held securely to prevent movement, but to allow for comfort of the individual as the data collection was a relatively lengthy process. A post-surgical boot which had a rigid plastic leg/foot back plate and a fleece lining was thought to be the optimum choice as it was firm enough to hold the limb securely as well as providing comfort for the subject. A metal plate was then devised and attached to the base of the boot. This was adjustable in two directions, in height so that the position of the pressure delivery system and the LDF probe could be altered according to the foot size of the subject, and in depth to ensure that the probe was held in the correct position against the skin surface. Once in place the boot ensured that the equipment and the lower limb of the subject would not move, so ensuring the correct and stable positioning for the data gathering process. The customised boot can be seen in Figure 3.5 and the equipment in situ in Figure 3.8.

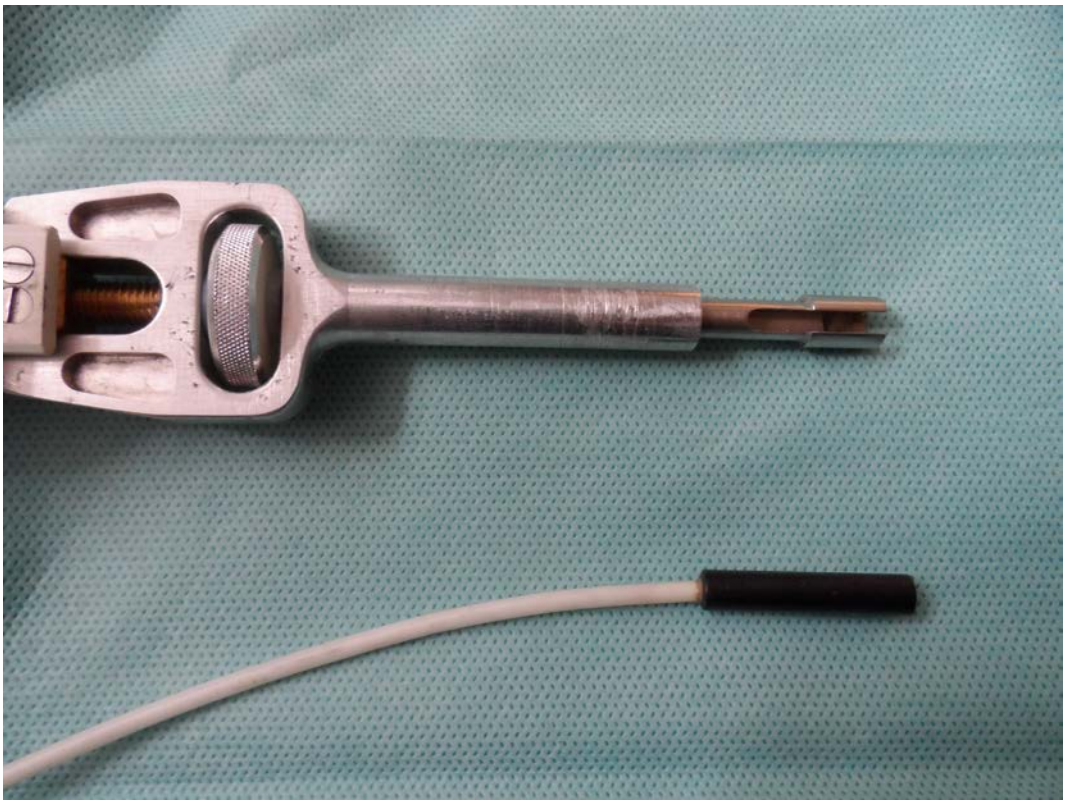

Figure 3.1 The pressure delivery system displaying the housing 'cuff' and the LDF probe

Figure 3.2 DP2 probe held within the base of the pressure delivery system

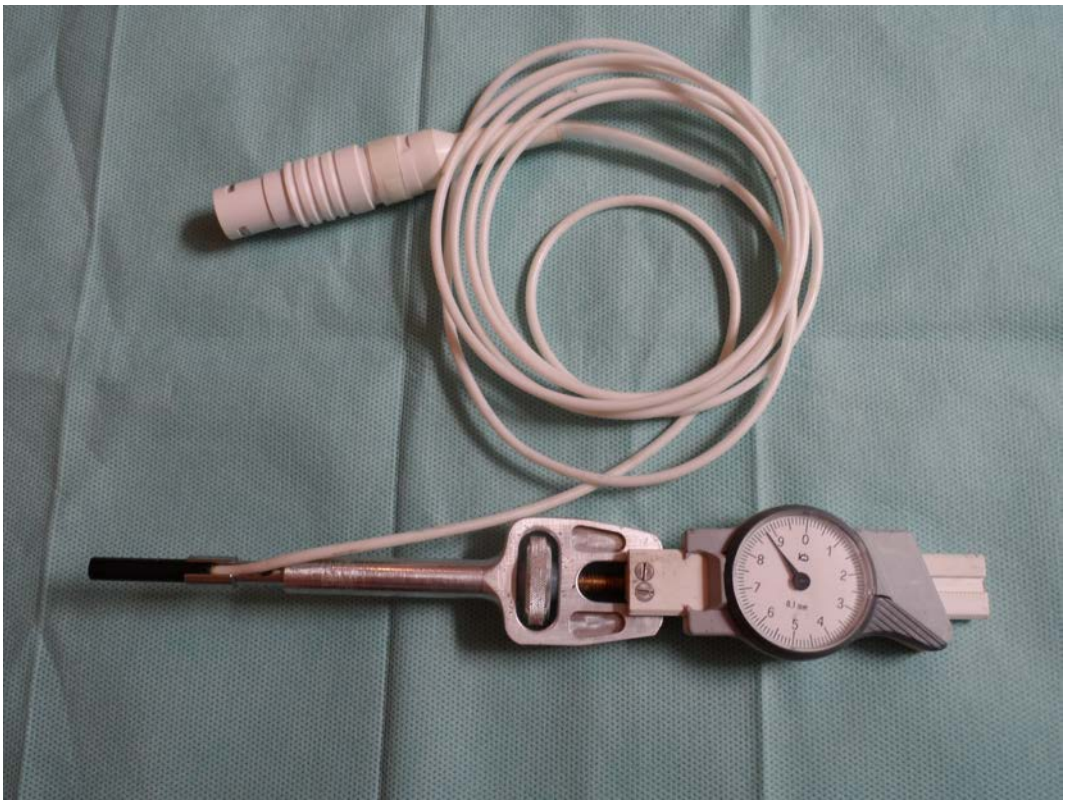

Figure 3.3 The pressure delivery system with DP2 probe attached allowing freedom for the optic fibres to function unimpeded

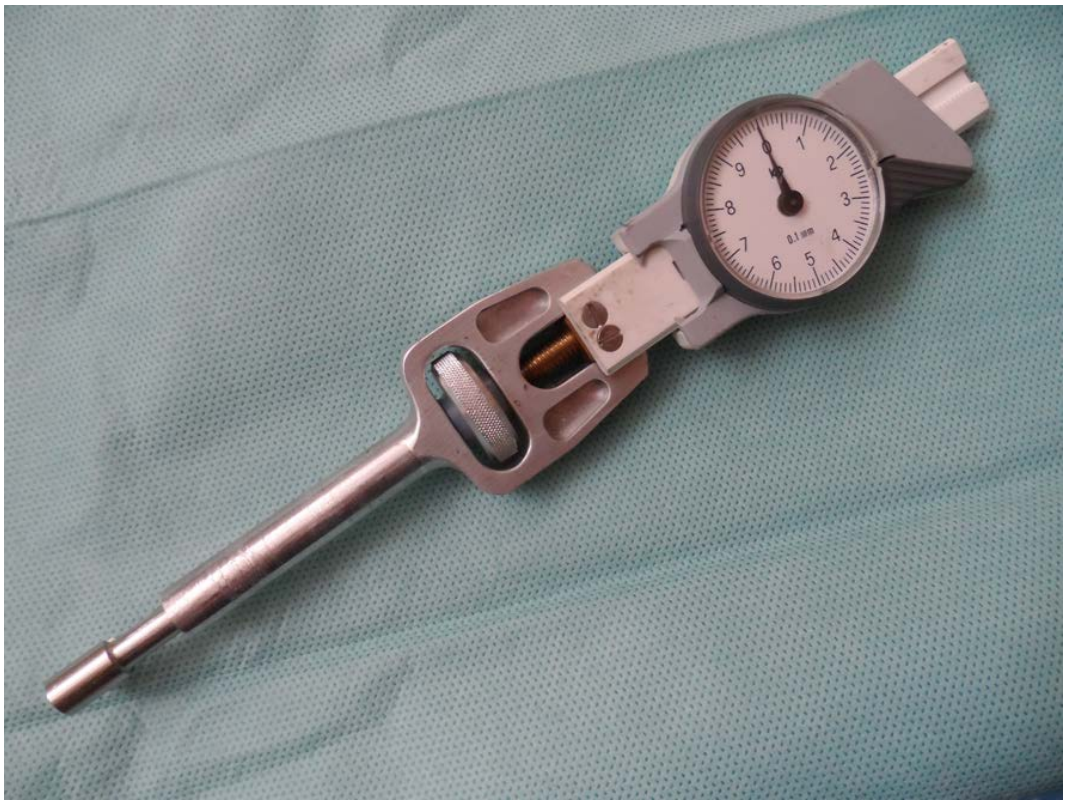

Figure 3.4 The pressure delivery system displaying the measuring gauge and control dial

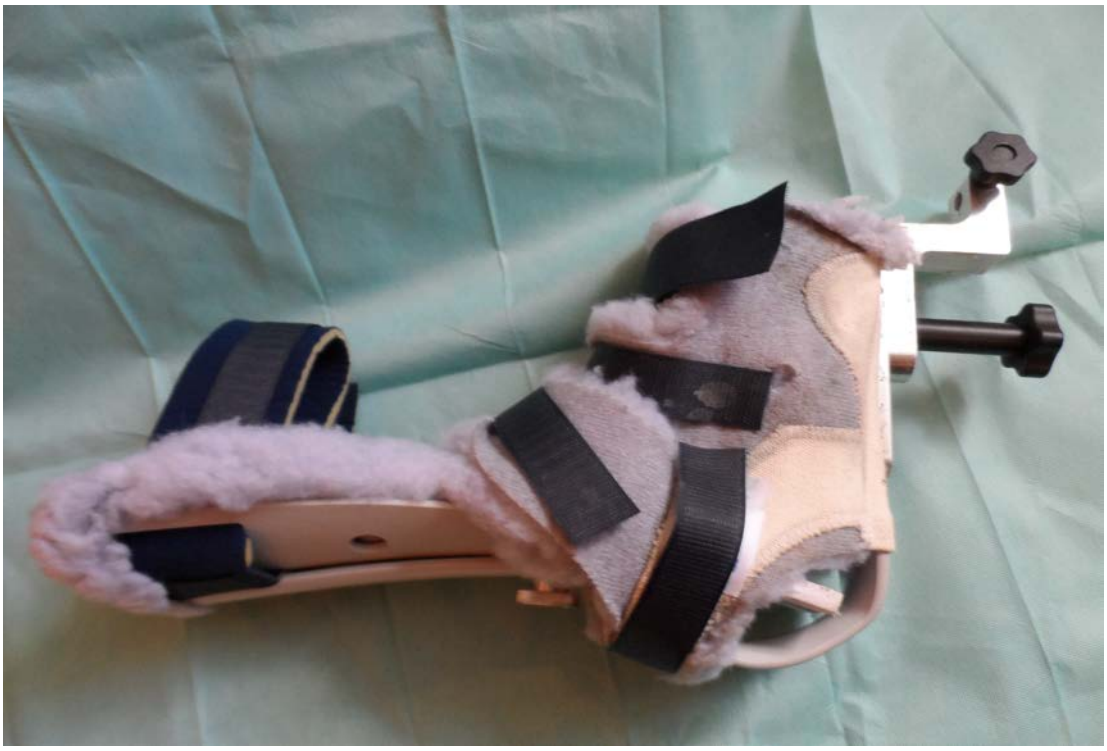

Figure 3.5 The customised boot with adjustable baseplate

3.1.3 In vitro repeatability (test-retest reliability) of the pressure delivery system

The pressure delivery system was tested for repeatability over 14 individual sessions during a period of 3 weeks, utilising a universal testing machine as illustrated in Figure 3.6. The testing equipment manufactured by Zwick Roell measures tensile stress as well as compressive strength of materials, thus a known force can be exerted on the material under test. The universal testing machine consists of a load frame which is the two supports for the equipment, a force transducer affording measurement of the load required, a cross head which is controlled to move up or down with the force as well as a means of measuring extension of deformation. The pressure delivery system was placed in the machine between the grips and the required force was exerted upon it.

Throughout the testing sessions a series of accurate and known forces were applied to the pressure delivery device and measurements taken as noted in Table 3.1 below. The interclass Correlation Coefficent (ICC) with absolute agreement and average measure was utilised to assess repeatability for the data. The ICC was 0.999, 95% C.I. (0.997-1.000), $F = 13381.911$, $p < 0.0001$. The results indicated good repeatability of the device and Figure 3.7 graphically displays the linear relationship between the values obtained and reflects the repeatability of the equipment.

Table 3.1 Mean forces applied with standard deviations to achieve the pressure device dial readings displayed during the testing of the delivery system

Pressure device dial readings	Force means	Std. Dev.
0.5	2.32	0.097
1.0	4.21	0.192
1.5	6.36	0.122
2.0	8.46	0.122
2.5	10.57	0.131
3.0	12.62	0.164
3.5	14.86	0.210
4.0	16.99	0.267
ICC	**0.999**	**(0.997 - 1.000)**
ANOVA	**F = 13381.911**	**p < 0.0001**

Figure 3.6 Universal testing machine (Zwick Roell) with the pressure delivery device in situ

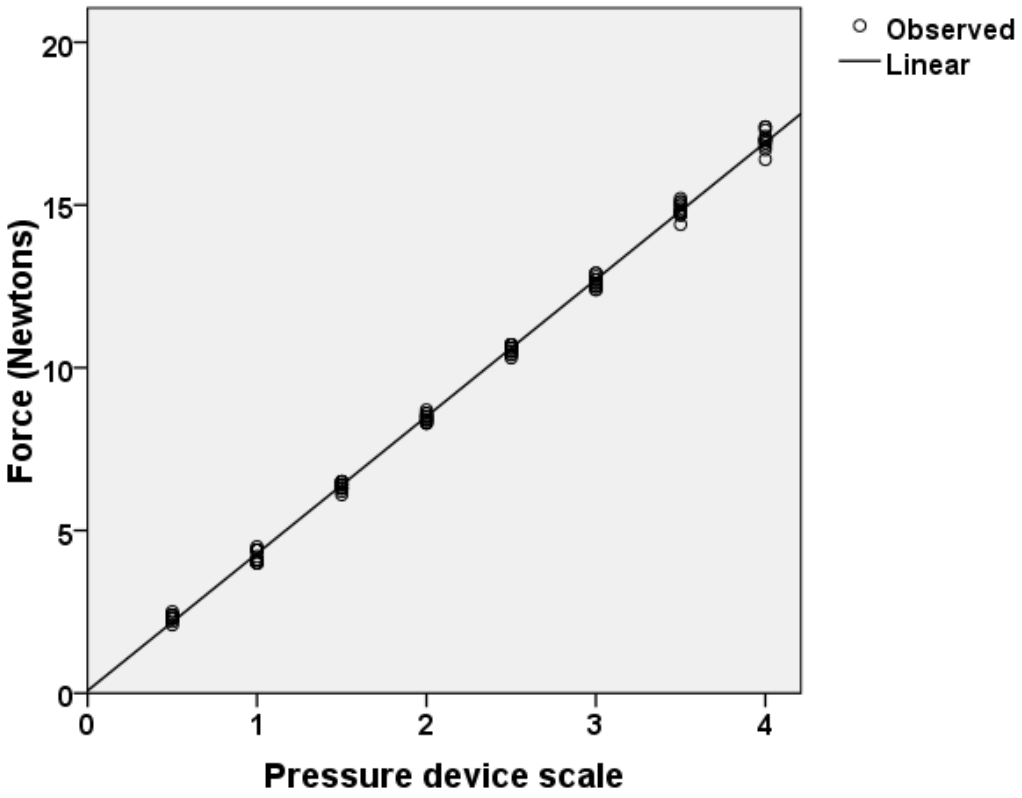

Figure 3.7 Linear relationship of the pressure device scale and applied forces

3.1.3.1 Conversion of force to pressure for utilisation of the pressure delivery device

The DRT4® DP2 probe (Figures 3.1, 3.2 and 3.3) which was the surface contact for pressure delivery had a radius of 3mm, thus an area of 29.16mm^2. As pressure is force/area then e.g. 17.4 Newtons /29.16mm^2 delivers a pressure of 0.59 N/mm^2 or 590 kiloPascals (kPa). Table 3.2 provides a conversion of the readings obtained from the pressure device, and thus the pressures delivered via the system to the plantar aspect of the foot.

Table 3.2 Conversion from force to pressure readings for the pressure delivery system

Pressure device dial readings	Force means	N/mm^2	kPa
0.5	2.32	0.0799	79.9
1.0	4.21	0.1444	144.4
1.5	6.36	0.2181	218.1
2.0	8.46	0.2901	290.1
2.5	10.57	0.3625	362.5
3.0	12.62	0.4331	433.1
3.5	14.86	0.5096	509.6
4.0	16.99	0.5826	582.6

3.1.4 In vivo repeatability of the pressure delivery system

Utilising a single subject, and setting the pressure delivered to 1.5 and 3 on the scale of the device (an equivalent of 218.1 and 433.1 kPa, respectively), a series of 8 testing sessions was carried out on separate occasions assessing the inter-day repeatability of the equipment (Figure 3.8). During each measurement session, the subject was in a supine position on a plinth under the same temperature conditions and pre-test abstinence from food and caffeine for 2 hours as the main study. The tests were carried out in a quiet, temperature controlled room of 22° ± 1° C following a 20 minute acclimatisation period. On each occasion baseline blood flow was recorded under the 3rd metatarsophalangeal joint of the right foot, followed by 1.5 and then 3 scale pressures delivered in conjunction with the laser Doppler flowmetry as illustrated in Figure 3.7. ICC was carried out and a value of 0.976 obtained with 95% C.I. (0.809 - 1.000), $F = 42.187$, $p < 0.0001$, which indicates good repeatability as illustrated in Table 3.3. Thus the system delivered repeatable in vivo measurements when utilised over a period of 8 separate sessions carried out over a period of 3 weeks under consistent external conditions.

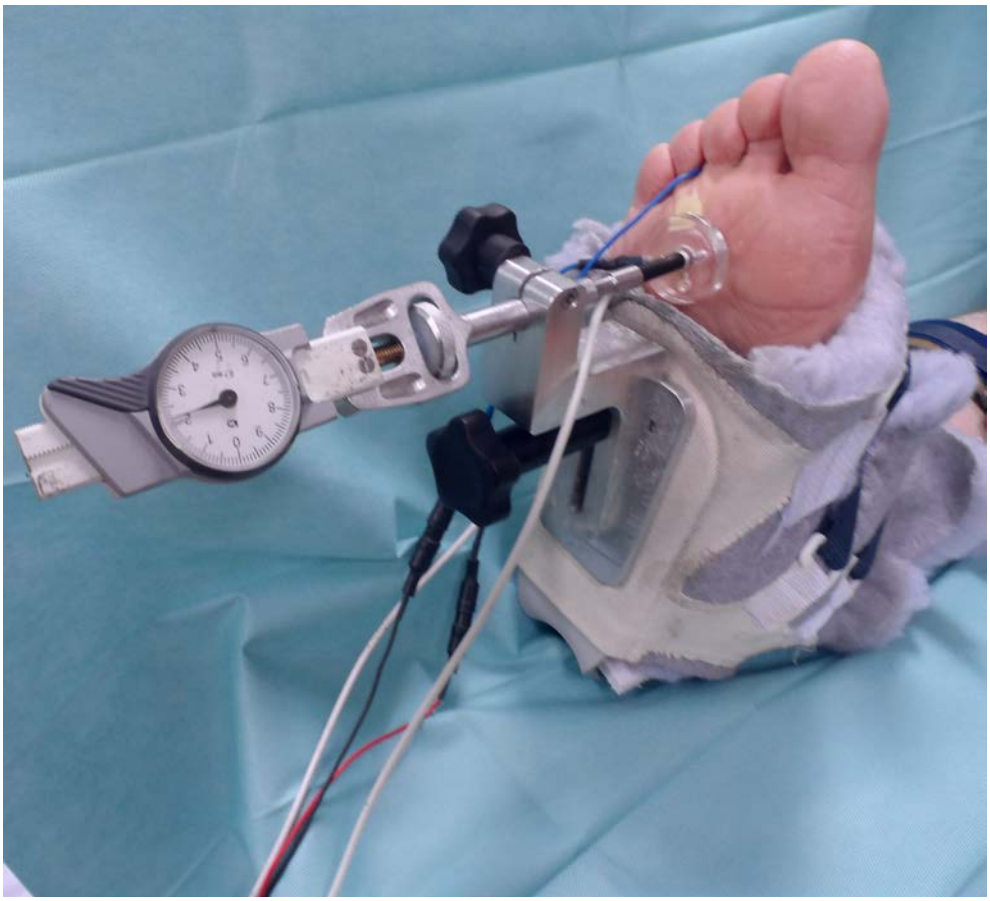

Figure 3.8 Equipment in situ measuring blood flow under the 3rd metatarsophalangeal joint of the right foot, using laser Doppler flowmetry in conjunction with the pressures delivery system simultaneously delivering pressure to the area.

Table 3.3 Inter-day repeatability of the pressure delivery system

Baseline Perfusion (AU)	Perfusion with 1.5 scale pressure added (AU)	Perfusion with 3 scale pressure added (AU)
83		11.2
144.3	40.4	9.4
216.5	42.2	9.9
51.8	20.2	8.2
125.4		11.5
159.6	57.3	11.7
168	28.1	8.2
101.9	42.2	14.2
ICC	**0.976**	**C.I. (0.809 – 1.000)**
	F = 42.187	**p < 0.0001**

On each occasion baseline perfusion was measured under the 3rd metatarsophalangeal joint, and perfusion again measured at the same site with the addition of pressure to a value of 1.5 on the scale (218.1 kPa) followed by pressure to a value of 3 on the scale (433.1 kPa). ICC = 0.976 (0.809 - 1.000) F = 42.187 with p < 0.0001 indicated good repeatability.

3.2 UTILISATION OF LASER DOPPLER FLOWMETRY (LDF)

3.2.1 Introduction

While there are many techniques available for indicating tissue perfusion, LDF is one of the few systems which can directly assess the microcirculation non-invasively, and can achieve this function relatively easily (Khan & Newton 2003; Rossi *et al.* 2006, 2008; Fredriksson *et al.* 2010). The laser Doppler flowmetry system has been extensively validated as an assessment tool for superficial skin blood flow, being found to be user-independent by utilising established protocols which help by controlling environmental temperature, reducing movement and reflection artefacts and refraining from ingesting cigarette smoke, caffeine and food for at least a period of 2 hours prior to assessment (Fullerton *et al.* 2002; Khan & Newton 2003). The DRT4® utilises a helium-neon laser light source operating at around 780 nm (Moor Instruments Ltd.). This provides an estimated perfusion depth of 1-1.5 mm into the dermis, detecting dermal perfusion without deeper influence from the circulation to the underlying skeletal muscle (Eun 1995; Morris & Shore 1996; Fullerton 2002; Khan & Newton 2003; Kvandal *et al.* 2006; Turner *et al.* 2008). While laser Doppler perfusion imaging (LDI) has advantages over the traditional LDF system, having the capability of providing a two dimensional matrix of the blood flux of a larger skin surface area producing a detailed perfusion map, it would be difficult to add pressure directly to the area with this type of system, thus the decision was made to utilise LDF as the DP2 probe could simultaneously measure flux as well as being durable enough to act as a component of the pressure delivery system as described in Section 3.1.2.

3.2.2 Calibration of the DRT4® LDF system with DP2 probe

Prior to utilisation and on a regular basis i.e. every 6 months or following movement of the equipment the DP2 probe was calibrated. Manufacturer recommendations are that recalibration is carried out on an annual basis, although the calibration solution has a shelf life of 6 months (Moor Instruments Ltd.). The probe was calibrated for the channel of the system chosen for use. The calibration solution uses a thermal (Brownian) motion of polystyrene microspheres in water which produces the reference signals required and is thus temperature dependent. The calibration process was carried out in a temperature-controlled environment of between 20 and 22 degrees centigrade. The probe head was positioned in the calibration solution near the centre of the fluid without contact with the container, and the fibre optic lead was unsupported during the calibration process. Successful calibration is registered as 'Calibration OK' on the system.

3.2.3 Choice of chamber for iontophoresis

The chambers utilised for iontophoresis are constructed of Perspex with an internal platinum wire electrode. As this work was to be carried out on the plantar aspect of the foot with the ion chamber in a vertical position, trials were carried out using different chamber sizes to find the optimal size of the ion chamber to use. The ion chamber had to be small enough to be located on the metatarsophalangeal joint plantar aspect of the forefoot, ensuring total contact to prevent surface burning due to uneven current distribution, as well as being strong enough to endure pressure from the delivery system and to house the DP2 probe. Several iontophoresis chambers were investigated, offering more area for solution retention versus affording contact and an element of control and accurate positioning for the DP2 probe. Smaller chambers have been associated with an

increase in skin perfusion when applying either positive or negative charges referred to as a 'galvanic response' (Ferrell *et al.* 2002; Turner *et al.* 2008), however overall the decision was made to utilise a smaller chamber with a central aperture of 6.1 mm diameter and the MIC1-Ion1 chamber was chosen as illustrated in Figure 3.9.

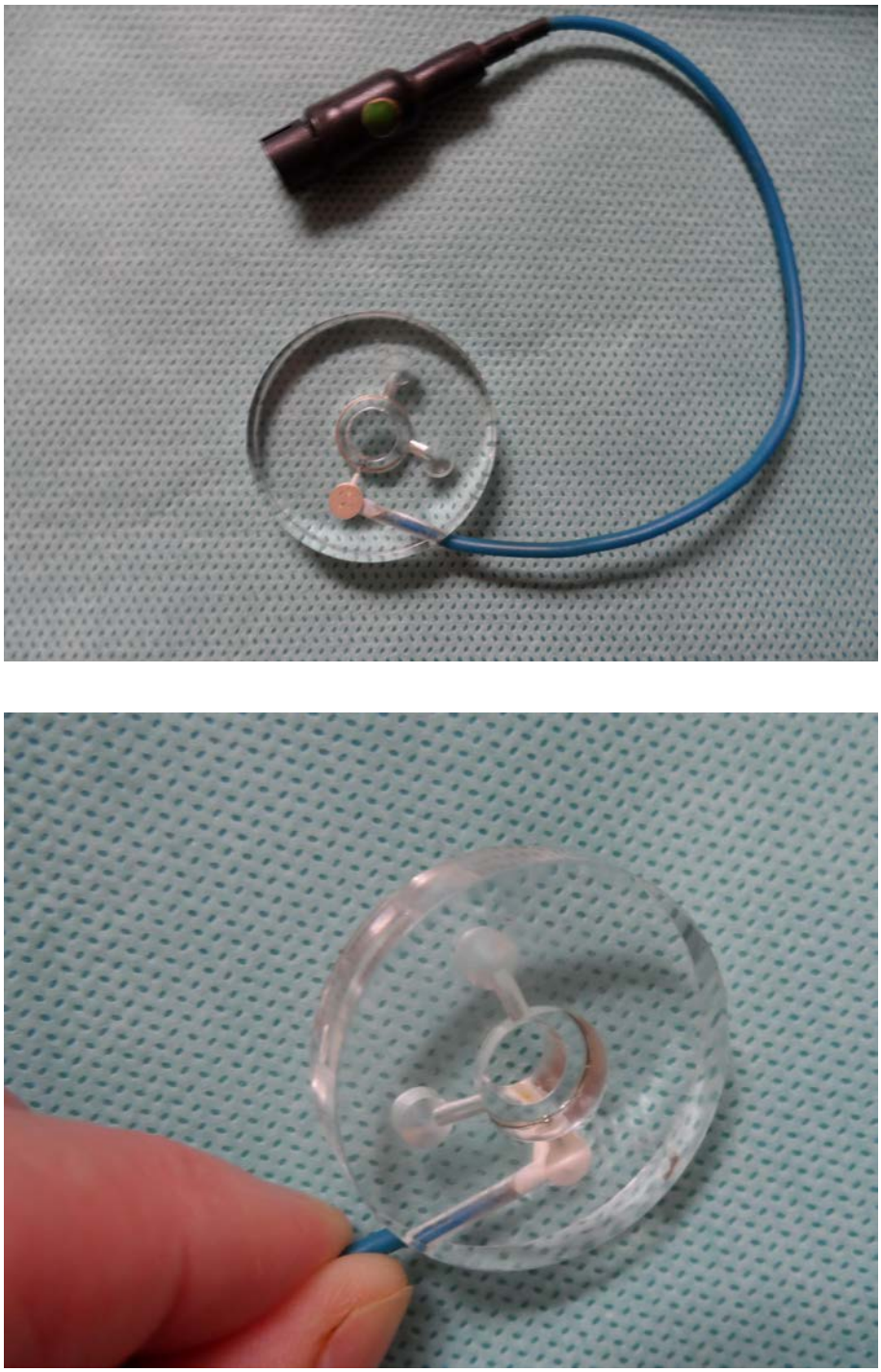

Figure 3.9 The chosen MIC1-Ion1 chamber. Dimensions of the elements of the chamber are a central aperture of 6.1mm and three connecting apertures each 4mm in diameter connected by a 2mm drill which allows flow of solution and top up when required.

3.2.4 Iontophoresis solutions

Acetylcholine (ACh) was the chosen endothelium-dependent agonist and sodium nitroprusside (SNP) as an endothelium-independent vasodilator for comparison (Khan & Newton 2003). Several vehicles have been utilised to dissolve the substances used during iontophoresis, commonly deionised water, tap water, and sodium chloride have been used (Ferrell *et al.* 2002). In fact Morris & Shore (1996) actually found that vehicle alone could lead to an increase in skin perfusion i.e. the 'galvanic response'. Deionised water was the original vehicle of choice; however the dimensions of the chamber then lead to the issue of retaining enough solution in contact with the skin for iontophoresis with the equipment being held in a vertical position and with the addition of the pressure delivery system preventing the utilisation of a cover to prevent spillage. During piloting, difficulties were experienced ensuring the chosen vasodilator solutions containing ACh and SNP remained in sufficient quantities in the iontophoresis chamber. It was decided that some form of inert thickening agent would help prevent leakage and spillage from the chamber. Noon *et al.* (1998) had successfully utilised 2% methylcellulose, and found it to prevent a current induced vasodilator response. The manufacturers also recommend the use of methylcellulose to help with contoured sites (Moor Instruments Ltd.). Methylcellulose is a water soluble derivative of pine pulp which dissolves quickly and is generally used as a thickener, binder or for water retention in pharmaceutical applications. It is stable at room temperatures, colourless, odourless and non-ionic (Dow Wolff 2012).

3.2.5 Ionotophoresis protocol development

Most studies utilising iontophoresis have been carried out on the upper limbs, or the dorsum of the foot (Pitei *et al.* 1997; Caballero *et al.* 1999; Khan *et al.* 2000; Kelly *et*

al. 2001; Pfutzner *et al.* 2001; Koitka *et al.* 2004a; Rossi *et al.* 2005). The area under investigation in this study was the plantar skin of the foot which in comparison to skin elsewhere on the body is relatively thick, with a lack of sebaceous glands but many sweat glands, and the proposed position of the foot was such that the equipment was to be held vertically on the plantar aspect. Thus, a suitable protocol had to be developed and the possibility of utilising methylcellulose as a thickening agent investigated.

Initially 2% and 4% solutions of methylcellulose in deionised water were constituted and mixed with 2% solutions of ACh and SNP. The 4% mixture was found to be fairly viscous and proved too thick to top up the iontophoresis chamber easily via the 2 mm channels connecting the central aperture which housed the DP2 probe. This allowed some air pockets to develop and if bubbling at the probe end had occurred it could have significantly reduced the effect of the drug. Thus, the decision was made to carry out further testing of protocols utilising 2% solution of methylcellulose. It has been noted that the choice of vehicle does not have an impact on the voltage being applied (Khan *et al.* 2004), however as the vehicle was also thickening the fluid, analysis of the impact of different solutions on protocols was carried out. It was decided that a gradual increase in current over time would be utilised, this method is thought to prevent possible non-specific, current induced galvanic response and in light of the small size of chamber coupled with the thickened solution it was felt this would offer the optimum opportunity to monitor the response at low currents (Turner *et al.* 2008).

5 gradual increasing protocols comparing a 2% solution of ACh and SNP with a thickened solution with added 2% methylcellulose were investigated, with the aim of finding the optimum protocol as detailed in Table 3.4. Each protocol was tested with both ACh and SNP in 2% solutions and again with the 2% solution combined with 2%

methylcellulose on 3 occasions. The testing took place utilising the same subject for each protocol.

Table 3.4 The 5 protocols tested using both 2% solution of ACh and SNP with added 2% methylcellulose and a graduated increase in currents from 0 to 100μA over time in seconds.

Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5
0μA 120secs	0μA 60secs	0μA 120secs	0μA 120secs	0μA 60secs
10μA 120secs	20μA 120secs	20μA 120secs	10μA 240secs	20μA 120secs
15 μA 120secs	40 μA 240secs	40 μA 240secs	20 μA 240secs	40 μA 120secs
20 μA 120secs	50 μA 240secs	50 μA 240secs	50 μA 240secs	50 μA 240secs
40 μA 120secs	75 μA 120secs	75 μA 240secs	75 μA 240secs	75 μA 240secs
50 μA 120secs	100 μA 300secs	100 μA 240secs	100 μA 240secs	100 μA 120secs
75 μA 120secs	0μA 300secs	0μA 300secs	0μA 240secs	0μA 240secs
100 μA 120secs				
0 μA 300secs				

Testing was carried out over a period of 2 months, in a temperature controlled environment of 22° $^{\pm}$ 1°C under the same conditions which would be followed for the main study; i.e. avoiding food ingestion and caffeine based drinks for at least 2 hours beforehand and with a 20 minute period of acclimatisation. Mean perfusion values were obtained for each substance with and without thickening agent, and ICC analysis was carried out on the data. There was a high degree of repeatability for the protocols, and Table 3.5 displays the findings.

Table 3.5 Results obtained from testing the 5 protocols with and without methylcellulose thickening agent as a vehicle for SNP and ACh used in the iontophoresis process

Protocol	ICC values	95% C.I.	ANOVA F value	P values
Protocol 1-SNP	0.978	(0.867-0.996)	38.434	< 0.0001
Protocol 1-ACh	0.885	(0.008-0.978)	19.657	< 0.0001
Protocol 2-SNP	0.735	(-0.271-0.960)	5.117	0.049
Protocol 2-ACh	0.957	(0.512-0.993)	44.116	< 0.0001
Protocol 3 -SNP	0.784	(-0.228-0.977)	15.759	0.010
Protocol 3-ACh	0.747	(-0.267-0.957)	8.713	0.009
Protocol 4-SNP	0.982	(0.857-0.998)	49.761	0.001
Protocol 4-ACh	0.955	(0.216-0.994)	56.109	< 0.0001
Protocol 5-SNP	0.988	(0.928-0.998)	86.638	< 0.0001
Protocol 5-ACh	0.982	(0.749-0.998)	106.338	< 0.0001

The results indicated that all protocols could be utilised with methylcellulose as a vehicle without having a significant impact on the perfusion values obtained. Protocols 4 and 5 displayed the highest repeatability and as protocol 5 displayed the highest repeatability and also was shorter in duration, it was chosen as the preferred protocol for the main study. Figure 3.10 displays the mean flux obtained in the testing of protocol 5 for SNP and Figure 3.11 displays the mean flux obtained for ACh, both with and without methylcellulose as a thickening agent.

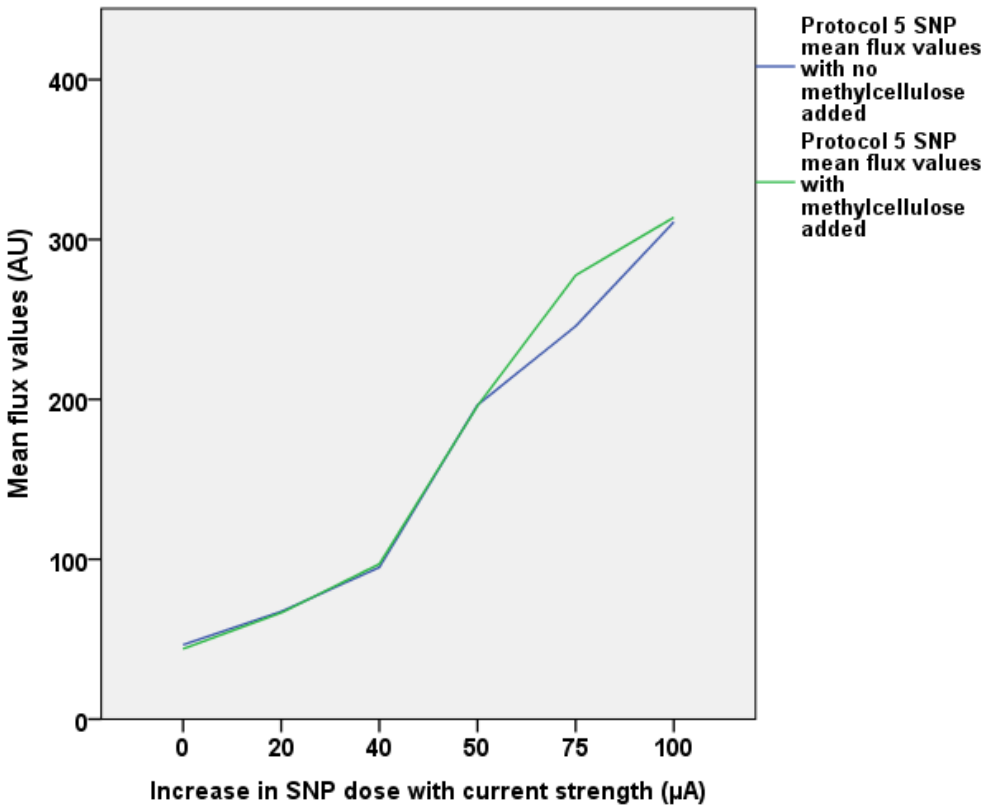

Figure 3.10 Graph of the mean flux values obtained for SNP with and without the addition of the thickening agent methylcellulose

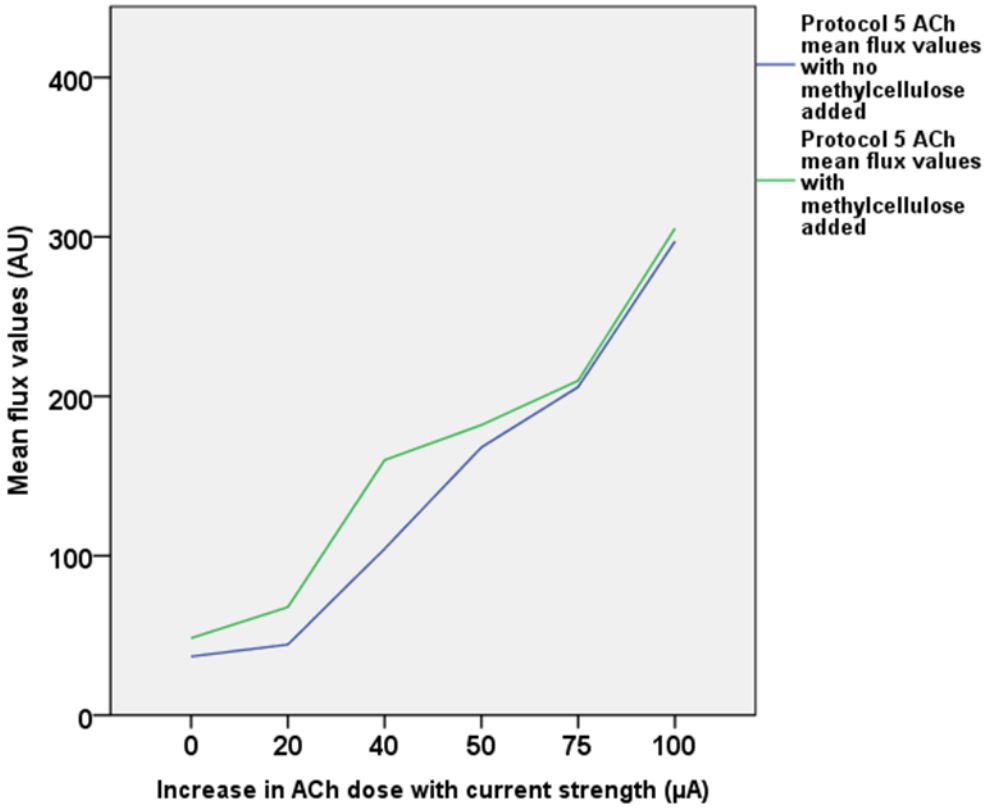

Figure 3.11 Graph of the mean flux values obtained for ACh with and without the addition of the thickening agent methylcellulose

3.2.6 In vivo inter-day repeatability testing of the laser Doppler flowmeter

Iontophoresis utilising 2% acetylcholine and 2% methylcellulose was carried out on the plantar aspect of the third metatarsophalangeal joint with 8 healthy volunteers on two separate occasions and at least two days apart. Each test was held in a temperature controlled environment of 22° ± 1° C following a 20 minute acclimatisation period, at the same time of day for both runs of the test and on each occasion the individual subject had followed the same protocol re abstaining from food and caffeine ingestion prior to testing. ICC analysis was carried out with a value of 0.933, 95% C.I. (0.674-0.987) F = 13.732, p = 0.001 indicative of good repeatability.

3.3 LASER SPECKLE CONTRAST IMAGING (LSCI)

Laser speckle contrast imaging is a relatively new method of measuring blood flow; however the reproducibility of the equipment when compared with other methods such as laser Doppler has been found to be very good, including inter-day reproducibility when assessing forearm post occlusive reactive hyperaemia (Roustit *et al.* 2010; Tew *et al.* 2011). As this equipment had not been used to measure the dynamics of perfusion in the form of a perfusion map on the plantar aspect of the foot before, a pilot study was conducted on 22 healthy subjects to assess feasibility for use to assess reactive hyperaemia on the plantar aspect of the foot.

3.3.1 Inter-day repeatability of LSCI-full field perfusion imager FLPI® (Moor Inst. Ltd)

Prior to carrying out a pilot study utilising the LSCI-FLPI® with a single subject under the experimental protocol conditions, repeated measurements were taken on the plantar aspect of the forefoot with the area of interest being the 3rd metatarsophalangeal joint area. The testing was carried out on 5 consecutive days, at the same time of day with the

pre-test conditions of no ingestion of food or caffeine for a period of 2 hours. ICC analysis was carried out and a value of 0.982, 95% C.I. (0.906-1.000) $F = 55.187$, $p < 0.0001$ obtained indicative of good repeatability.

3.3.2 Pilot study with LSCI-FLPI® equipment, methodology and recruitment

Initially ethical approval was sought and granted for this pilot study from Queen Margaret University research ethics committee. Recruitment was via an electronic poster distributed through the internal mail system (Appendix 1). Participants were healthy adult volunteers between the ages of 20 and 48 years of age (mean 30.3 years) and consisted of 32% (7) males and 68% (15) females. The exclusion criteria consisted of any underlying lower limb vascular or neurological conditions; or any underlying pathology of the spine/lower limbs which results in an inability to walk unaided. All subjects were provided with a written information sheet (Appendix 2) and indicated their consent by signing a consent form (Appendix 3).

3.3.3 Pilot study protocol

The volunteers were initially acclimatised in a temperature controlled room of 22° ±1°C for a period of 20 minutes in a supine position. During the initial 5-10 minutes details of the procedure were explained to the volunteer and information sheets provided, and if the volunteer was happy to proceed then consent forms were signed off. It was clearly outlined to all volunteers that they could stop the procedure at any stage. Using the LSCI-FLPI® system (Moor Instruments Ltd.) the perfusion for the plantar aspect of the foot was recorded for 4 minutes providing baseline resting blood flow data as seen in Figure 3.12. The FLPI® system does not require being in direct contact with the skin but

sits approximately 10 cms away from the skin surface. Five areas were chosen at the peripheral aspect of the plantar aspect of the foot for analysis. They consisted of three toes, and the metatarsophalangeal joint area of the foot directly behind the toes, thus covering the peripheral plantar aspect of the foot. Following this initial resting blood flow period of measurement, a pressure cuff was placed around the calf area and inflated to 200 mmHg for a period of 4 minutes providing an occlusive stage during which measurements of flux continued across the same areas of the foot. Following the occlusive stage, recordings were taken again for a period of up to 15 minutes to record the reactive hyperaemia which consisted of a peak of maximum blood flow followed by a slower return to baseline resting perfusion.

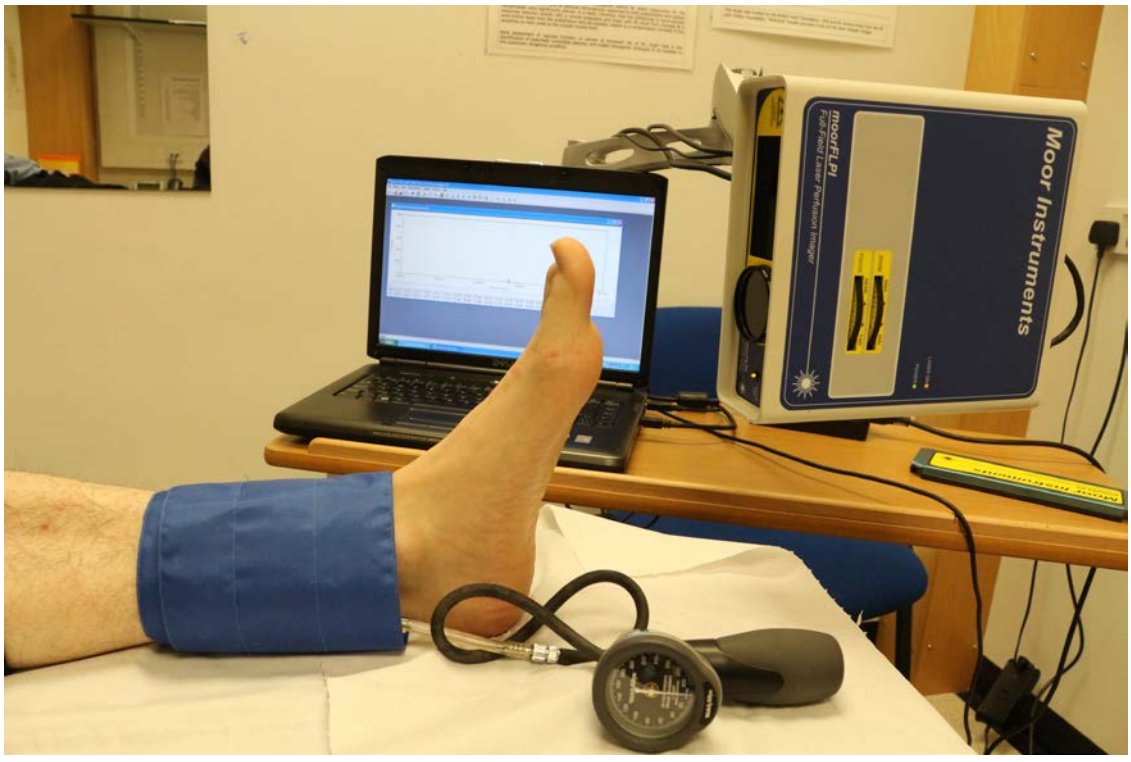

Figure 3.12 LSCI-FLPI® system with the pressure cuff utilised for the pilot study

3.3.3.1 Resting perfusion for the group

Resting perfusion for the group was measured at the first, third and fifth toes as well as the first and lesser (2^{nd}, 3^{rd} and 4^{th}) MPJs on the plantar aspect of the foot. Generally each area displayed similar readings, with the exception of the third toe which displayed a much smaller range of values than the other forefoot areas under study which can be noted in Table 3.6 and displayed in Figure 3.13.

Table 3.6 Descriptive statistics for resting flux obtained at the areas under study

	1^{st} toe	3^{rd} toe	5^{th} toe	Mid MPJs	1^{ST} MPJ
Mean	13.86	14.09	13.23	18.00	18.68
Median	14.00	12.00	13.00	16.50	17.00
St. Dev	5.72	8.06	9.32	9.50	8.05

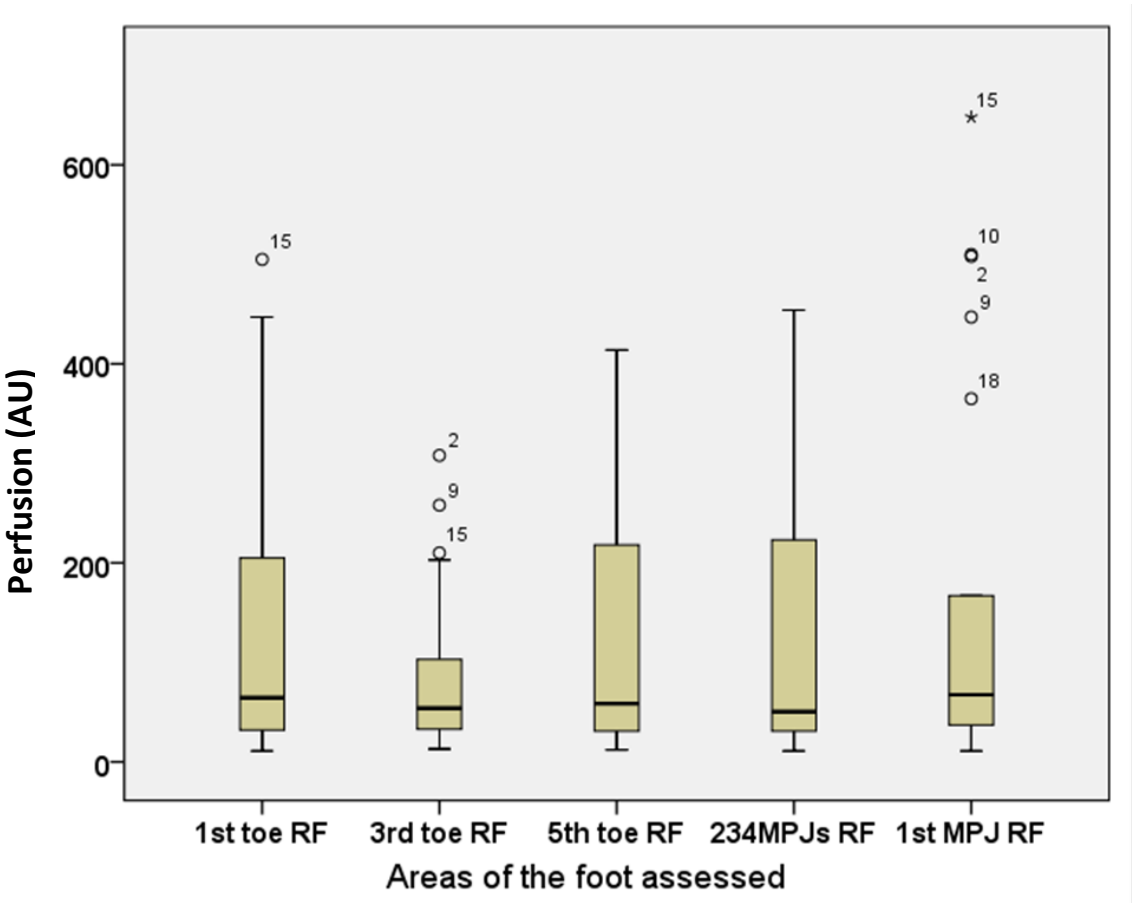

Figure 3.13 Box plots of resting perfusion for first, third, and fifth toes as well as the 2^{nd}, 3^{rd} and 4^{th} metatarsophalangeal joints and the 1^{st} metatarsophalangeal joint on the plantar aspect of the foot

3.3.3.2 Occlusive perfusion for the group

Occlusive perfusion or flux for the group was measured for the same areas of interest as the resting perfusion. As expected on occlusion, the perfusion registered over each area under study was very much reduced in comparison to the resting perfusion. The values obtained for each area of interest can be seen in Table 3.7 and displayed in Figure 3.14.

Table 3.7 Descriptive statistics for occlusion at the areas under study

	1st toe	3rd toe	5th toe	Mid MPJs	1ST MPJ
Mean	13.86	14.09	13.23	18.00	18.68
Median	14.00	12.00	13.00	16.50	17.00
St. Dev	5.72	8.06	9.32	9.50	8.05

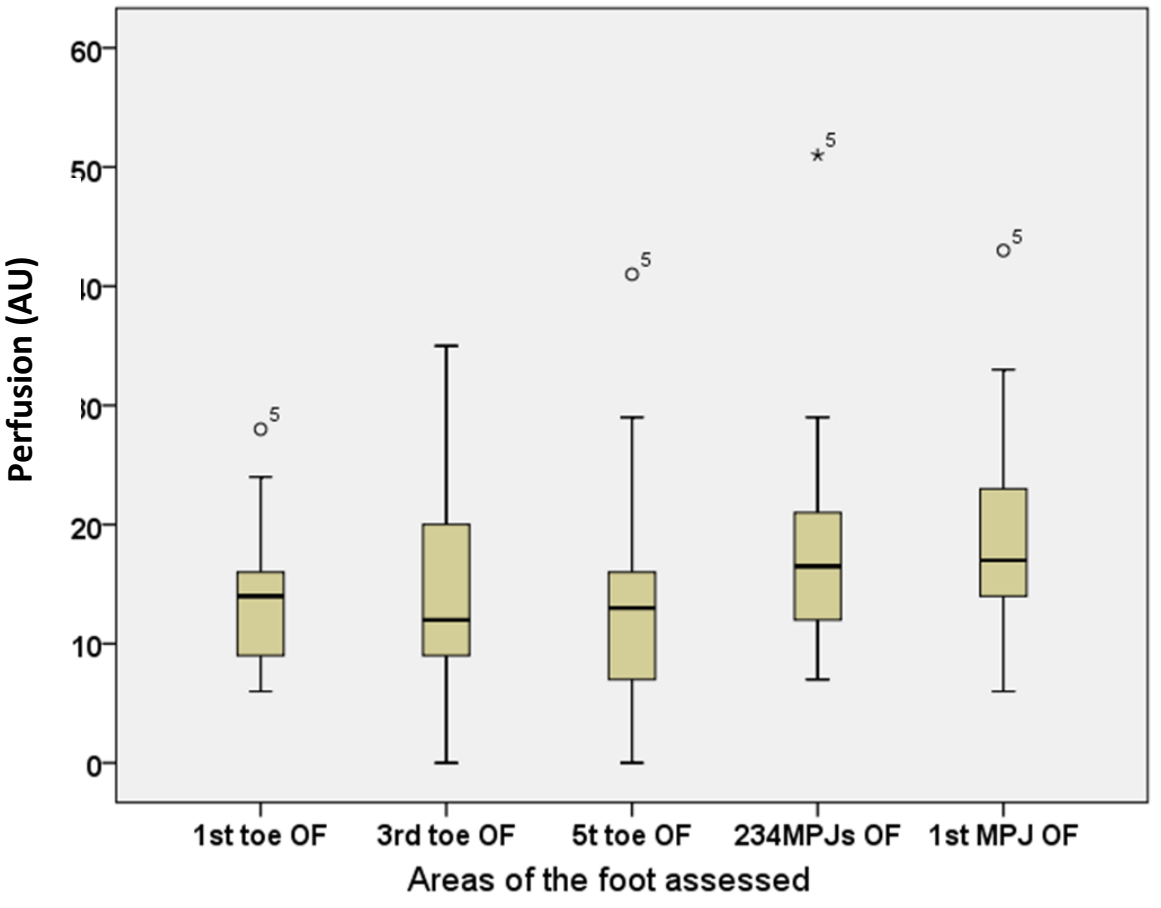

Figure 3.14 Box plots of occlusive perfusion for first, third, and fifth toes as well as the 2nd, 3rd and 4th metatarsophalangeal joints and the 1st metatarsophalangeal joint on the plantar aspect of the foot

3.3.3.3 Post occlusion peak perfusion values

Peak perfusion values registered a large increase in volume on release of the occlusion, with up to ten times the value of the resting flux being registered. Peak perfusion statistics can be seen in Table 3.8 and are displayed in Figure 3.15.

Table 3.8 Descriptive statistics for peak flux immediately following occlusion for the areas under study

	1st toe	3rd toe	5th toe	Mid MPJs	1ST MPJ
Mean	810.05	776.41	811.91	1024.23	1029.36
Median	804.00	759.50	848.50	1031.50	1002.50
St. Dev	282.35	230.37	311.79	348.84	414.42

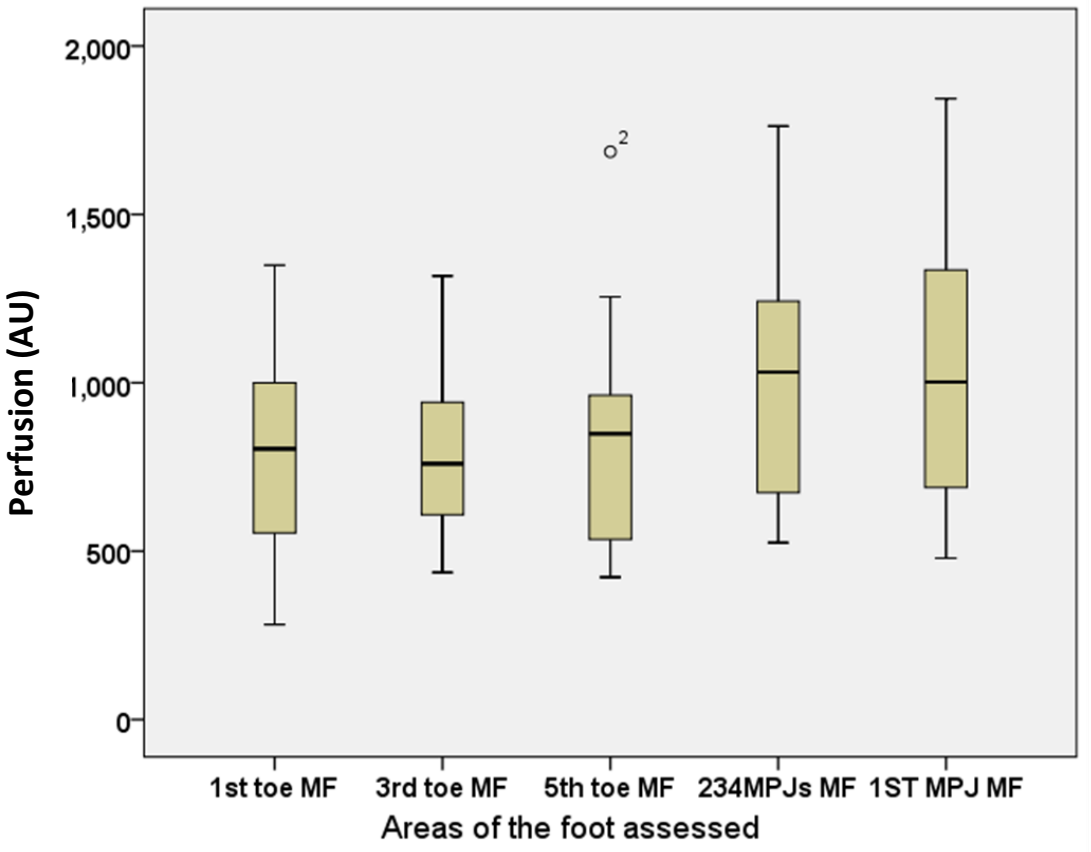

Figure 3.15 Box plots of peak perfusion following occlusion for first, third, and fifth toes as well as the 2nd, 3rd and 4th metatarsophalangeal joints and the 1st metatarsophalangeal joint on the plantar aspect of the foot

3.3.3.4 Time taken to return to resting perfusion

The time taken to return to baseline resting flux displayed a mean value of 2.17 minutes with standard deviation of 0.82 and median value 2.10 minutes. The range was from 1.25 to 4.92 minutes.

3.4 SUMMARY

The plantar pressure delivery system was specifically developed for this study and was found to produce repeatable values, and thus could be utilised for the main study to add pressure when investigating blood flow in conjunction with laser Doppler flowmetry. The process of iontophoresis was fully investigated for utilisation, with assessment of the specific chamber dimensions, investigation of the optimum thickness for the solutions and the protocol for use on the plantar surface of the foot. Repeatability studies were carried out on the laser Doppler flowmeter to ensure both equipment and protocols were suitable for use in the main study.

The LSCI-FLPI® equipment performed well under testing and during a pilot study when utilised on the lower limb, with no adverse results or issues raised during or after testing, thus it was also found to be a suitable method of assessing the post occlusive hyperaemic response for the main study.

Chapter Four

Application of the developed protocol

4 APPLICATION OF THE DEVELOPED PROTOCOL

4.1 INTRODUCTION

Investigating the function of the microvasculature supplying the plantar surface of the foot when under the normal pressures exerted during ambulation, could be key to understanding why the tissues are compromised in conditions such as diabetes mellitus, leading to breakdown and the development of ulceration. Assessment of endothelial function during ambulation has proven to be extremely difficult to achieve due to the requirements to house blood flux analysis equipment for the plantar aspect of the foot within a pressure analysis base or insole, and to date no commercial equipment is available to carry out this analysis. The rationale for the methods employed in this study was to develop an in-vivo, reliable, and repeatable, as well as user friendly, method for simultaneous assessment of endothelial function of the microvasculature while simulating the normal pressures exerted on the forefoot area during ambulation.

4.2 CHOICE OF ENVIRONMENT FOR THE STUDY

The equipment developed and chosen for this study was portable; however standardisation of the external conditions was important to minimise fluctuations in skin perfusion. Consequently, all procedures were conducted in a temperature controlled laboratory in the Vascular Unit at Ninewells Hospital, Dundee.

4.3 ETHICAL APPROVAL

Prior to any development work and the actual procedure being carried out, ethical approval was sought and granted from Tayside Committee on Medical Research Ethics

B. The laser speckle imaging equipment is a relatively new piece of equipment which was not available at the start of this study. Following exploratory work utilising this equipment it was thought to be particularly useful for the assessment of the post occlusive hyperaemia (PORH) component of this study, and a substantial amendment application was submitted and further approval for this amendment to the original protocol sought, which was approved by the committee.

4.4 PROCEDURE

4.4.1 Background

The study group consisted of 60 volunteers based in and around the city of Dundee. This group was further divided into 30 subjects with Type 2 diabetes mellitus (19 males and 11 females), with an age range from 36 to 81 years (mean of 68 years). The duration of diabetes ranged from 5 years to 23 years (mean duration 11.3 years).

The control group was also made up of 30 volunteers, of which 18 were male and 12 female. The range of ages was from 37 to 80 years, with the mean age being 60.1 years. The control subjects were questioned about their medical history, with a particular emphasis on diabetes mellitus and vascular problems. However, testing of blood glucose levels was not carried out, thus it should be noted that it is possible that some of the controls may have been undiagnosed with asymptomatic diabetes.

4.4.2 Volunteers with diabetes

Ninewells hospital in Dundee has an established reputation for the care of diabetes and thus attracts patients from many areas in and around Dundee. Therefore it was chosen for the source of volunteers with Type 2 diabetes and permission to approach patients to

become volunteers was given by Honorary Professor Graham Leese, Consultant Physician in Diabetes.

4.4.2.1 Invitation to participate

Subjects with diabetes who could be suitable for inclusion in the study were identified from adult patients on the diabetes centre database Scottish Care Information-Diabetes Collaboration (SCI-DC) (Diabetes in Scotland 2010). SCI-DC provides a centralised source of detailed information for all people in Scotland who have diabetes. It was set up originally for clinical use and designed to improve the quality of care for those with diabetes by allowing shared information across the health care team. A random selection of 46 possible subjects was made from the database, living in and around Dundee. Letters of invitation to take part in the study, along with subject information sheets (Appendix 4) were initially sent out with return slips included. Individuals who had returned favourable return slips were then contacted via telephone and further details and explanations provided with an invitation to attend for the study issued. Of an initial list of 46 possible volunteers contacted, 32 agreed to participate with 2 proving to be not suitable for the study. One having open wounds on the plantar aspect of the foot and with the possibility of cross infection and alteration of the normal gait pattern was declined. The second had a pacemaker fitted and following discussion, the individual did not feel comfortable in taking part in the study. The exclusion criteria for the group were underlying pathology of the spine/lower limbs which results in an inability to walk unaided. Thus, of the original 46 possible volunteers approached 65% agreed to participate who were suitable for the study.

4.4.3 Control volunteers

The control group consisted of 30 healthy adults with no known symptomatic vascular complications who were recruited from posters placed on Ninewells Hospital and University of Dundee electronic notice boards (Appendix 5). Word of mouth also gained some of the more mature volunteers such as retired staff members and relatives of current staff who had noted the electronic recruitment request.

The exclusion criteria in the control group were any underlying lower limb vascular or neurological conditions, or any underlying pathology of the spine/lower limbs which could have resulted in an inability to walk unaided. The individuals were questioned about their general health and a lower limb assessment was carried out checking skin appearance for colour, temperature, and lower limb pulses. Ankle reflexes and lower limb muscle power was assessed as well as sensation in the form of touch/pressure, temperature and proprioception. Of the volunteers who came forward only 1 withdrew from the study due to lower back pain which prevented the individual lying in a supine position for any length of time.

4.5 ASSESSMENT PROTOCOL

Prior to the procedure being carried out, following a discussion and full explanation of the study, written informed consent was obtained (Appendix 6). This was followed by a short interview with each volunteer and details such as age, sex, general health, medication and, where appropriate, duration of diabetes and mode of management for diabetes, were obtained. Initial assessment of the lower limbs was then carried out for the control group to ensure suitability of the volunteer for the study as detailed in Section 4.4.3.

4.5.1 Procedure

4.5.1.1 Initial plantar pressure analysis

Initial barefoot peak plantar pressure analysis of the individual's right foot was carried out using the Emed-x/E system. This was to provide the peak pressure exerted under the forefoot of each subject when walking as displayed in Figure 4.1. It is a computerised, dynamic, portable pressure analysis system which consists of a footplate containing 6,080 sensors with a sensor area of $0.25cm^2$ set into a portable walkway (Novel Instruments) as illustrated in Figure 4.2. The portable walkway consisted of a lightweight, rigid material, with a cut out section which allowed the plate to sit flush within the walkway itself. Each individual was asked to walk across the walkway and plate at their own walking pace, looking ahead to prevent targeting of the plate which could alter their natural gait and as such might impact on the pressure measurement. Two full steps were taken prior to stepping onto the plate, which is an accepted protocol to achieve a natural strike of the foot on the pressure plate (Myers-Rice *et al.* 1994). Three strikes were recorded for each subject and the mean peak pressure was used for the 6 areas of the forefoot included in this study i.e. 1^{st}, 3^{rd} and 5^{th} toes and 1^{st}, 3^{rd} and 5^{th} MPJs of the right foot.

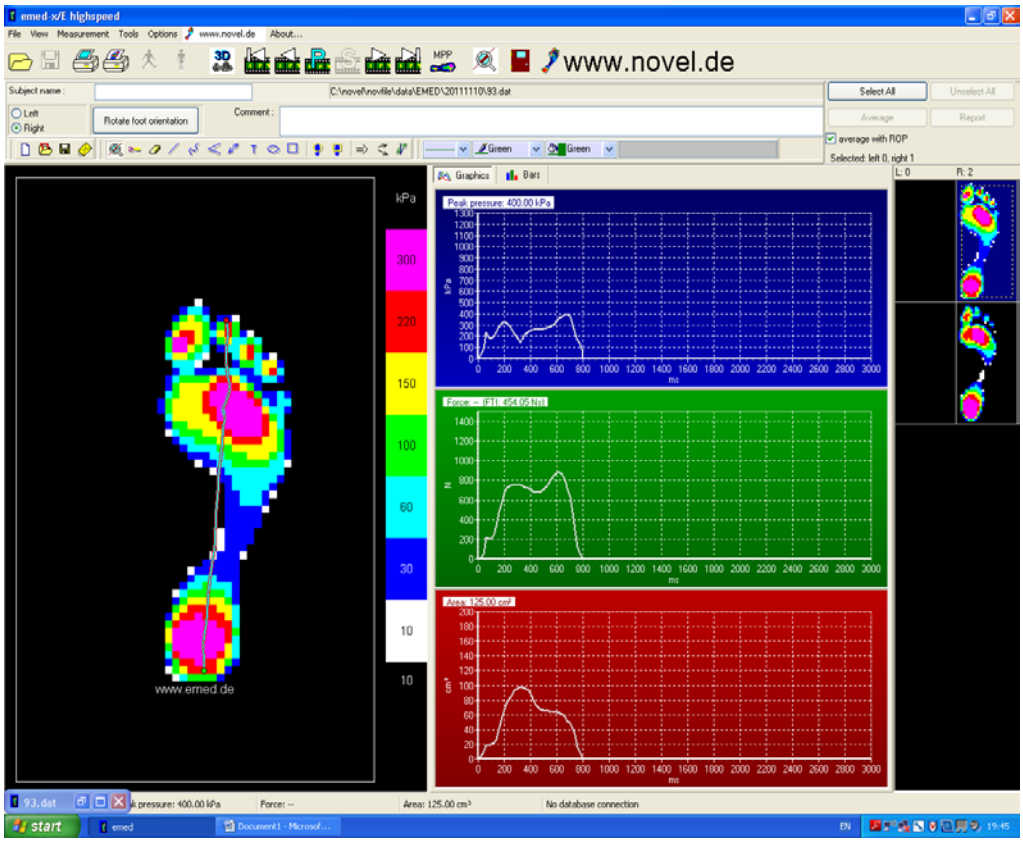

Figure 4.1 Emed-x/E pressure analysis system with peak plantar pressures displayed in kPa during walking

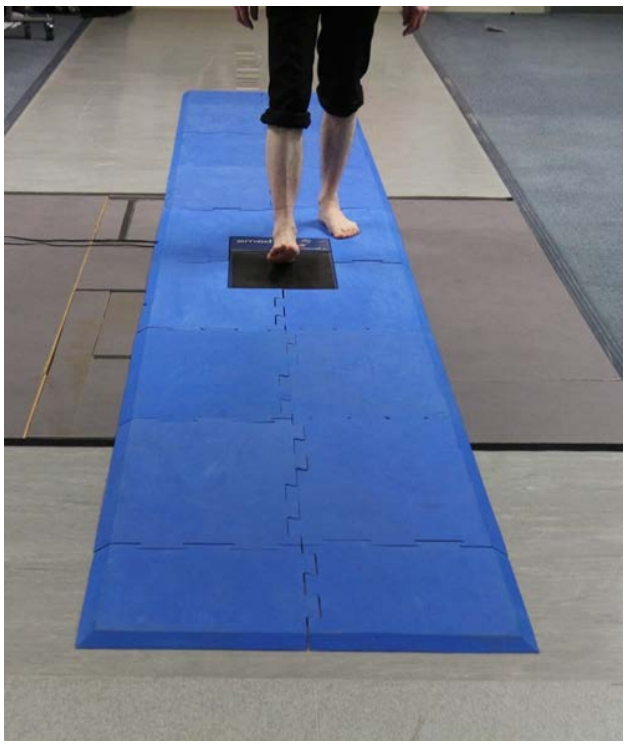

Figure 4.2 Emed-x/E pressure analysis system set into the portable walkway

4.5.1.2 Recording of reactive hyperaemia

Subjects were then taken to the temperature controlled laboratory in the vascular unit and asked to remove their shoes and socks/tights, and then to rest on the plinth in a supine position. Any overlying callous found in the locality under study was reduced by debridement using a sterile scalpel blade to allow effective penetration of the iontophoresis solutions for the experiments described in Section 4.5.1.3.

Following acclimatisation in the temperature controlled laboratory of 22° ± 1°C for 20 minutes, 6 areas of interest on the forefoot were marked out using the laser speckle imaging system. The areas of interest were the 5^{th} toe, 3^{rd} toe, 1^{st} toe and the 5^{th}, 3^{rd} and 1^{st} metatarsophalangeal joint areas on the plantar aspect of the foot. As foot sizes varied across the subjects, a set size of area of interest was not possible but the area was chosen to ensure flux measurements only over the areas under study. Baseline data were recorded for 2 minutes and following this, a pressure cuff placed around the calf area was then inflated to 200 mmHg for a period of 5 minutes providing an occlusive stage as illustrated in Figure 3.12. Following release of the occlusive cuff, the reactive hyperaemia was recorded continuously for 5 minutes and readings taken at 2 and 5 minutes post occlusion to enable analysis of the recovery period as illustrated in Figures 4.3 and 4.4.

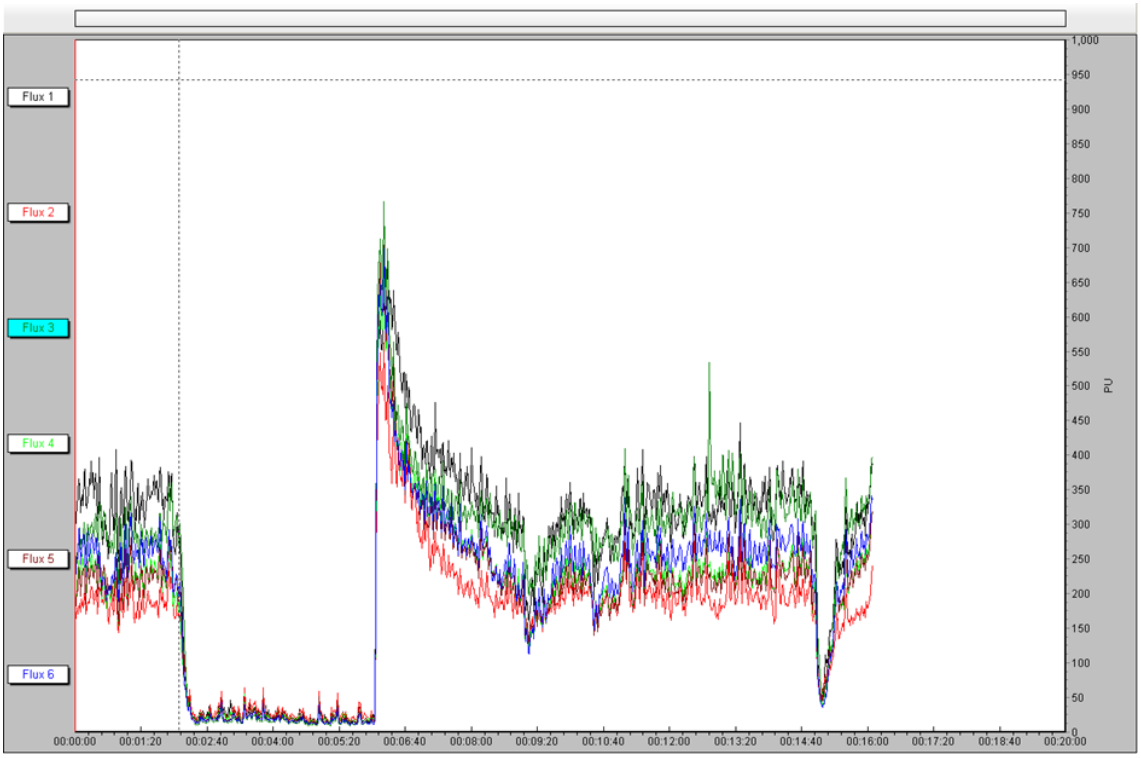

Figure 4.3 Superimposed trace of the 6 areas under study displayed together with initial baseline resting flux, followed by occlusion and the release of the cuff

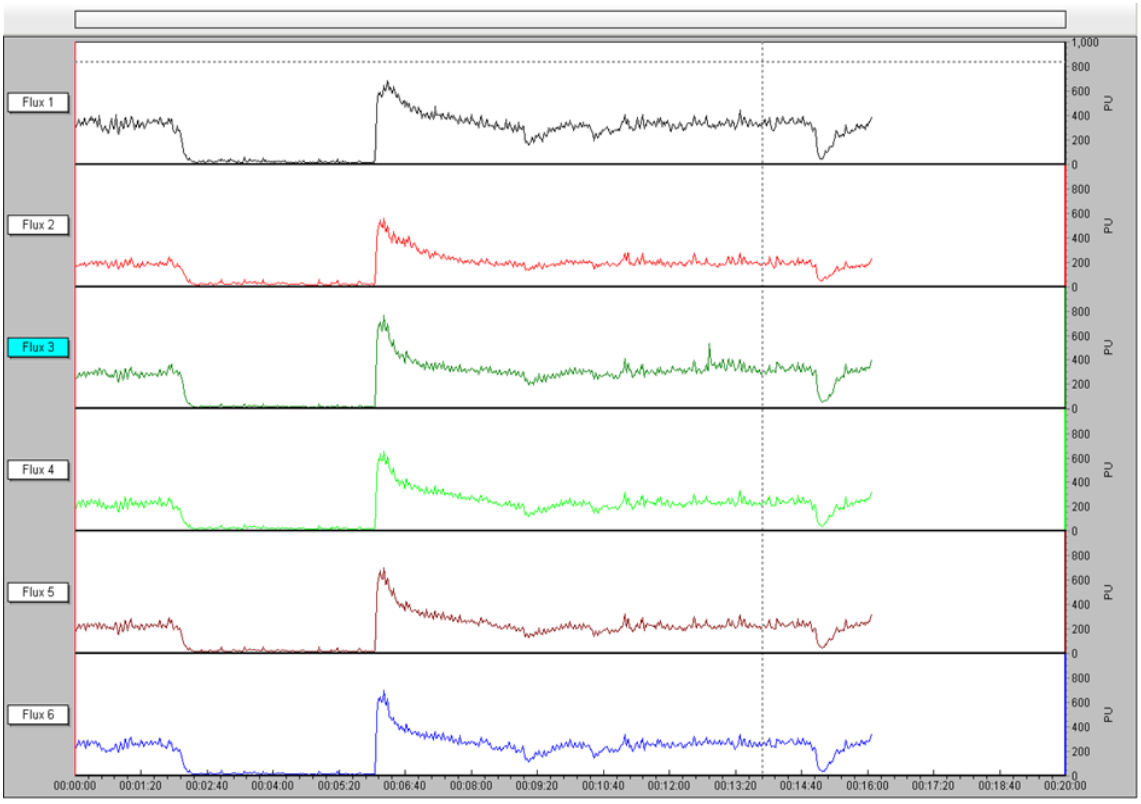

Figure 4.4 Individual traces from each of the 6 forefoot areas under study during the assessment of reactive hyperaemia displaying the 3 stages of baseline flux, occlusion and release of the cuff with resultant hyperaemia

4.5.1.3 Measurement of blood flux with the application of vasodilator solutions and simultaneous addition of pressure

The right foot was then placed in the customised boot designed to support the lower part of the limb and foot comfortably for the time required for gathering data, as well as holding it in a stable position for delivery of pressure as illustrated in Figure 4.5. The skin over the area of interest, the third metatarsophalangeal joint area on the plantar aspect of the foot (chosen as an area which sustains high pressure during ambulation across the forefoot), was cleansed with an alcohol swab. The iontophoresis chamber was attached to the skin using double sided adhesive pads specifically designed for use with the system, and the 2% solution of ACh with 2% methylcellulose added to the chamber. The DRT4® DP2 probe and pressure delivery system were then placed in position with the chamber and secured. Local blood flow to the third metatarsophalangeal joint area of the plantar aspect of the right forefoot was measured using a laser Doppler flowmeter while iontophoresis was being carried out as displayed in Figure 4.6. Iontophoresis was carried out using the protocol detailed in Section 3.2.5, with 2% acetylcholine in a vehicle of 2% methylcellulose, with the process being repeated using 2% sodium nitroprusside also in a vehicle of 2% methylcellulose. During the process, application of differing pressures were delivered to the area starting with no pressure, followed by 50% and then 100% of the subject's individual peak pressure while measuring blood flow. The pressures were calculated for each subject utilising the mean peak walking pressure found under the third metatarsophalangeal joint area of the plantar aspect utilising the Emed-x/E pressure analysis system and delivered during iontophoresis using the pressure delivery system as illustrated in Figure 4.5. A second probe was positioned on the 5^{th} metatarsophalangeal joint area of the same foot as a control measure of blood flux.

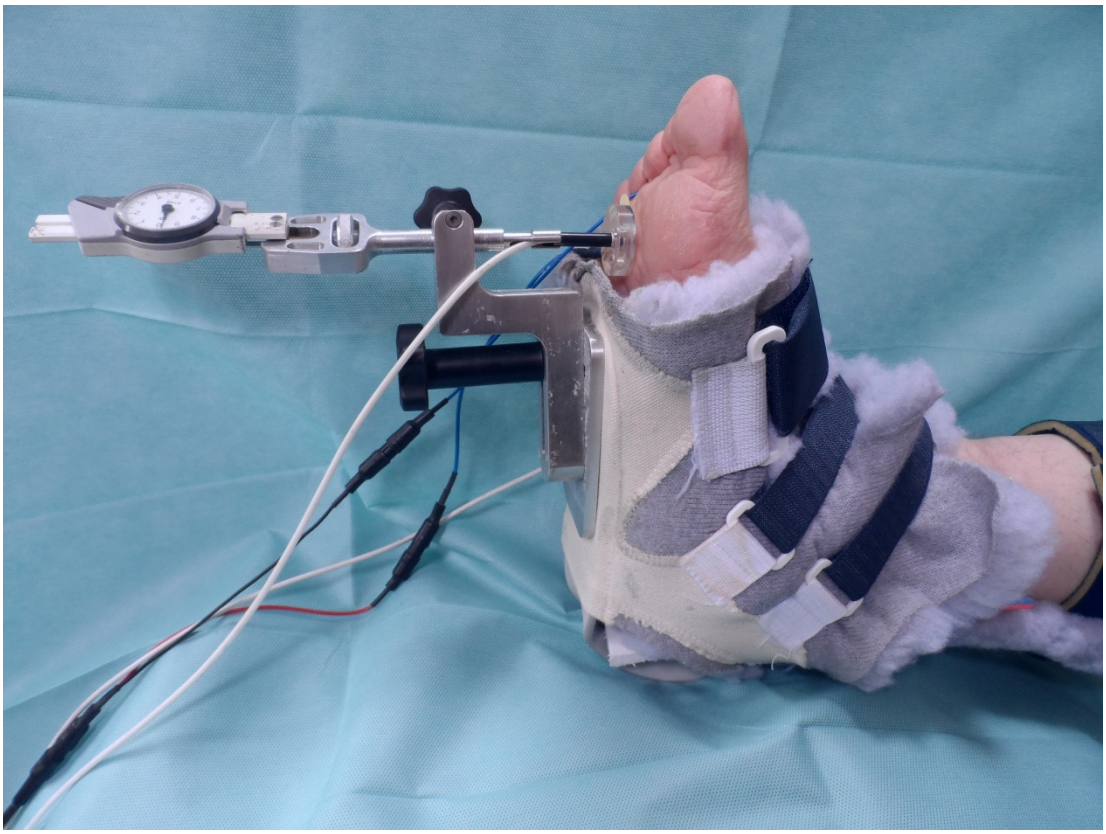

Figure 4.5 The pressure delivery system held in position via the adapted boot with the iontophoresis chamber housing the laser Doppler probe and the solutions with the vasodilators

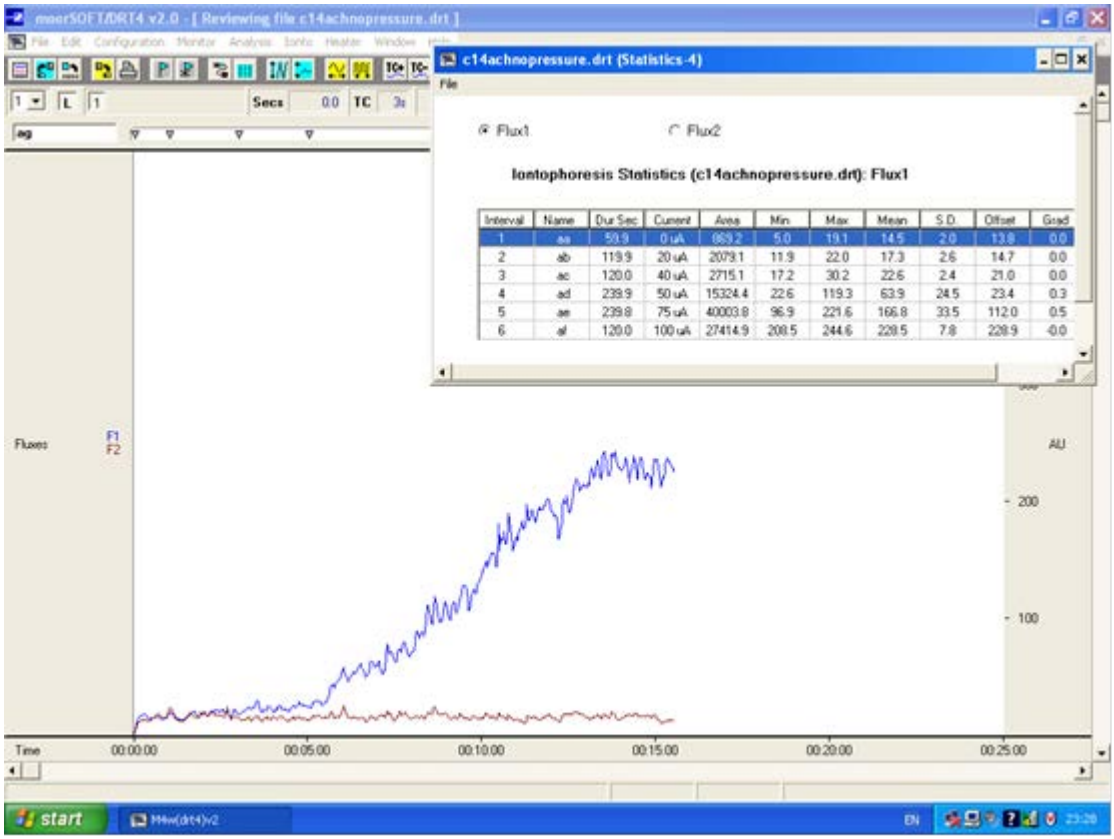

Figure 4.6 Laser Doppler flowmeter trace during the process of iontophoresis with ACh with the active iontophoresis statistics displayed. F1 is the active probe flux and F2 is the control probe trace

4.6 STATISTICAL METHODS

The data collected from this study was analysed using the Statistical Package for the Social Sciences (SPSS) version 19. The significance level adopted for all analysis carried out was $p < 0.05$. Assessment of the repeatability of equipment during the development stage of the project was carried out utilising the Interclass Correlation Coefficient (ICC) with 95% confidence interval. Spearman's correlation coefficient has been utilised to display associations between the different areas of the forefoot under study with the LSCI, and between the data obtained from laser Doppler flowmetry and laser speckle imaging, as well as the association between the actual forefoot pressures and baseline blood flux. Repeated measures ANOVA with Bonferonni correction was used to investigate the impact of pressure on endothelial function when utilising ACh and SNP with iontophoresis on the subjects with diabetes and the healthy control group, and for the laser speckle data when comparing the group with diabetes and the control group. This was followed with Mann-Whitney U test to further analyse the data in the assessment of pressure on endothelial function when utilising ACh and SNP with iontophoresis on the subjects with diabetes and the healthy control group.

4.7 RESULTS OF THE APPLICATION OF THE PROTOCOL

4.7.1 Results of post occlusive reactive hyperaemia (PORH) assessment using LSCI

4.7.1.1 Introduction

PORH was measured in the two groups of subjects to assess for any differences in the response across the groups, as well as to compare the response across the plantar pressure areas in the 30 volunteers who had Type 2 diabetes and 30 age and sex matched healthy control subjects. Laser speckle contrast imaging (LSCI) data was

available for 94.24% of the overall group with a breakdown of 95.38% of the subjects with Type 2 diabetes and 93.11% of the control subjects when measuring the resting flux, occlusion values, peak and time to peak as well as 2 minutes and 5 minutes post peak parameters. 100% data was not achieved due to slight movement of the limbs in a small number of individuals during the data gathering process. Initial testing of the data showed that it did not follow a normal distribution pattern, which was revealed by calculating the z value for the data utilising the skewness and kurtosis values with a skewness value of 1.96 or greater indicative of significance at $p < 0.05$. As can be seen in Table 4.1 the majority of z values obtained were in excess of 1.96, thus non-parametric statistical analysis was utilised with the exception of repeated measures ANOVA where the F value is thought to be robust, and as such can be utilised without a normal distribution (Field 2013).

4.7.1.2 Post occlusive reactive hyperaemia findings between the two groups

Six stages of reactive hyperaemia, over six areas of interest on the plantar aspect of the forefoot were investigated in both groups. The six elements were:-

- an initial baseline resting blood flux measurement
- an occlusive stage with reduction in blood flux
- the peak blood flux on removal of the occlusion
- the time to reach peak flux
- 2 minutes following removal of the occlusion blood flux as an indicator of recovery of blood flow
- 5 minutes following removal of the occlusion blood flux as an indicator of recovery of blood flow

Comparisons between the stages of the hyperaemic response were made between the 2 groups using repeated measures ANOVA with post hoc Bonferroni analysis. Mauchly's test indicated that the assumption of sphericity had been violated therefore the degrees of freedom were corrected with Huynh-Feldt. As the data did not follow a normal distribution pattern as indicated in Section 4.7.1.1, and noted in Table 4.1 a non-parametric statistical analysis was chosen for the follow up investigation of the individual areas of study, thus Mann-Whitney U test was used.

Table 4.1 Descriptive statistics indicating skewness of LSCI data for n=60

Resting Flux measured in arbitrary units (AU)

Area	Mean	St.Dev.	Skewness Statistic	St.error	Z value	Kurtosis Statistic	St.error	Z value
5th toe	167.00	125.99	1.273	0.319	3.99	1.334	0.628	2.12
3rd toe	149.60	115.51	2.051	0.314	6.53	5.770	0.618	9.34
1st toe	149.56	126.00	1.914	0.314	6.10	4.093	0.618	6.62
5th MPJ	157.02	139.13	2.308	0.327	7.06	7.108	0.644	11.04
3RD MPJ	159.42	151.07	2.417	0.314	7.70	7.114	0.618	11.51
1ST MPJ	146.72	113.07	1.489	0.314	3.18	2.273	0.618	3.68

Flux during occlusion measured in arbitrary units (AU)

Area	Mean	St.Dev.	Skewness Statistic	St.error	Z value	Kurtosis Statistic	St.error	Z value
5th toe	26.90	18.09	3.086	0.319	9.67	15.223	0.628	24.24
3rd toe	28.80	16.48	1.811	0.314	5.77	3.690	0.618	5.97
1st toe	25.15	16.40	1.925	0.314	6.13	3.824	0.618	6.19
5th MPJ	27.18	16.39	2.045	0.327	6.25	5.080	0.644	7.89
3RD MPJ	28.89	17.27	2.329	0.314	7.42	6.290	0.618	10.18
1ST MPJ	25.18	13.34	1.642	0.314	5.23	4.413	0.618	7.14

Table 4.1 Descriptive statistics indicating skewness of LSCI data for n=60 continued

Peak flow measured in arbitrary units (AU)

Area	Mean	St. Dev.	Skewness Statistic	St.error	Z value	Kurtosis Statistic	St.error	Z value
5th toe	440.19	242.05	0.968	0.322	3.01	1.061	0.634	1.67
3rd toe	423.84	221.70	1.121	0.314	3.57	1.334	0.618	2.16
1st toe	417.04	238.27	0.881	0.316	2.79	0.050	0.623	0.08
5th MPJ	471.10	224.67	0.463	0.327	1.42	-0.029	0.644	0.05
3RD MPJ	485.74	231.60	0.470	0.314	1.50	-0.070	0.618	0.11
1ST MPJ	457.27	239.06	0.875	0.314	2.79	0.617	0.618	1.00

Time to peak flow measured in seconds

Area	Mean	St.Dev.	Skewness Statistic	St.error	Z value	Kurtosis Statistic	St.error	Z value
5th toe	14.22	9.09	2.189	0.322	6.80	7.976	0.634	12.58
3rd toe	15.19	9.93	1.711	0.314	5.45	4.076	0.618	6.60
1st toe	14.04	9.29	2.240	0.314	7.13	7.312	0.618	11.83
5th MPJ	15.15	12.26	2.615	0.325	8.05	8.576	0.639	13.42
3RD MPJ	14.39	9.69	2.069	0.314	6.59	5.822	0.618	9.42
1ST MPJ	15.17	11.97	2.451	0.314	7.81	7.572	0.618	12.25

2 minutes post peak flow (AU)

Area	Mean	St.Dev.	Skewness Statistic	St.error	Z value	Kurtosis Statistic	St.error	Z value
5th toe	172.55	115.90	1.379	0.319	4.38	2.587	0.628	4.12
3rd toe	168.17	110.08	0.916	0.311	3.22	0.078	0.613	0.13
1st toe	148.61	114.31	1.317	0.322	4.09	1.570	0.634	2.48
5th MPJ	152.27	110.79	1.215	0.319	3.81	1.318	0.628	2.10
3rd MPJ	171.36	121.89	1.125	0.314	3.58	.854	0.618	1.38
1st MPJ	141.82	101.13	1.365	0.316	4.32	1.586	0.623	2.55

Table 4.1 Descriptive statistics indicating skewness of LSCI data for n=60 continued

5 minutes post peak flow (AU)

Area	Mean	St.Dev.	Skewness Statistic	St.error	Z value	Kurtosis Statistic	St.error	Z value
5th toe	139.81	104.32	0.900	0.322	2.80	-0.226	0.634	0.36
3rd toe	142.33	106.87	1.281	0.311	4.12	1.395	0.613	2.28
1st toe	134.38	108.13	1.031	0.325	3.17	0.380	0.639	0.60
5th MPJ	133.54	104.68	1.376	0.319	4.31	1.764	0.628	2.81
3rd MPJ	144.25	109.96	1.087	0.314	3.46	0.593	0.618	0.96
1st MPJ	124.95	103.07	1.324	0.322	4.11	1.125	0.634	1.77

The majority of z values are over 1.96 indicative of a skewed distribution thus requirement generally for non-parametric analysis.

4.7.1.3 Analysis of the resting flux measurements between the group with Type 2 diabetes and the control group

The resting blood flux values obtained for the group with Type 2 diabetes and the control group were comparable, although the group with Type 2 diabetes displayed slightly higher values as can be seen in Table 4.2 and illustrated in Figure 4.7. There was no significant difference found within the subjects between the sites under study for resting flux ($F = 1.354$, $p = 0.264$) with no significant difference across the groups ($F = 0.211$, $p = 0.651$) and no significant interaction effect for resting flux and the groups ($F = 0.536$, $p = 0.584$). With follow up analysis utilising Mann Whitney U test, no significant difference was found between the groups across any of the six individual sites under study as illustrated in Table 4.2.

Table 4.2 Comparison of the resting flux across the two groups

Area	T2DM Mean flux	T2DM St. Dev.	Controls Mean flux	Controls St. Dev.	Significance p value
5th toe	178.79	123.92	154.34	129.31	0.254
3rd toe	154.06	105.17	145.14	126.73	0.489
1st toe	164.98	128.57	134.14	123.68	0.142
5th MPJ	161.94	110.54	151.90	165.84	0.206
3rd MPJ	152.64	103.10	166.19	189.04	0.194
1st MPJ	149.23	97.24	144.22	128.67	0.363

T2DM is the group with Type 2 diabetes and Controls are the control subjects, Mann-Whitney U test was used for the follow up analysis and p values obtained. There was no significant difference found within subjects between the sites under study for resting flux (p = 0.264, ANOVA), or the groups (p = 0.651, ANOVA) or for the interaction effect between resting flux and the group (p = 0.584 ANOVA).

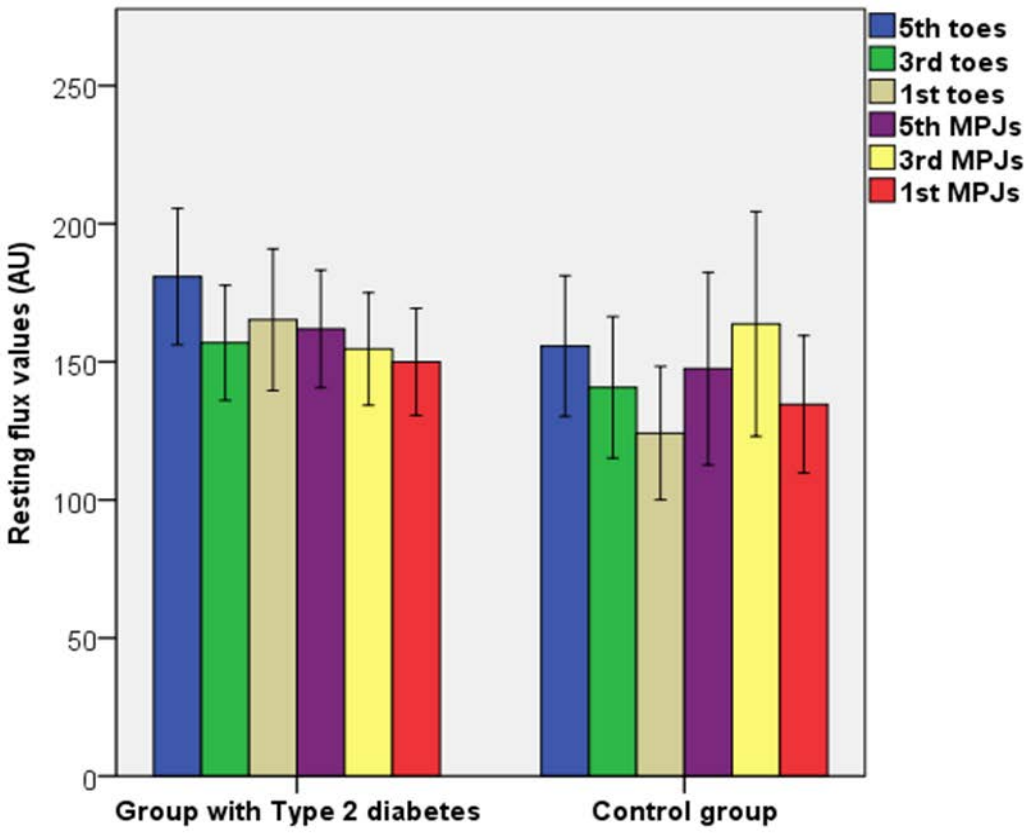

Figure 4.7 Comparison of the resting flux (AU) for the two groups

4.7.1.4 Comparison of the occlusive stage blood flux values for the two groups

During the occlusive stage of the PORH assessment the blood flow in both groups was reduced substantially from resting flux. No significant difference was found with occlusion across the sites under study ($F = 1.654$, $p = 0.165$) or across the groups ($F = 0.930$, $p = 0.345$) and no significant interaction was found between occlusion and the groups ($F = 0.683$, $p = 0.567$). With follow up analysis, there was no significantly different between the flux values obtained for the groups during the occlusive stage at any of the individual areas under study as can be seen in Table 4.3 and illustrated in Figure 4.8.

Table 4.3 Comparison of the occlusion flux values across the two groups

Area	T2DM Mean flux	T2DM St. Dev.	Controls Mean flux	Controls St. Dev.	Significance p value
5th toe	26.57	12.86	27.25	22.67	0.676
3rd toe	28.89	15.56	28.71	17.63	0.859
1st toe	23.83	13.10	26.46	19.30	0.675
5th MPJ	27.95	14.88	26.38	18.09	0.499
3rd MPJ	28.96	14.88	28.82	19.65	0.592
1st MPJ	24.83	10.29	25.52	16.00	0.913

T2DM is the group with Type 2 diabetes and Controls are the control subjects, Mann-Whitney U test was used for the follow up analysis and p values obtained. There was no significant difference found within subjects for occlusion (p = 0.165, ANOVA), or the groups (p = 0.345, ANOVA) or for the interaction effect between occlusion and the group (p = 0.567, ANOVA). Follow up analysis indicated no significant difference between the groups in any of the areas under study.

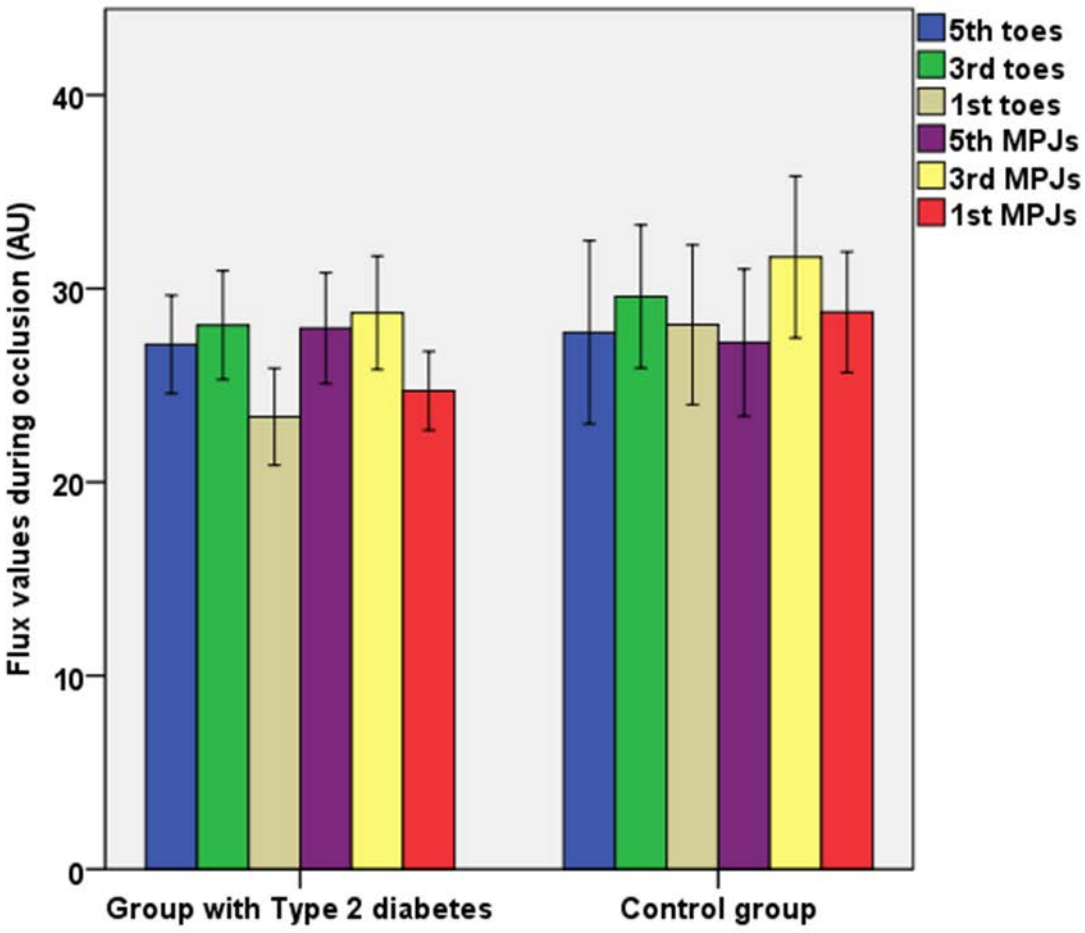

Figure 4.8 Blood flux during occlusion (AU), a comparison between the two groups

4.7.1.5 Comparison of the peak flux achieved post occlusion between the two groups

A significant difference was found for peak flux achieved on release of the occlusion ($F = 4.560$, $p = 0.004$), and for the group ($F = 6.337$, $p = 0.020$) as well as a significant interaction found between the peak flux and the groups ($F = 0.235$, $p = 0.032$). As can be noted in Table 4.4 and illustrated in Figure 4.9, the trend was for the group of subjects with Type 2 diabetes to have a higher peak flux value than the control group. With follow up analysis the peak flux following occlusion found on the plantar aspect of the 5th toe was significantly higher in the group with Type 2 diabetes than in the control group ($p = 0.049$). The value for the peak flux following occlusion found on the plantar aspect of the 3rd toe was significantly higher in the group with Type 2 diabetes than found in the control group ($p = 0.013$). There was no significant difference found

between the groups on the plantar aspect of the hallux, however the trend indicated in Figure 4.9 clearly displayed that the actual mean values obtained across the group at the hallux were higher than found in the control group, although statistical significance was not achieved (p = 0.175. N.S.). There was a significant difference found on the plantar aspect of the 5th MPJ between the groups, again with the subjects with diabetes displaying a higher peak flux on release of occlusion in comparison with the control group (p = 0.004). On the plantar aspect of the 3rd MPJ a significant difference was noted with higher values achieved in peak flux in the group with type 2 diabetes (p = 0.014) and a significant difference was found on the plantar aspect of the 1st MPJ with the diabetic subjects displaying a higher peak flux on release of occlusion than the control group (p = 0.019).

Table 4.4 Comparison of peak flux values (AU) following release of occlusion between the two groups

Area	T2DM Mean flux	T2DM St. Dev.	Controls Mean flux	Controls St. Dev.	Significance p value
5th toe	486.58	225.02	388.45	254.09	0.049
3rd toe	482.51	210.06	365.17	220.90	0.013
1st toe	452.99	236.97	382.33	238.45	0.175 N.S.
5th MPJ	561.62	215.21	377.10	196.93	0.004
3rd MPJ	569.77	225.51	401.71	209.09	0.014
1st MPJ	532.47	250.39	382.06	204.76	0.019

T2DM is the group with Type 2 diabetes and Controls are the control subjects, Mann-Whitney U test was used for the follow up analysis and p values obtained. There was a significant difference found within subjects for peak flux (p = 0.004, ANOVA), for the groups (p = 0.020, ANOVA) and for the interaction effect between peak flux and the group (p = 0.032, ANOVA). On follow up analysis, there was a significant difference found between the groups in the areas under study with the exception of the 1st toe (p = 0.175, M.W.U).

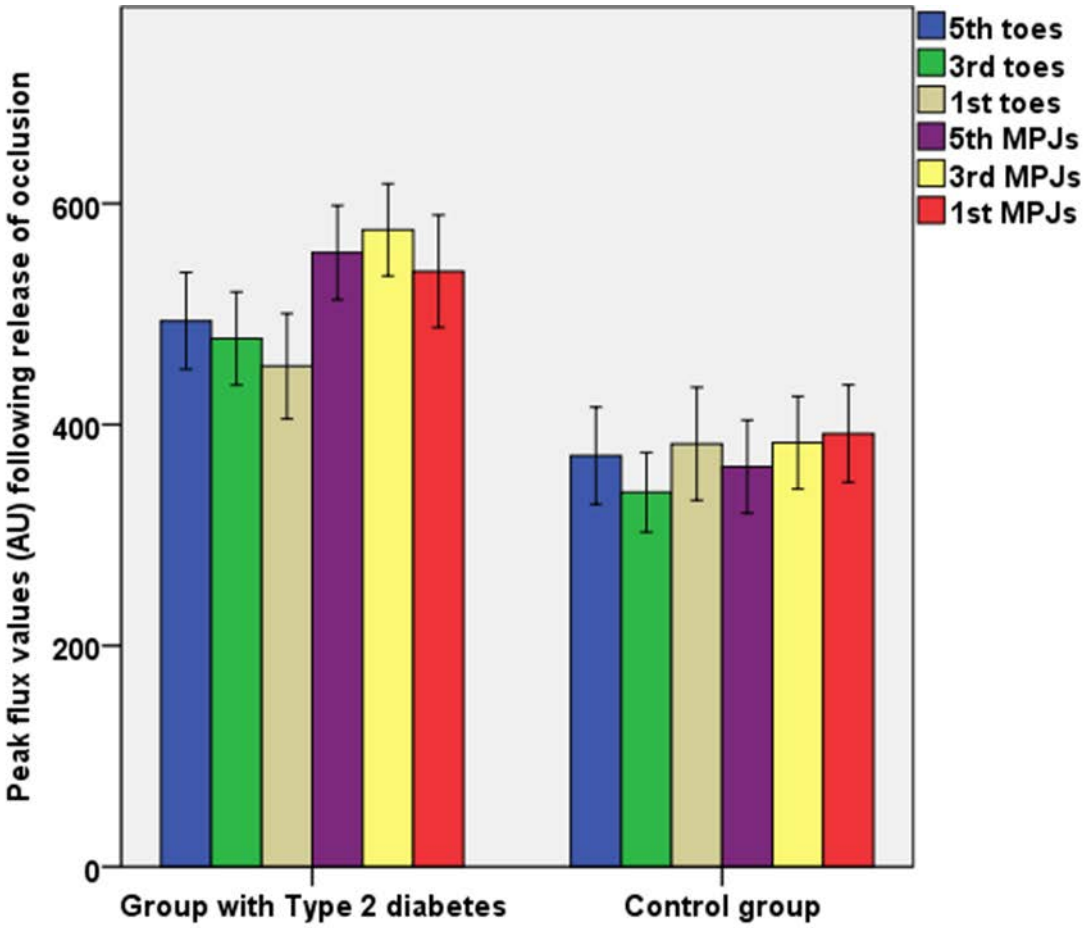

Figure 4.9 Comparison of peak flux values (AU) following release of occlusion in the two groups

4.7.1.6 Comparison of the time to reach peak flux following occlusion between the group with Type 2 diabetes and the control group

The time taken for each of the groups (measured in seconds) to reach the peak flux following release of the occlusion to the area was very similar, and no significant difference was found with the time taken to reach peak flux ($F = 0.973$, $p = 0.417$) or for the effect of group ($F = 0.130$, $p = 0.722$) and no significant interaction between time to reach peak flux and the group ($F = 0.235$, $p = 0.868$). With the follow up analysis using Mann-Whitney U test, there was no significant difference found between the time taken to reach peak flux on the plantar aspect of the 5th toe ($p = 0.561$), and as measured on the plantar aspect of the 3rd toe ($p = 0.858$) or as measured on the plantar aspect of the hallux ($p = 0.714$). Again no significant difference was found in the time taken to reach peak flux when measured on the plantar aspect of the 5th MPJ ($p = 0.938$), or the 3rd

MPJ (p = 0.889) nor on the plantar aspect of the 1st MPJ (p = 0.895) as can be seen in Table 4.5 and illustrated in Figure 4.10.

Table 4.5 Comparison of the time to reach peak flux values across the two groups

Area	T2DM Mean flux	T2DM St. Dev.	Controls Mean flux	Controls St. Dev.	Significance p value
5th toe	14.49	8.54	13.92	9.82	0.561
3rd toe	14.58	8.47	15.79	11.32	0.858
1st toe	14.05	8.08	14.03	10.51	0.714
5th MPJ	15.40	13.12	14.88	11.53	0.938
3rd MPJ	14.34	8.64	14.45	10.79	0.889
1st MPJ	15.24	12.59	15.10	11.54	0.895

T2DM is the group with Type 2 diabetes and Controls are the control subjects, Mann-Whitney U test was used for the follow up analysis and p values obtained. There was no significant difference found within subjects for time to peak flux (p = 0.417, ANOVA), or the groups (p = 0.722, ANOVA) or for the interaction effect between time to peak flux and the group (p = 0.868, ANOVA). No significant difference was found between the groups in any of the areas under study in the follow up analysis.

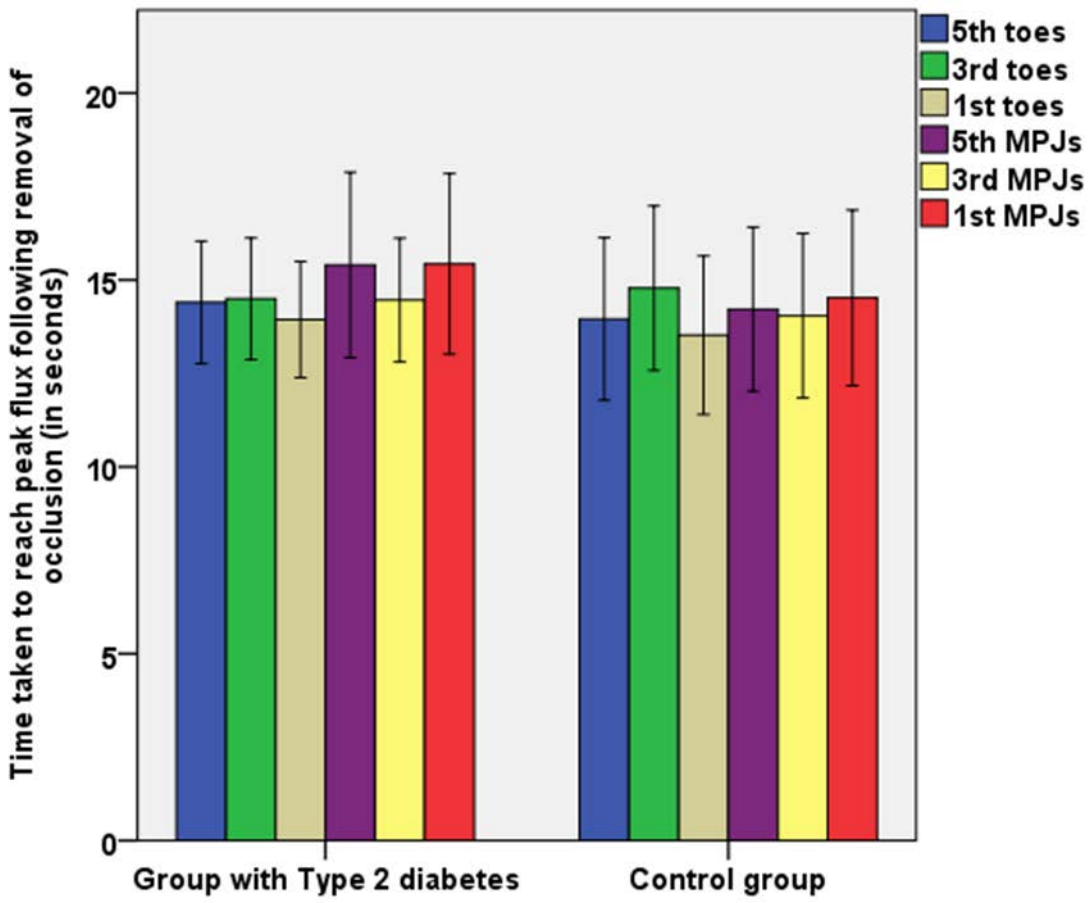

Figure 4.10 Comparison of the time to peak flux (seconds) following removal of occlusion between the two groups

4.7.1.7 Comparison of the blood flux values registered 2 minutes post peak values between the two groups

Although the group with Type 2 diabetes registered slightly higher values across the areas of study than the control group, there was no significant difference found within subjects for blood flux values obtained two minutes after the peak flux following occlusion ($F = 1.324$, $p = 0.261$) or for the group ($F = 0.362$, $p = 0.555$) or for the interaction between 2 minutes post peak flux values and the group ($F = 0.685$, $p = 0.487$). As can be noted in Table 4.6 and illustrated in Figure 4.11, there was no significant difference found on follow up analysis between the blood flux on the plantar aspect of the 5^{th}, 3^{rd} and 1^{st} toes or 5^{th}, 3^{rd} or 1^{st} MPJ areas between the groups at a period of 2 minutes post maximum or peak flux.

Table 4.6 Comparison of 2 minutes post peak flux values (AU) following release of occlusion between the two groups

Area	T2DM Mean flux	T2DM St. Dev.	Controls Mean flux	Controls St. Dev.	Significance p value
5th toe	170.60	95.67	174.80	137.57	0.681
3rd toe	170.87	117.01	165.38	104.42	0.940
1st toe	163.50	118.92	135.27	110.38	0.345
5th MPJ	175.24	127.09	127.59	85.73	0.222
3rd MPJ	186.74	128.15	155.97	115.45	0.273
1st MPJ	146.07	93.61	137.09	110.50	0.598

T2DM is the group with Type 2 diabetes and Controls are the control subjects, Mann-Whitney U test was used for the follow up analysis. There was no significant difference found within subjects for 2 mins. post peak flux (p = 0.261, ANOVA), or the groups (p = 0.555, ANOVA) or for the interaction effect between 2 mins. post peak flux and the group (p = 0.487, ANOVA) and no significant difference between the groups in any of the sites on follow up analysis.

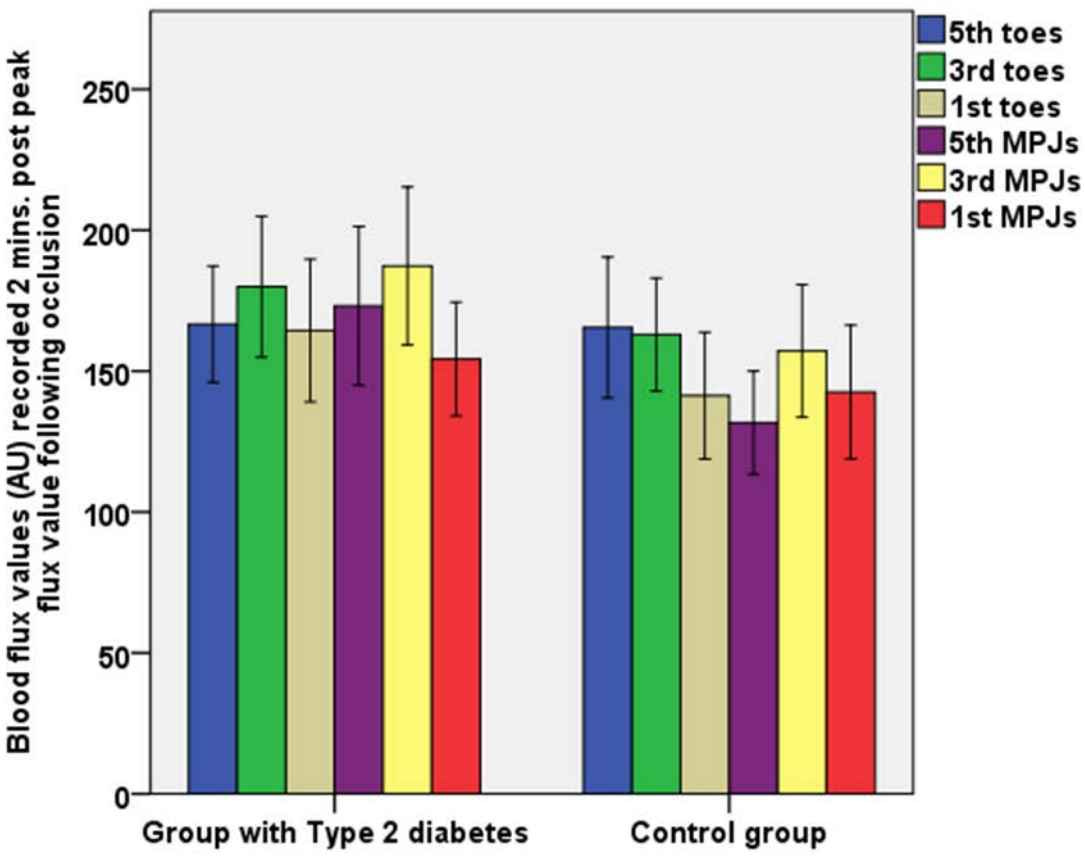

Figure 4.11 Comparison of the blood flux values at 2 minutes post peak flux (AU) between the two groups

4.7.1.8 Five minutes post peak flux values, comparison between the two groups

At five minutes post peak flux values the flux values obtained are slightly higher in the group with Type 2 diabetes when compared with the control group. There was no significant difference found for 5 minutes post peak flux ($F = 1.197$, $p = 0.320$) or for the group ($F = 1.206$, $p = 0.288$) and no significant interaction was found between the 5 minutes post peak flux values and the groups ($F = 0.740$, $p = 0.573$). However, on follow up analysis there was a significant difference found between two of the areas under study i.e. in the areas of the first toe and the 5th MPJ, it should be noted that the significance levels obtained were marginal. The results can be seen in Table 4.7, and are illustrated in Figure 4.12. There was no significant difference noted between the groups on the plantar aspect of the 5th toe ($p = 0.749$), or on the plantar aspect of the 3rd toe ($p = 0.317$). However, on the plantar surface of the hallux a significant difference in blood flux measured 5 minutes post peak flux was found between the groups, although marginal in value ($p = 0.047$), and on the plantar aspect of the 5th MPJ area of study, 5 minutes post peak pressure a significant difference was found between the groups ($p = 0.045$) however as in the hallux the value of the level of significance was marginal. There was no significant difference found between the groups on the plantar aspect of the 3rd MPJ area of the foot at 5 minutes post peak flux ($p = 0.062$) or at the plantar aspect of the 1st MPJ area ($p = 0.207$).

Table 4.7 Comparison of 5 minutes post peak flux values (AU) following release of occlusion between the two groups

Area	T2DM Mean flux	T2DM St. Dev.	Controls Mean flux	Controls St. Dev.	Significance p value
5th toe	145.63	110.62	133.30	98.58	0.749 N.S.
3rd toe	157.88	122.38	126.25	87.297	0.317 N.S.
1st toe	165.07	123.93	105.89	83.57	0.047
5th MPJ	162.79	120.67	102.13	74.30	0.045
3rd MPJ	171.53	120.26	116.97	92.79	0.062 N.S.
1st MPJ	138.25	98.34	111.16	107.86	0.207 N.S.

T2DM is the group with Type 2 diabetes and Controls are the control subjects, Mann-Whitney U test was used for the follow up analysis. There was no significant difference found within subjects for 5 mins. post peak flux (p = 0.320, ANOVA), or the groups (p = 0.288, ANOVA) or for the interaction effect between 5 mins. post peak flux and the group (p = 0.573, ANOVA). On follow up analysis, there was no significant difference found between the groups in the areas under study with the exception of the 1st toe (p = 0.047, M.W.U) and 5th MPJ areas (p = 0.045, M.W.U).

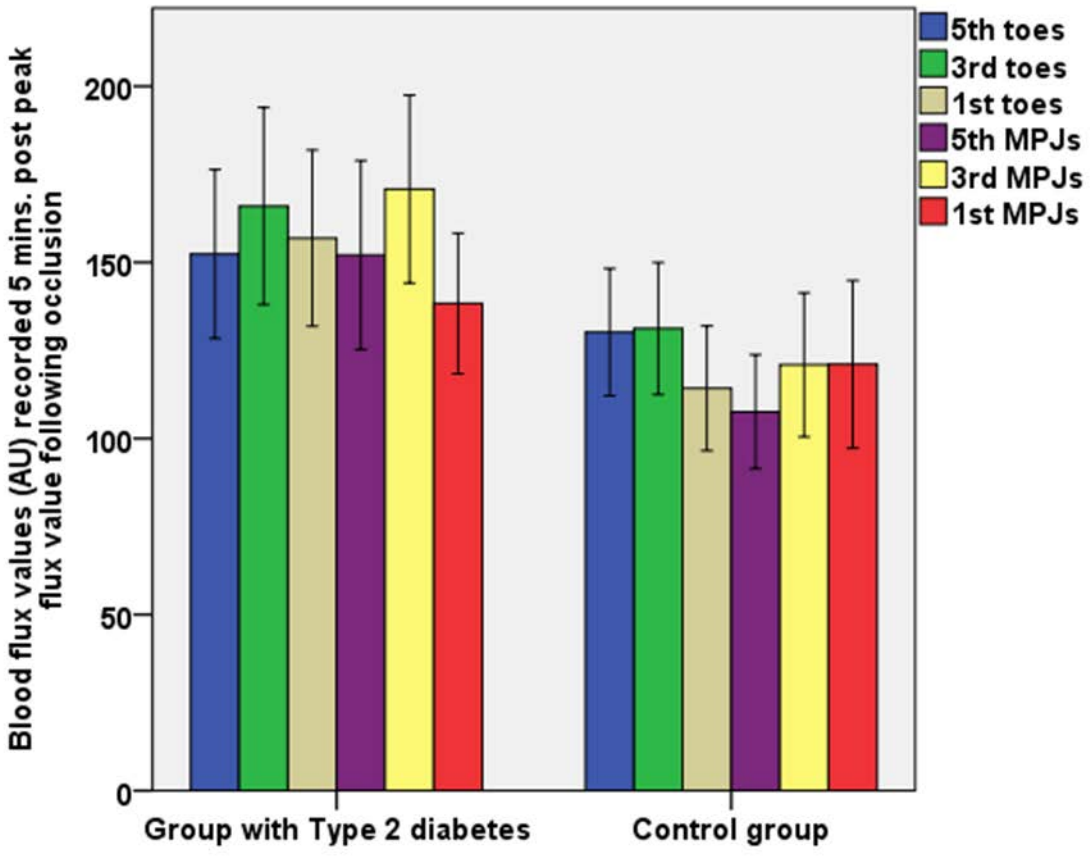

Figure 4.12 Comparison of the blood flux values (AU) 5 minutes post peak following release of occlusion between the groups

4.7.2 Investigation of the association between values obtained for the different areas under study with reactive hyperaemia and the LSCI –FLPI® system

There was a strong association found between the 6 areas of the plantar aspect under study when assessed across the group as a whole, and also across both the group with Type 2 diabetes and the control group (with the exception of 3 associations during occlusion) when assessed as individual groups, however the correlations were stronger in the group with Type 2 diabetes than found in the control group. A normal distribution was not found across the data obtained from the reactive hyperaemia assessment using LSCI as noted in Section 4.7.1 and Table 4.1 so a non-parametric method of analysis was used to analyse the data i.e. Spearman's correlation coefficient. The association found across the group as a whole can be found in Tables 4.8 and 4.9 and demonstrated in the scatterplots Figures 4.13 to 4.18. The association found between the areas in the group with diabetes can be found in Tables 4.10 and 4.11 and seen in scatterplots 4.19 to 4.24, with data for the control subjects in Tables 4.12 and 4.13 and Figures 4.25 to 4.30.

Table 4.8 Mean values and standard deviations for the areas for n=60 under study for association

Area	Resting flux (AU) Mean ± S.D.	Occlusion (AU) Mean ± S.D.	Peak flux (AU) Mean ± S.D.	Time to peak flux (secs.) Mean ± S.D.	2 mins post peak flux (AU) Mean ± S.D.	5 mins. post peak flux (AU) Mean ± S.D.
5th toe	167.00±125.99	26.90±18.09	440.19±242.05	14.22±9.09	172.55±115.90	139.81±104.32
3rd toe	149.60±115.51	28.80±16.48	423.84±221.70	15.19±9.93	168.17±110.08	142.33±106.87
1st toe	149.56±126.00	25.15±16.40	417.04±238.27	14.04±9.29	148.61±114.31	134.38±108.13
5th MPJ	157.02±139.13	27.18±16.39	471.10±224.67	15.15±12.26	152.27±110.79	133.54±104.68
3rd MPJ	159.42±151.07	28.89±17.27	485.74±231.60	14.39±9.69	171.36±121.89	144.25±109.96
1st MPJ	146.72±113.07	25.18±13.34	457.27±239.06	15.17±11.97	141.82±101.13	124.95±103.07

Table 4.9 Correlation found between the areas of the plantar aspect of the forefoot in the group where n=60, including both subjects with Type 2 diabetes and the control subjects

Areas	Resting Flux		Occlusion		Peak Flux		Time to Peak Flux		2 mins. post Peak Flux		5 mins. post Peak Flux	
	r	p	r	p	r	p	r	p	r	p	r	p
5tv3t	0.929	<0.0001	0.627	<0.0001	0.891	<0.0001	0.797	<0.0001	0.833	<0.0001	0.884	<0.0001
5tv1t	0.853	<0.0001	0.421	0.0001	0.600	<0.0001	0.812	<0.0001	0.802	<0.0001	0.801	<0.0001
5tv5m	0.864	<0.0001	0.465	0.0001	0.791	<0.0001	0.768	<0.0001	0.836	<0.0001	0.812	<0.0001
5tv3m	0.845	<0.0001	0.557	<0.0001	0.889	<0.0001	0.771	<0.0001	0.855	<0.0001	0.782	<0.0001
5tv1m	0.879	<0.0001	0.495	<0.0001	0.589	<0.0001	0.787	<0.0001	0.767	<0.0001	0.745	<0.0001
3tv1t	0.877	<0.0001	0.569	<0.0001	0.666	<0.0001	0.859	<0.0001	0.793	<0.0001	0.842	<0.0001
3tv5m	0.845	<0.0001	0.605	<0.0001	0.770	<0.0001	0.787	<0.0001	0.770	<0.0001	0.783	<0.0001
3tv3m	0.844	<0.0001	0.599	<0.0001	0.826	<0.0001	0.832	<0.0001	0.792	<0.0001	0.824	<0.0001
3tv1m	0.871	<0.0001	0.567	<0.0001	0.594	<0.0001	0.794	<0.0001	0.814	<0.0001	0.824	<0.0001
1tv5m	0.837	<0.0001	0.533	<0.0001	0.449	<0.0001	0.866	<0.0001	0.791	<0.0001	0.831	<0.0001
1tv3m	0.850	<0.0001	0.600	<0.0001	0.573	<0.0001	0.851	<0.0001	0.820	<0.0001	0.815	<0.0001
1tv1m	0.877	<0.0001	0.637	<0.0001	0.590	<0.0001	0.869	<0.0001	0.808	<0.0001	0.872	<0.0001
5mv3m	0.944	<0.0001	0.784	<0.0001	0.848	<0.0001	0.880	<0.0001	0.894	<0.0001	0.912	<0.0001
5mv1m	0.910	<0.0001	0.773	<0.0001	0.665	<0.0001	0.849	<0.0001	0.833	<0.0001	0.842	<0.0001
3mv1m	0.939	<0.0001	0.907	<0.0001	0.725	<0.0001	0.900	<0.0001	0.864	<0.0001	0.895	<0.0001

In Table 4.9 t = toe, m =MPJ, r = Spearman's correlation coefficient, p=significance
There was a significant association found between all areas under study in the group with both subjects with Type 2 diabetes and control subjects where n=60, with 5th toe n=56, 3rd toe n=58, 1st toe n=58, 5th MPJ n=53, 3rd MPJ n=58 and 1st MPJ n=58.

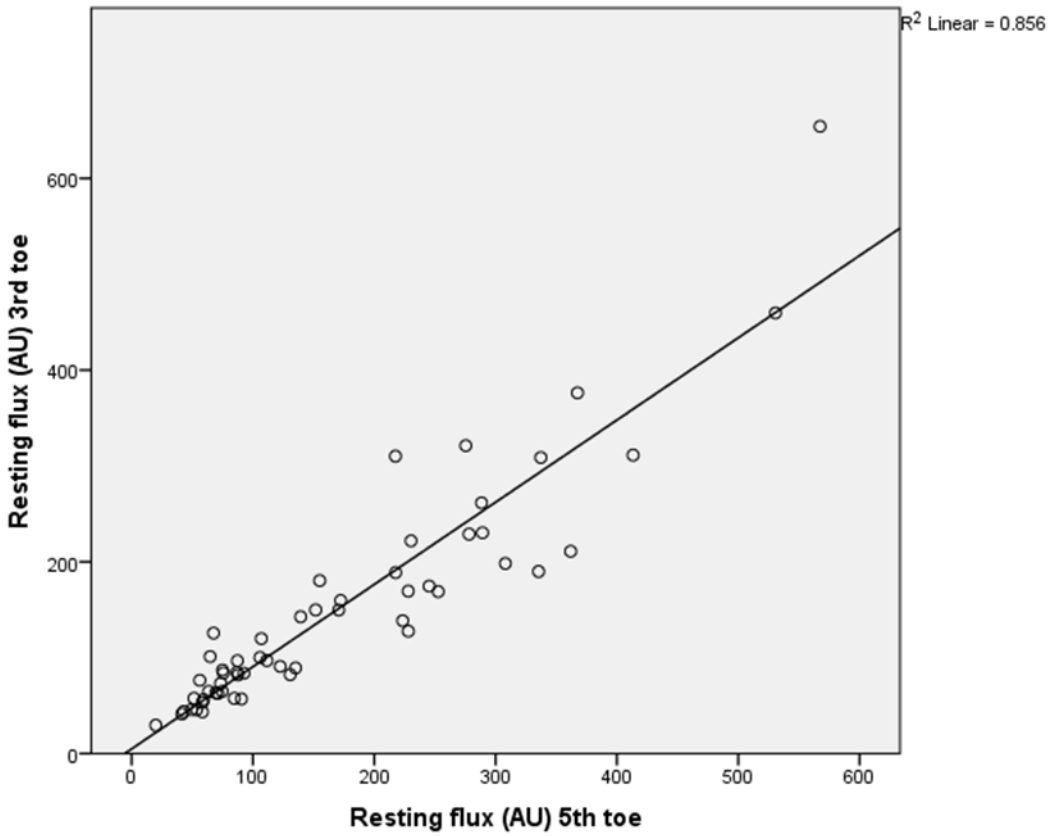

Figure 4.13 Scatterplot displaying the association between resting flux (AU) found at the 5th and 3rd toes

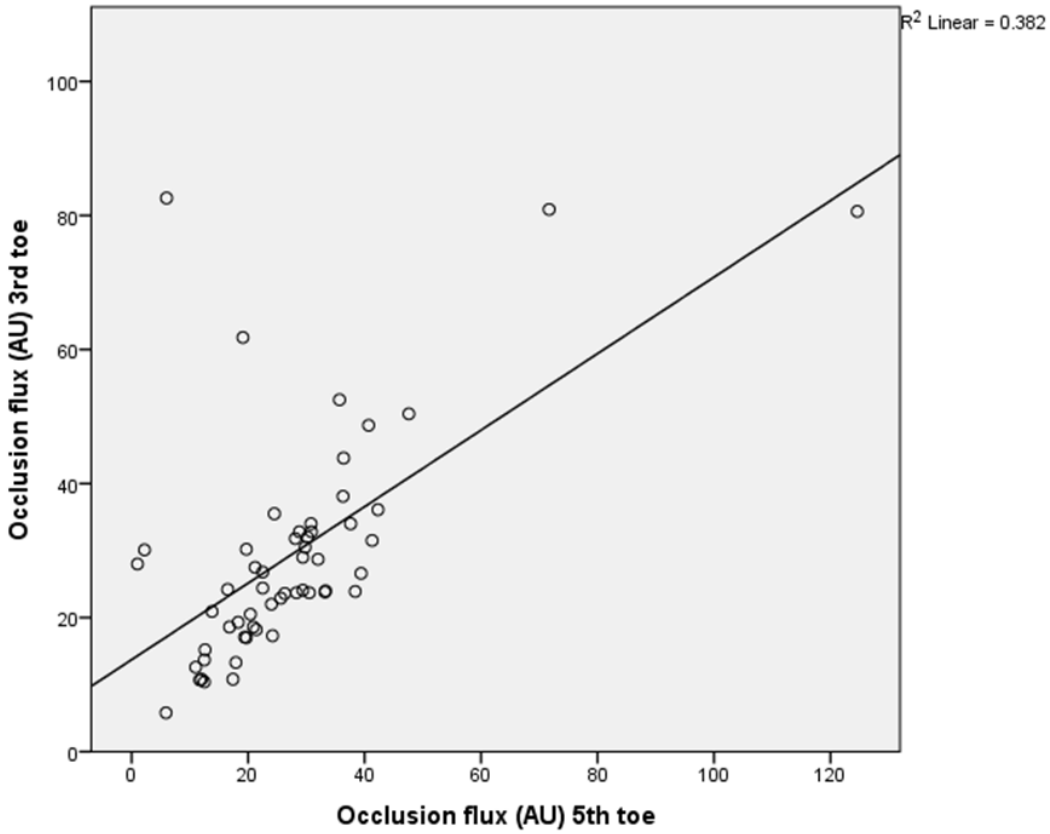

Figure 4.14 Scatterplot displaying the association between occlusion flux (AU) found at the 5th and 3rd toes

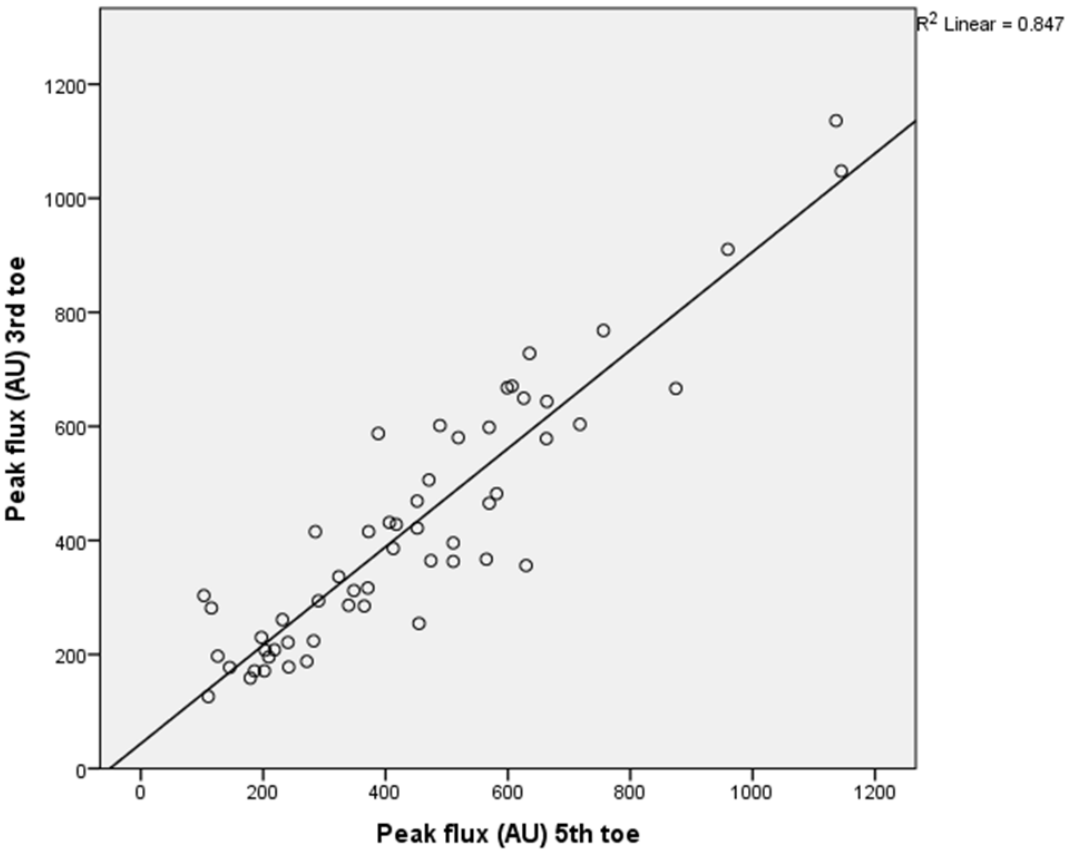

Figure 4.15 Scatterplot of the association of peak flux (AU) following occlusion for the 5th and 3rd toes

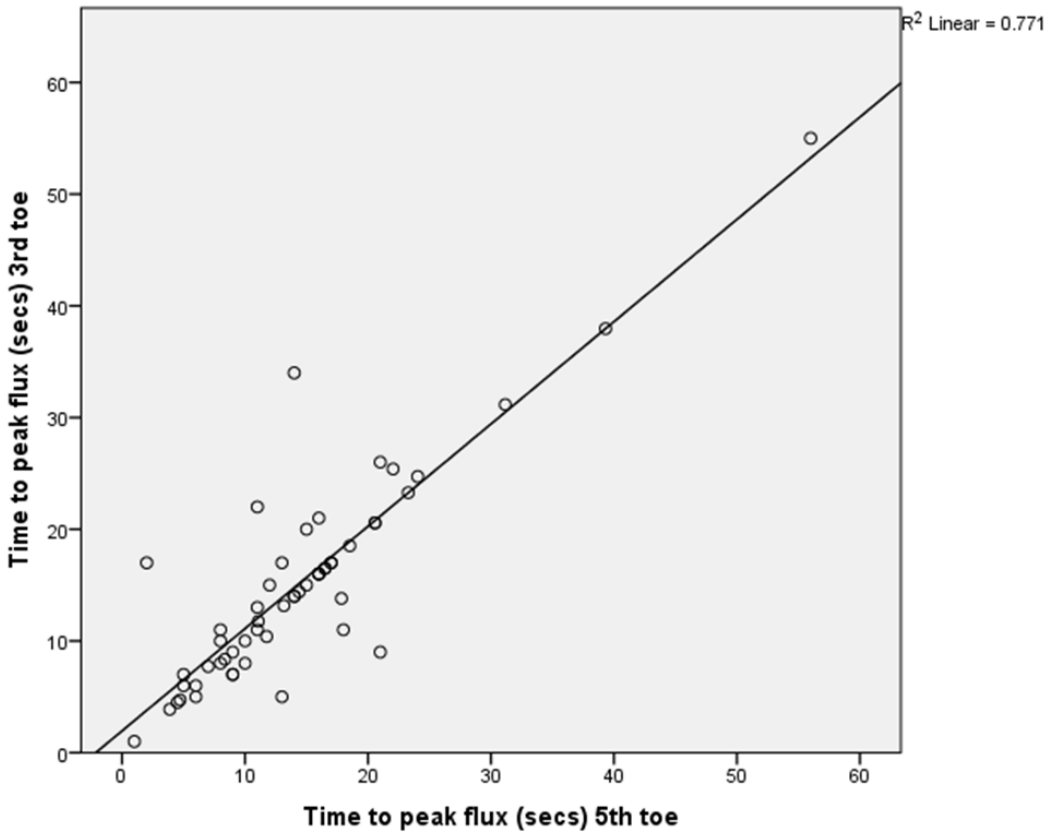

Figure 4.16 Scatterplot of the association of time to peak flux (seconds) following occlusion for the 5th and 3rd toes

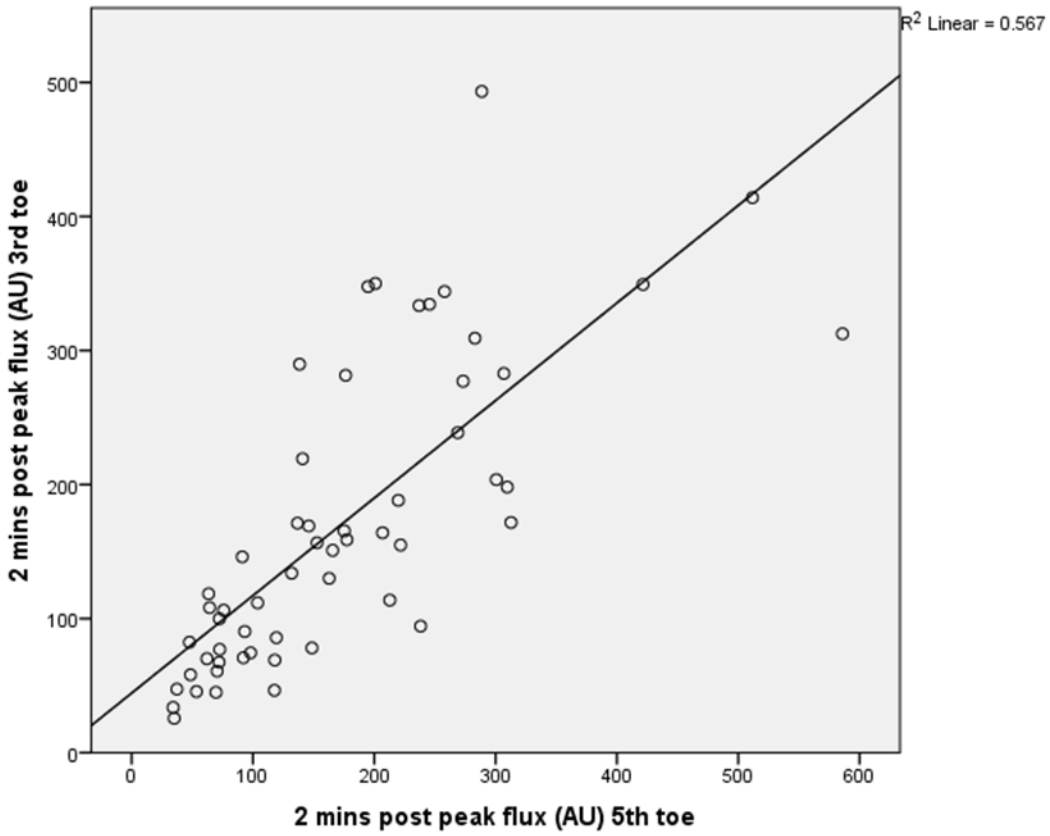

Figure 4.17 Scatterplot of the association for 2 minutes post peak flux (AU) between 5th and 3rd toes

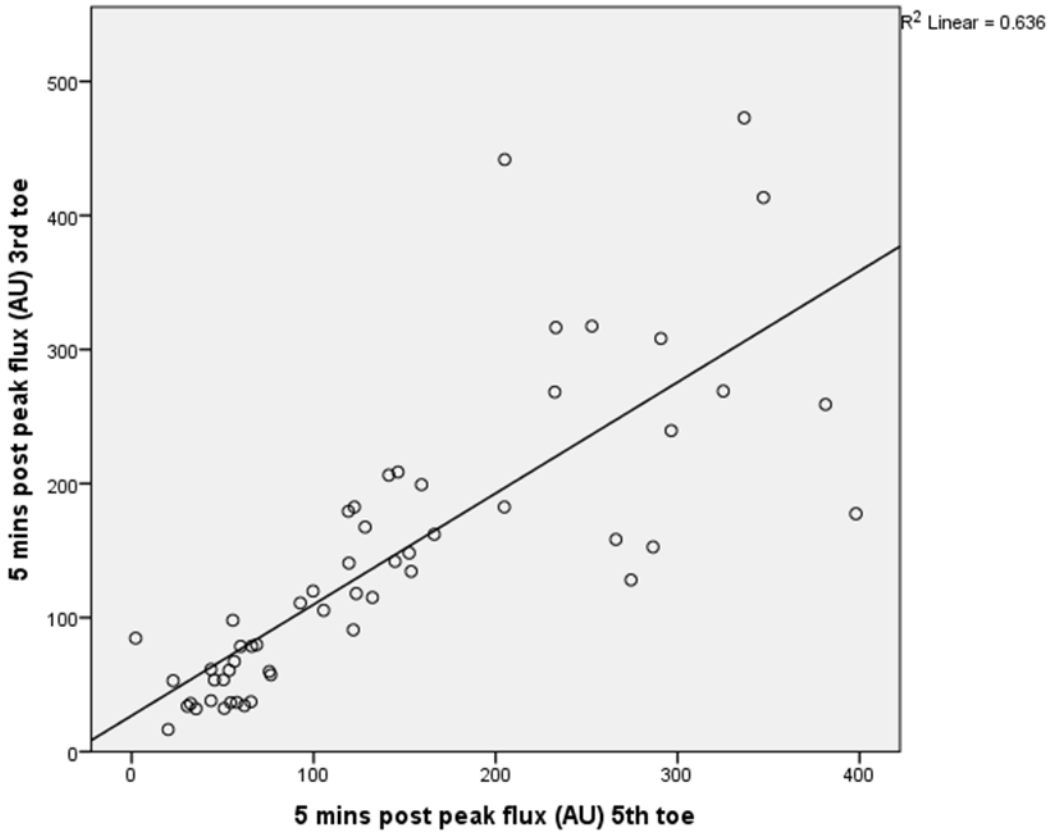

Figure 4.18 Scatterplot of the association for 5 minutes post peak flux (AU) between 5th and 3rd toes

Table 4.10 Mean values with standard deviations for the group with Type 2 diabetes found in the areas under study for association

Area	Resting flux (AU) Mean ± S.D.	Occlusion (AU) Mean ± S.D.	Peak flux (AU) Mean ± S.D.	Time to peak flux (secs.) Mean ± S.D.	2 mins post peak flux (AU) Mean ± S.D.	5 mins. post peak flux (AU) Mean ± S.D.
5th toe	178.79±123.92	26.57±12.86	486.58±225.02	14.49±8.54	170.60±95.67	145.63±110.62
3rd toe	154.05±105.17	28.89±15.56	482.51±210.06	14.58±8.47	170.87±117.01	157.88±122.38
1st toe	164.98±128.57	23.83±13.10	452.99±236.97	14.05±8.08	163.50±118.92	165.07±123.93
5th MPJ	161.94±110.54	27.95±14.88	561.62±215.21	15.40±13.12	175.24±127.09	162.79±120.67
3rd MPJ	152.65±103.10	28.96±14.88	569.77±225.51	14.34±8.64	186.74±128.15	171.53±120.26
1st MPJ	149.23±97.24	24.83±10.29	532.47±250.39	15.24±12.59	146.07±93.61	138.25±98.34

Table 4.11 Correlation found between the areas of the plantar aspect of the forefoot in the group with Type 2 diabetes (n=30)

Areas	Resting Flux		Occlusion		Peak Flux		Time to Peak Flux		2 mins. post Peak Flux		5 mins. post Peak Flux	
	r	p	r	p	r	p	r	p	r	p	r	p
5tv3t	0.912	<0.0001	0.677	<0.0001	0.862	<0.0001	0.867	<0.0001	0.774	<0.0001	0.862	<0.0001
5tv1t	0.835	<0.0001	0.442	0.016	0.596	0.001	0.824	<0.0001	0.830	<0.0001	0.804	<0.0001
5tv5m	0.936	<0.0001	0.686	<0.0001	0.738	<0.0001	0.895	<0.0001	0.817	<0.0001	0.814	<0.0001
5tv3m	0.863	<0.0001	0.741	<0.0001	0.845	<0.0001	0.891	<0.0001	0.815	<0.0001	0.767	<0.0001
5tv1m	0.870	<0.0001	0.720	<0.0001	0.595	0.001	0.867	<0.0001	0.701	<0.0001	0.717	<0.0001
3tv1t	0.878	<0.0001	0.578	<0.0001	0.694	<0.0001	0.961	<0.0001	0.877	<0.0001	0.941	<0.0001
3tv5m	0902	<0.0001	0.725	<0.0001	0.737	<0.0001	0.948	<0.0001	0.805	<0.0001	0.805	<0.0001
3tv3m	0.890	<0.0001	0.781	<0.0001	0.792	<0.0001	0.930	<0.0001	0.789	<0.0001	0.869	<0.0001
3tv1m	0.888	<0.0001	0.724	<0.0001	0.644	<0.0001	0.927	<0.0001	0.831	<0.0001	0.881	<0.0001
1tv5m	0.851	<0.0001	0.499	0.008	0.492	0.011	0.972	<0.0001	0.866	<0.0001	0.885	<0.0001
1tv3m	0.867	<0.0001	0.693	<0.0001	0.459	0.014	0.945	<0.0001	0.885	<0.0001	0.913	<0.0001
1tv1m	0.854	<0.0001	0.736	<0.0001	0.572	0.001	0.950	<0.0001	0.835	<0.0001	0.932	<0.0001
5mv3m	0.937	<0.0001	0.889	<0.0001	0.712	<0.0001	0.922	<0.0001	0.855	<0.0001	0.898	<0.0001
5mv1m	0.940	<0.0001	0.848	<0.0001	0.585	0.001	0.986	<0.0001	0.803	<0.0001	0.803	<0.0001
3mv1m	0.922	<0.0001	0.953	<0.0001	0.727	<0.0001	0.982	<0.0001	0.861	<0.0001	0.916	<0.0001

In Table 4.11 t = toe, m =MPJ, r = Spearman's correlation coefficient, p=significance
There was a significant association found between all areas under study in the group with Type 2 diabetes and n=30 for the group, for 5th toe, 3rd toe and 1st toe n=29, 5th MPJ n=27 and for 3rd and 1st MPJs n=29.

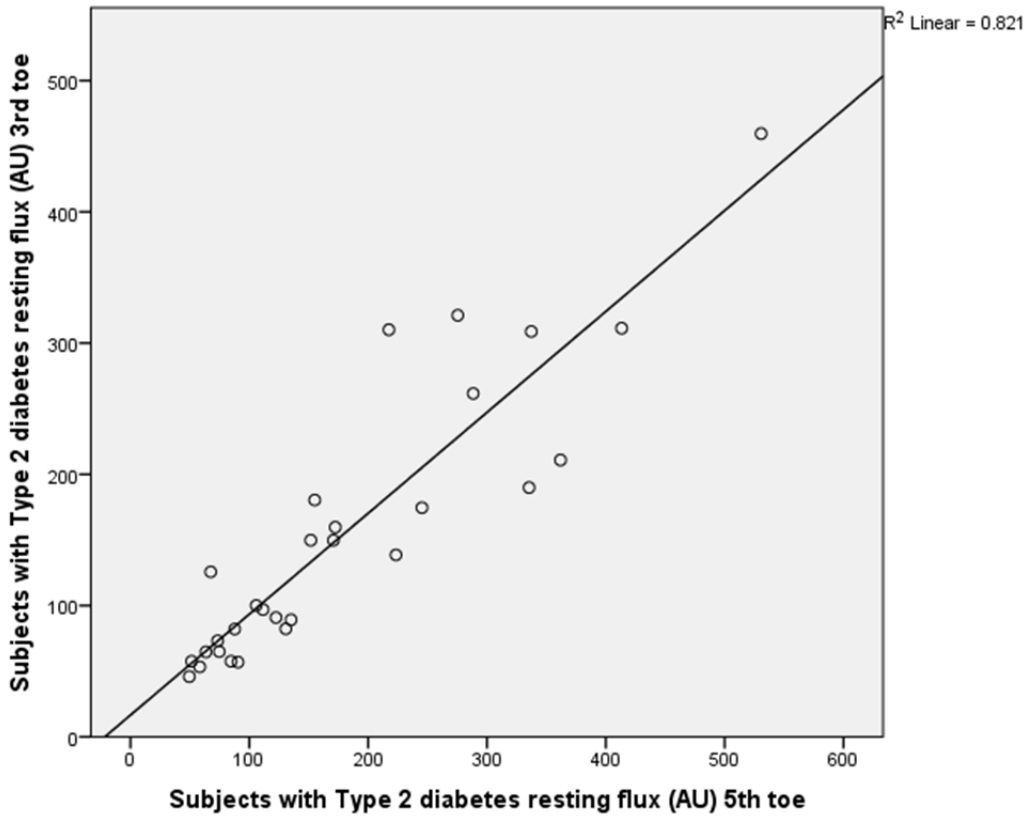

Figure 4.19 Scatterplot of the association between the resting flux (AU) at 5th and 3rd toes in the group with Type 2 diabetes

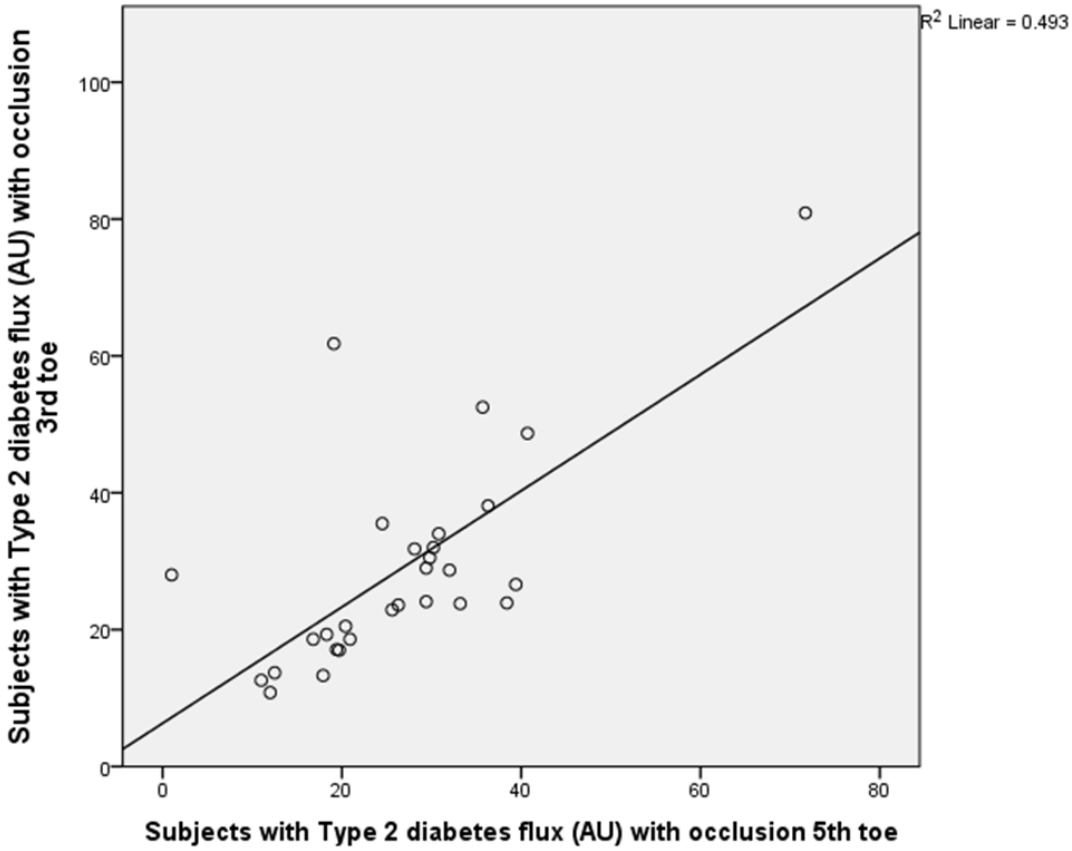

Figure 4.20 Scatterplot of the association between the 5th and 3rd toes (AU) with occlusion in the group with Type 2 diabetes

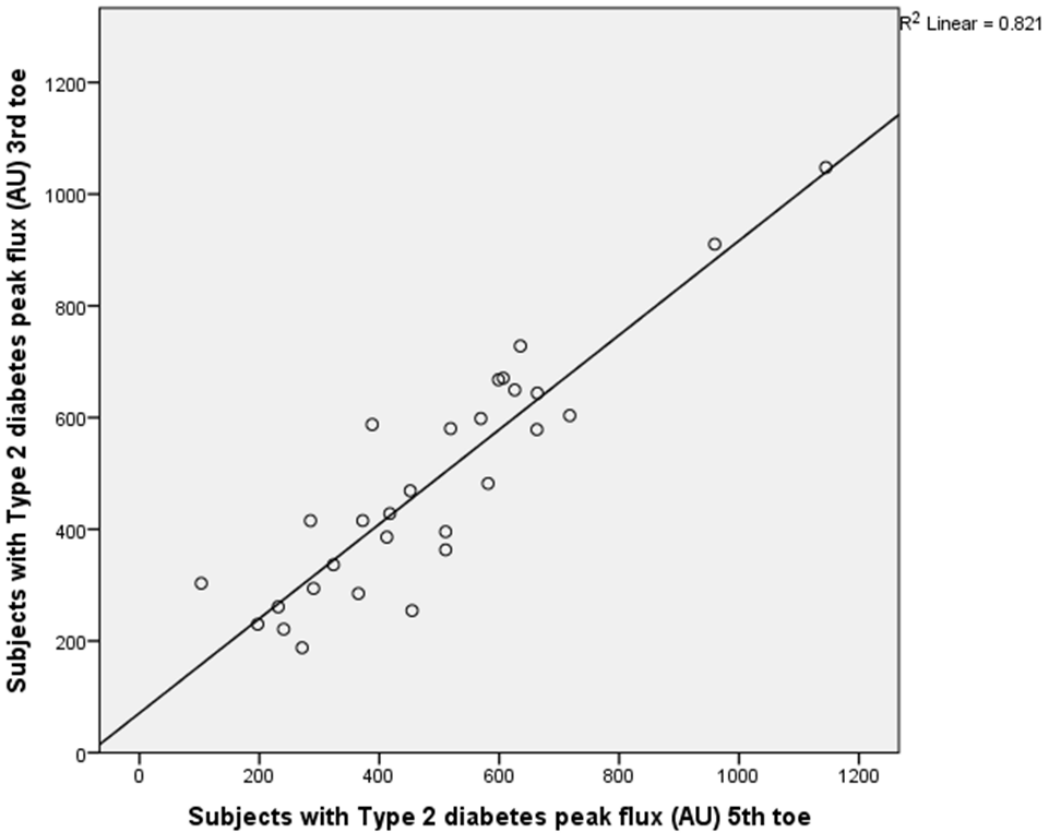

Figure 4.21 Scatterplot of the association between the peak flux at the 5th and 3rd toes (AU) in the group with Type 2 diabetes

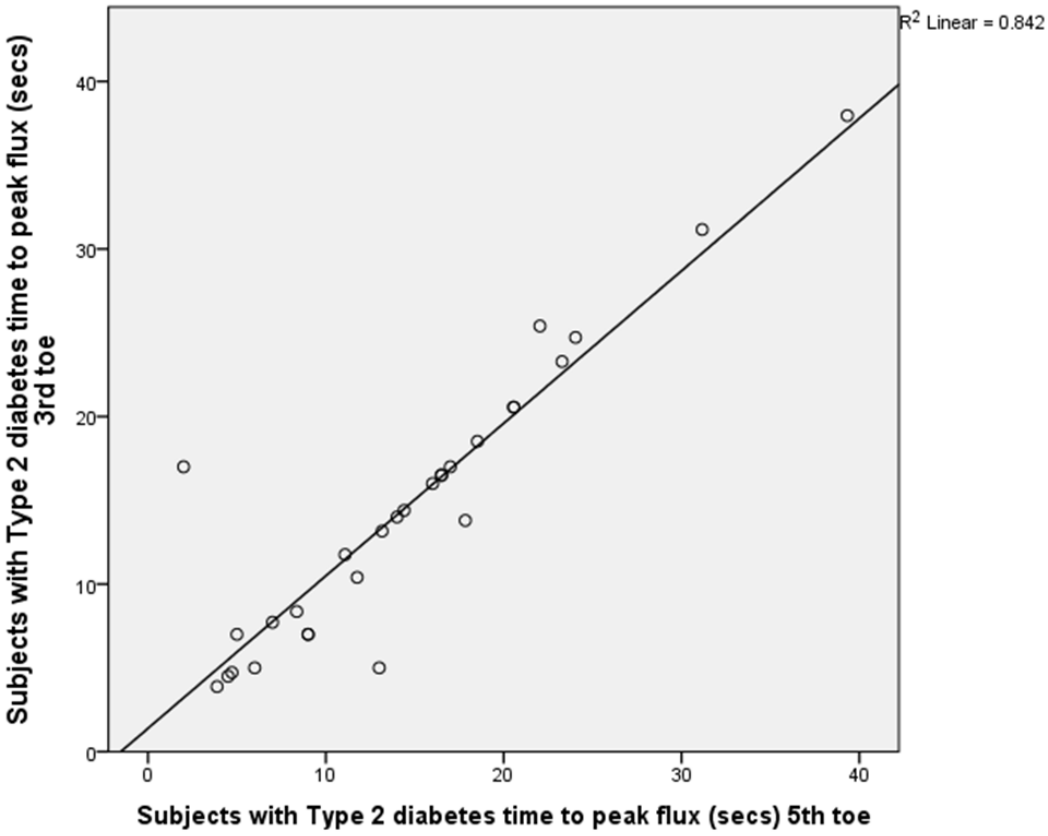

Figure 4.22 Scatterplot of the association between time to peak flux at the 5th and 3rd toes (seconds)) in the group with Type 2 diabetes

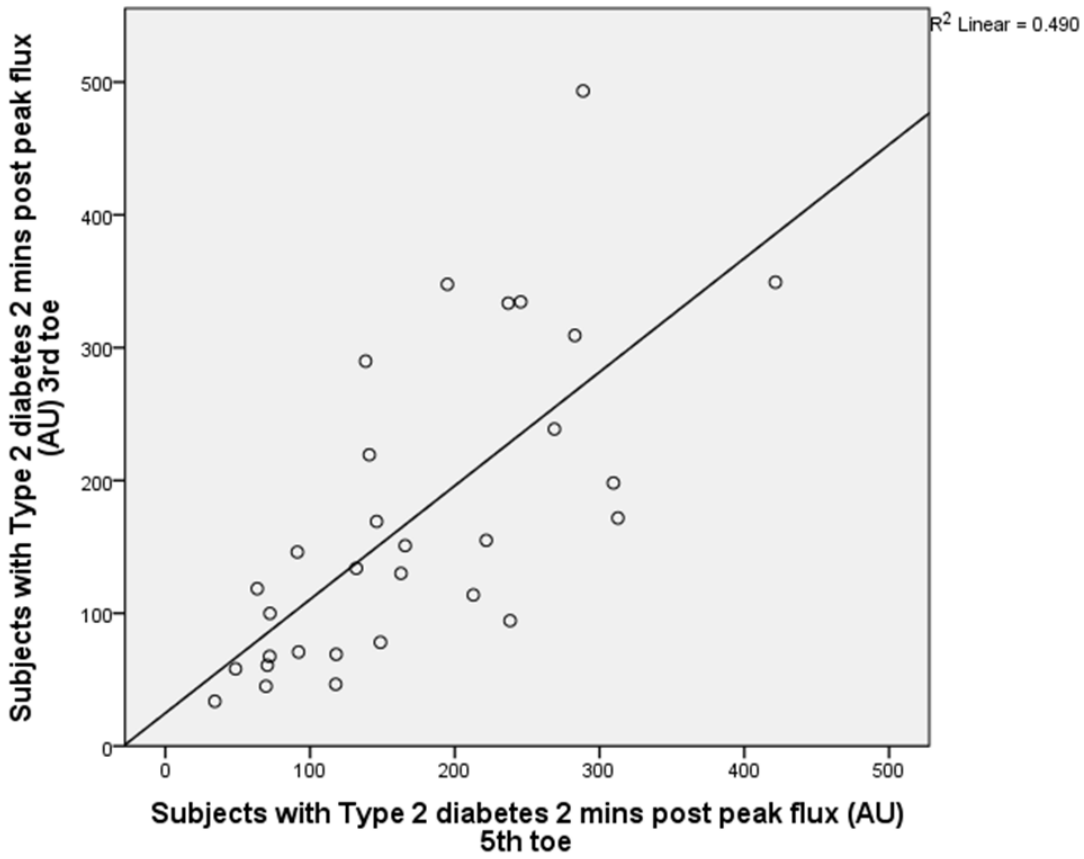

Figure 4.23 Scatterplot of the association found between 2 minutes post peak flux on the 5th and 3rd toes (AU) in the group with Type 2 diabetes

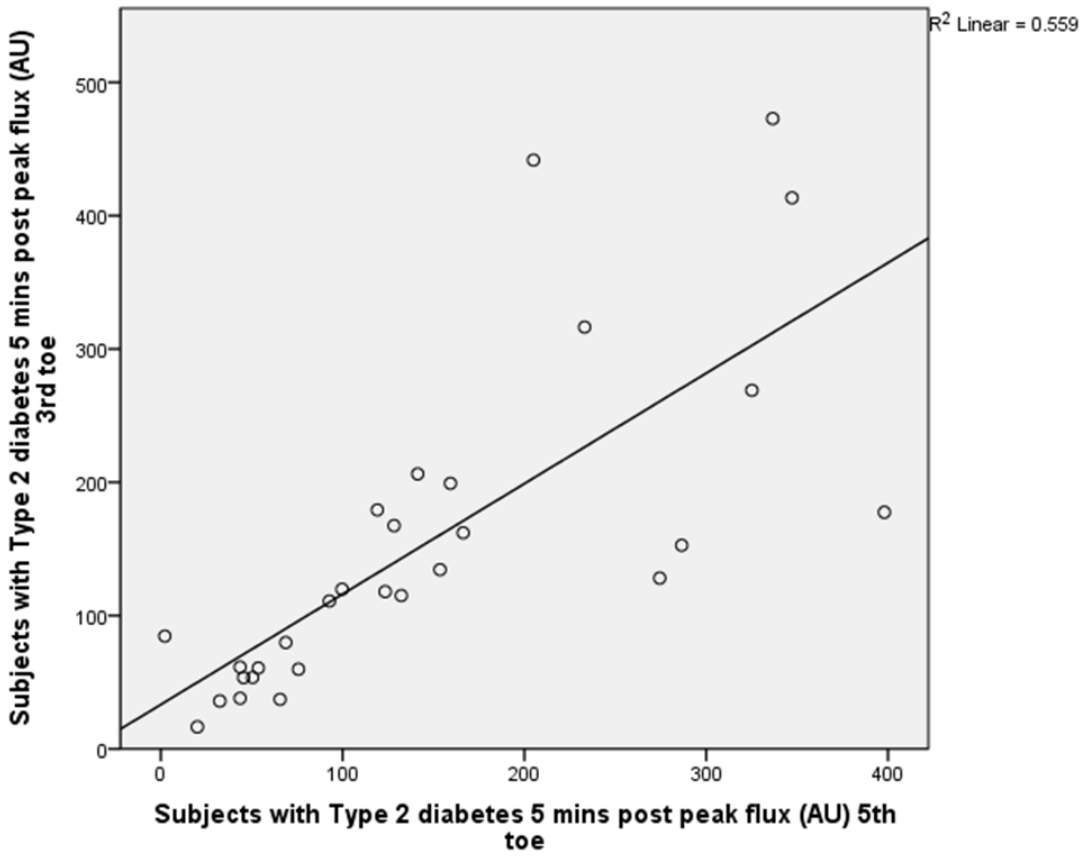

Figure 4.24 Scatterplot of the association between the post peak flux after 5 minutes for the 5th and 3rd toes in the group with Type 2 diabetes

Table 4.12 Mean values and standard deviations for the group of healthy control subjects n = 30 found in the areas under study for association

Area	Resting flux (AU) Mean ± S.D.	Occlusion (AU) Mean ± S.D.	Peak flux (AU) Mean ± S.D.	Time to peak flux (secs.) Mean ± S.D.	2 mins post peak flux (AU) Mean ± S.D.	5 mins. post peak flux (AU) Mean ± S.D.
5th toe	154.34±129.31	154.34±129.31	388.45±254.09	13.92±9.82	174.80±137.57	133.30±98.58
3rd toe	145.14±126.73	145.14±126.73	365.17±220.90	15.79±11.32	165.38±104.42	126.25±87.30
1st toe	134.14±123.68	134.14±123.68	382.33±238.45	14.03±10.51	135.27±110.38	105.88±83.57
5th MPJ	151.90±165.84	151.90±165.84	377.10±196.93	14.88±11.53	127.59±85.73	102.13±74.30
3rd MPJ	166.19±189.04	166.19±189.04	401.71±209.09	14.45±10.79	155.97±115.45	116.97±92.79
1st MPJ	144.22±128.67	144.22±128.67	382.06±204.76	15.10±11.54	137.09±110.50	111.16±107.86

4.13 Correlation found between the areas of the plantar aspect of the forefoot (n = 30) in the group of control subjects

Areas	Resting Flux		Occlusion		Peak Flux		Time to Peak Flux		2 mins. post Peak Flux		5 mins. post Peak Flux	
	r	p	r	p	r	p	r	p	r	p	r	p
5tv3t	0.951	<0.0001	0.577	0.002	0.889	<0.0001	0.708	<0.0001	0.912	<0.0001	0.911	<0.0001
5tv1t	0.812	<0.0001	0.370	**0.058N.S**.	0.575	0.002	0.795	<0.0001	0.772	<0.0001	0.824	<0.0001
5tv5m	0.838	<0.0001	0.196	**0.359N.S**.	0.806	<0.0001	0.510	0.013	0.867	<0.0001	0.841	<0.0001
5tv3m	0.834	<0.0001	0.413	0.032	0.891	<0.0001	0.581	0.002	0.867	<0.0001	0.822	<0.0001
5tv1m	0.858	<0.0001	0.291	**0.141N.S.**	0.495	0.010	0.684	<0.0001	0.849	<0.0001	0.786	<0.0001
3tv1t	0.887	<0.0001	0.534	0.003	0.628	<0.0001	0.723	<0.0001	0.733	<0.0001	0.739	<0.0001
3tv5m	0.814	<0.0001	0.468	0.016	0.772	<0.0001	0.503	0.009	0.801	<0.0001	0.768	<0.0001
3tv3m	0.847	<0.0001	0.452	0.014	0.806	<0.0001	0.705	<0.0001	0.795	<0.0001	0.799	<0.0001
3tv1m	0.876	<0.0001	0.446	0.015	0.504	0.005	0.636	<0.0001	0.828	<0.0001	0.765	<0.0001
1tv5m	0.767	<0.0001	0.571	0.002	0.426	0.03	0.679	<0.0001	0.673	<0.0001	0.768	<0.0001
1tv3m	0.817	<0.0001	0.527	0.003	0.685	<0.0001	0.698	<0.0001	0.750	<0.0001	0.692	<0.0001
1tv1m	0.864	<0.0001	0.553	0.002	0.611	<0.0001	0.754	<0.0001	0.751	<0.0001	0.785	<0.0001
5mv3m	0.939	<0.0001	0.691	<0.0001	0.850	<0.0001	0.708	<0.0001	0.902	<0.0001	0.930	<0.0001
5mv1m	0.892	<0.0001	0.729	<0.0001	0.646	<0.0001	0.674	<0.0001	0.897	<0.0001	0.890	<0.0001
3mv1m	0.936	<0.0001	0.876	<0.0001	0.676	<0.0001	0.815	<0.0001	0.911	<0.0001	0.893	<0.0001

In Table 4.13 t = toe, m =MPJ, r = Spearman's correlation coefficient, p=significance
There was a significant association found between all areas under study with the exception of associations between 5^{th} toe and 1^{st} toe, 5^{th} toe and 5^{th} MPJ and 5^{th} toe and 1^{st} MPJ which during occlusion only did not display a significant association in the control group with n=30 for the group, for 5^{th} toe n=27, 3^{rd} toe and 1^{st} toe n=29, 5^{th} MPJ n=26 and for 3^{rd} and 1^{st} MPJs n=29.

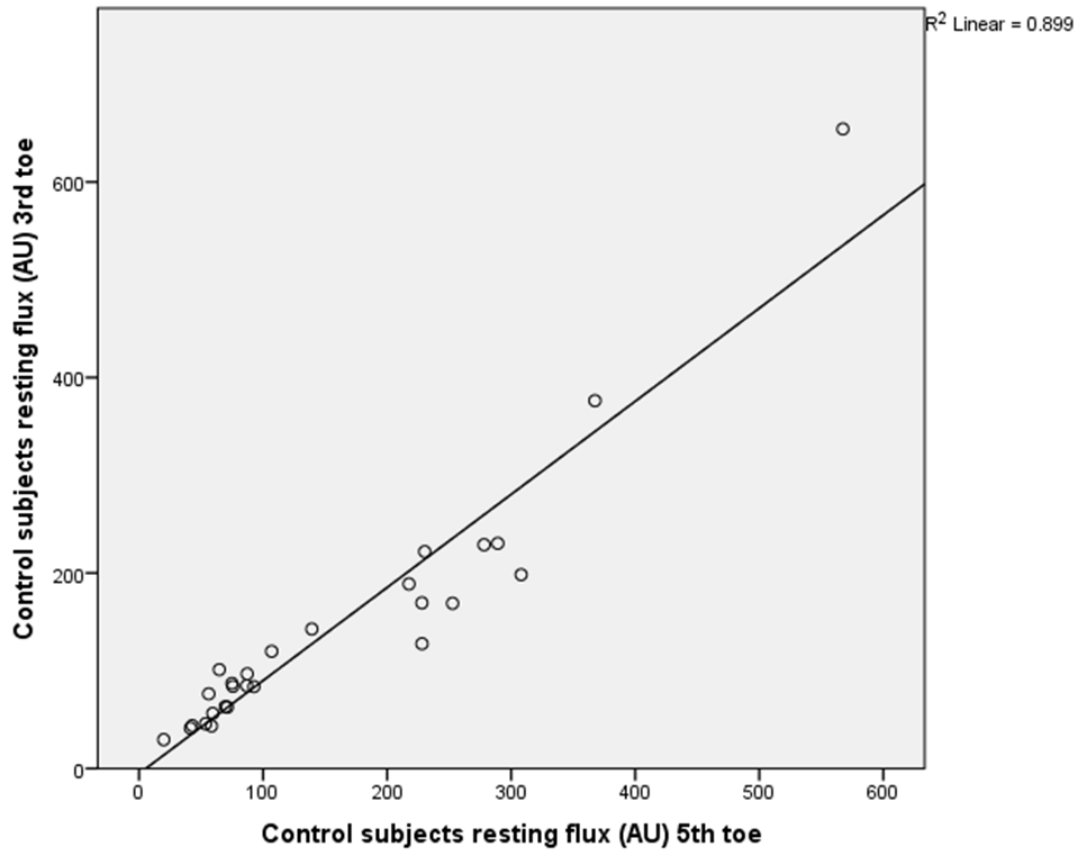

Figure 4.25 Scatterplot of the association between the resting flux (AU) found at the 5th and 3rd toes in the control subjects

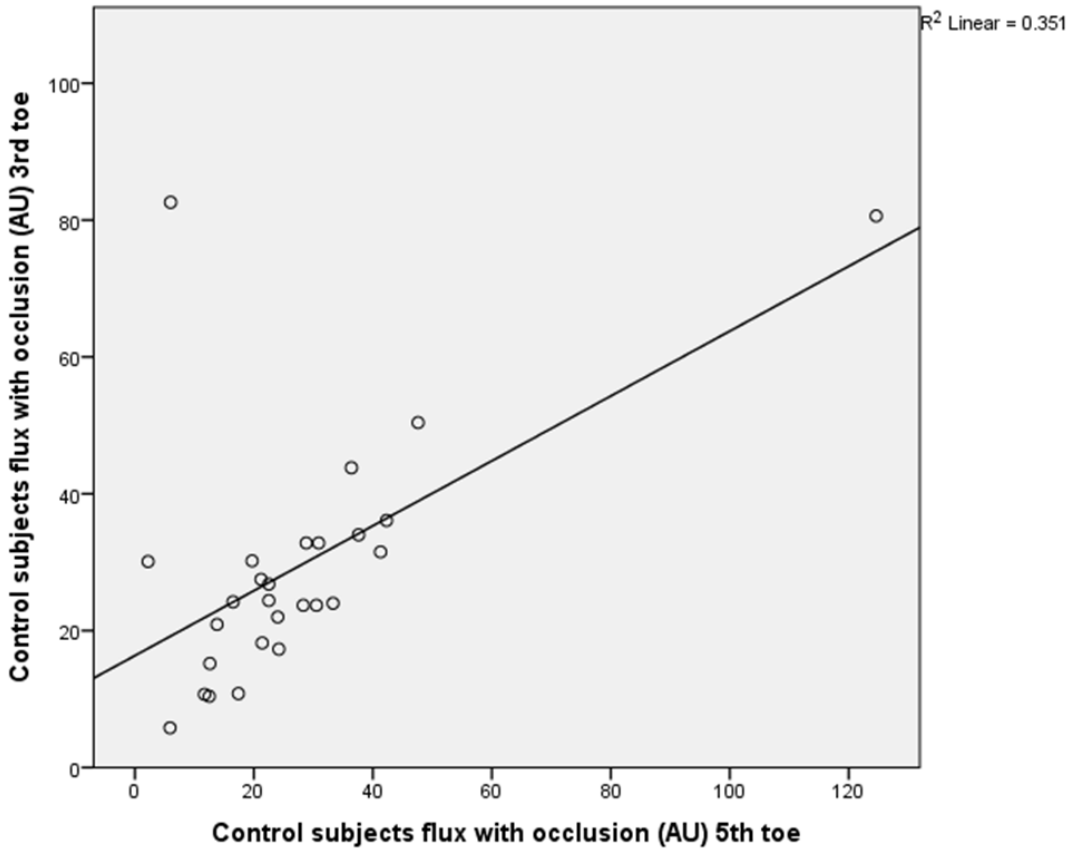

Figure 4.26 Scatterplot of the association between 5th and 3rd toe (AU) during occlusion in the control subjects

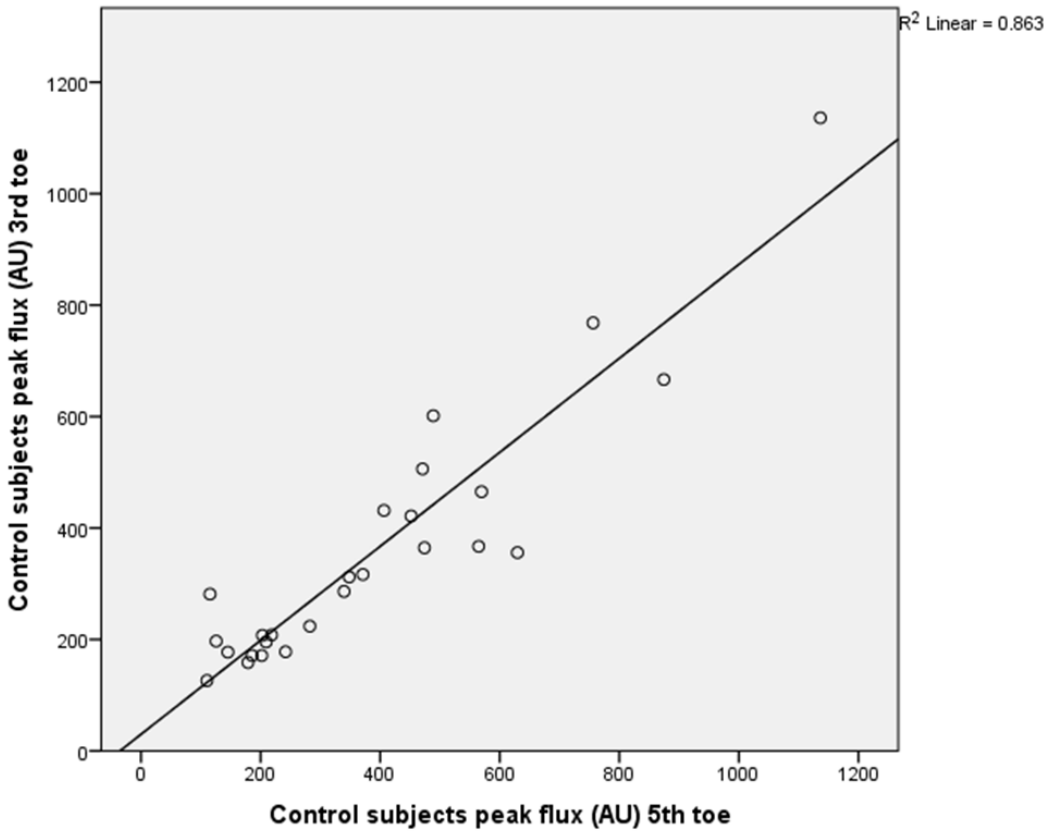

Figure 4.27 Scatterplot displaying the association between the peak flux (AU) found at the 3rd and 5th toes in the group of control subjects

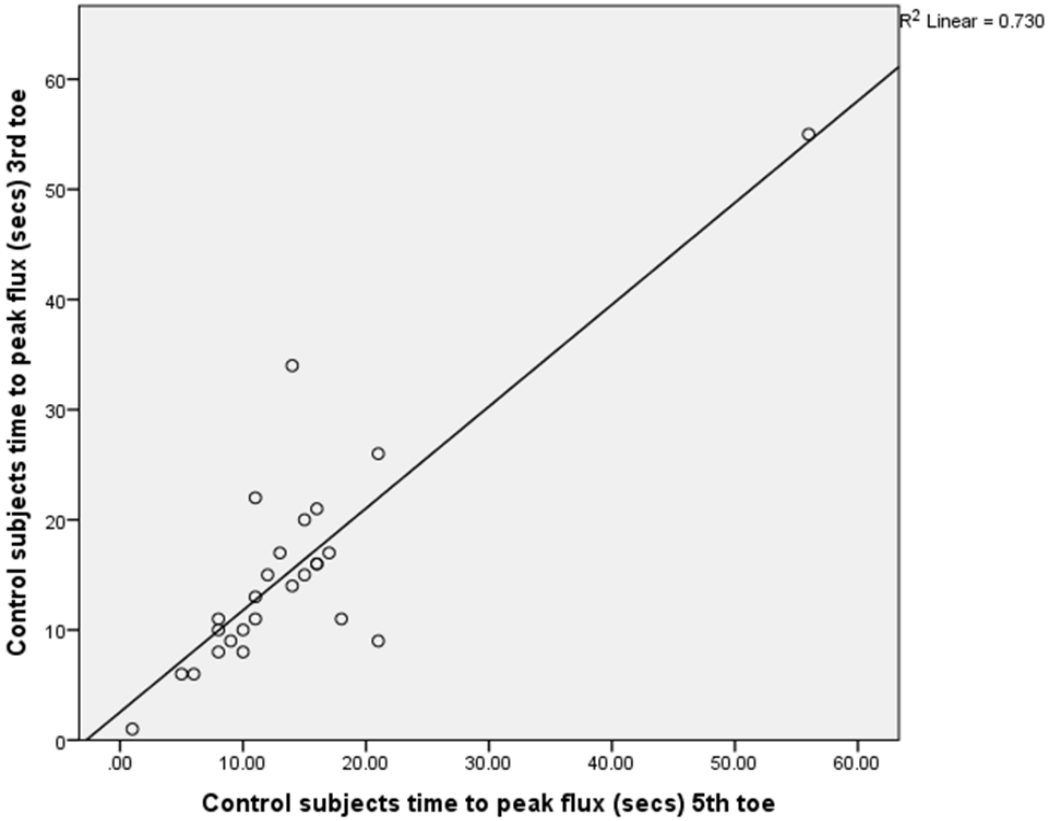

Figure 4.28 Scatterplot displaying the association between the time to peak flux found at the 5th and 3rd toes (seconds) in the group of control subjects

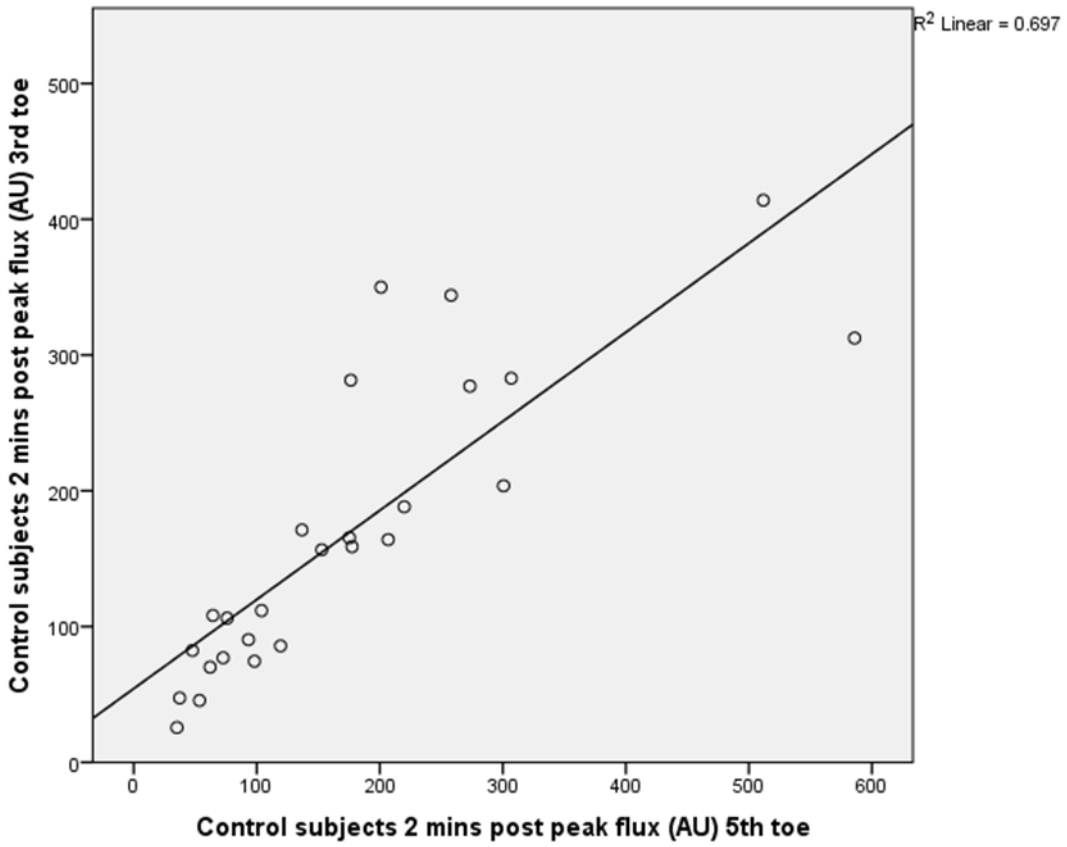

Figure 4.29 Scatterplot displaying the association found at 2 mins. post peak flux at the 5th and 3rd toes (AU) in the group of control subjects

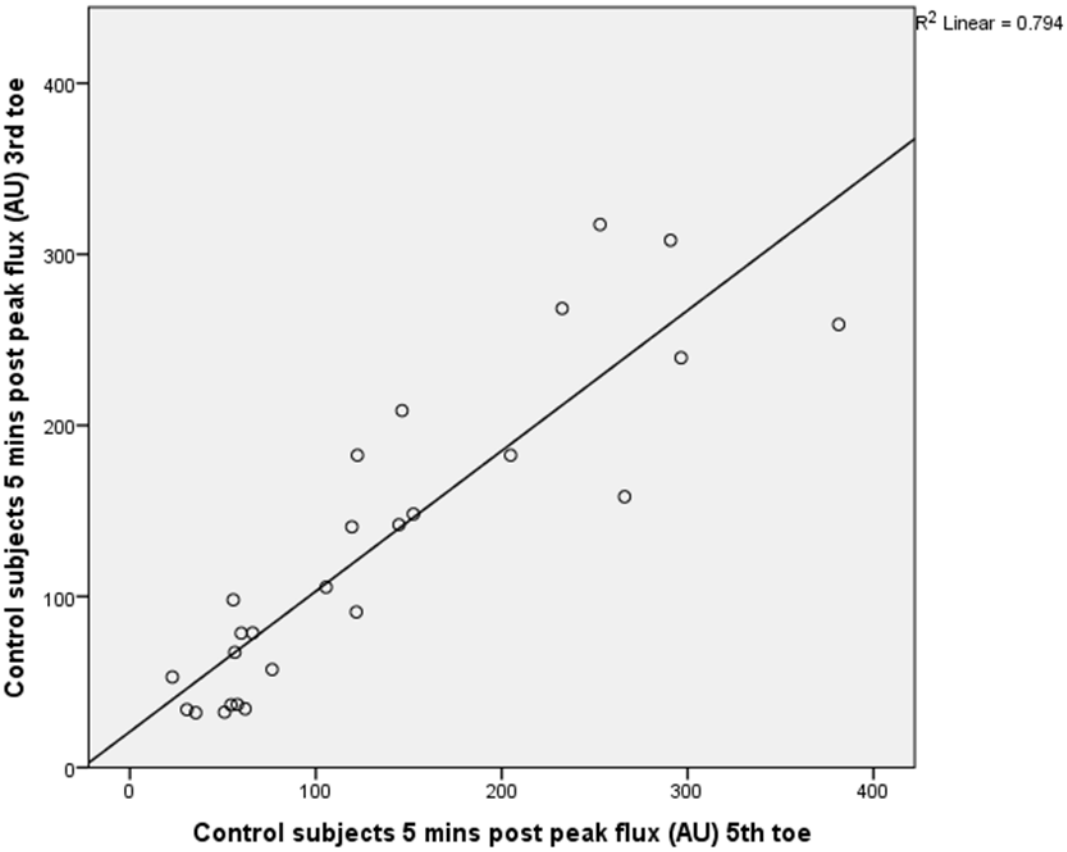

Figure 4.30 Scatterplot displaying the association found between the 5th and 3rd toes (AU) 5 mins. post peak flux in the group of control subjects

4.7.3 Comparison of LDF and LSCI when utilised to measure blood flux on the foot in this study

Both LSCI and LDF were used to assess the baseline resting flux on the plantar aspect of the 3rd MPJ and a strong correlation was found between the two systems. As the data did not display a normal distribution the non-parametric Spearman's correlation coefficient was used. With inclusion of all subjects with Type 2 diabetes and the control subjects, the correlation coefficient was 0.601 and $p < 0.0001$**,** thus a strong association was found between the resting flux at the 3rd MPJ. When the results obtained for the group with diabetes between the two methods were compared for resting blood flux prior to intervention with occlusion, or current with ACh there was a strong correlation ($r = 0.638$ $p < 0.0001$). For the group of control subjects there was also a strong association between the methods of assessment ($r = 0.549$, $p = 0.003$). The comparison was also carried out between the LSCI resting flux data found at 3rd MPJ, and the LDF data obtained with SNP prior to initiating the current for iontophoresis. For the group as a whole $r = 0.458$, $p = 0.001$ indicated a strong association, for the group with Type 2 diabetes the association again was strong ($r = 0.61$, $p = 0.001$) and for the control group data there was also a strong association found ($r = 0.742$, $p < 0.0001$). Thus in this particular study, a strong association was found between the resting flux as measured on the plantar aspect at the 3rd MPJ when using LSCI and LDF methods of assessing blood flux as can been noted in Table 4.14 and displayed in Figures 4.31 to 4.36.

Table 4.14 Results of the comparison between utilisation of LSCI and LDF when measuring baseline resting flux on the plantar aspect of 3rd MPJ

Group under study	Comparison	Corr. co. r	Significance p value
Whole group-n=60 subjects with Type 2 diabetes plus controls	**LSCI vs LDF-ACh**	**0.601**	**< 0.0001**
Subjects with type 2 diabetes-n=30	**LSCI vs LDF-ACh**	**0.638**	**< 0.0001**
Control subjects n=30	**LSCI vs LDF-ACh**	**0.549**	**0.003**
Whole group-n=60 subjects with Type 2 diabetes plus controls	**LSCI vs LDF-SNP**	**0.458**	**0.001**
Subjects with Type 2 diabetes n=30	**LSCI vs LDF-SNP**	**0.611**	**0.001**
Control subjects n=30	**LSCI vs LDF-SNP**	**0.742**	**< 0.0001**

There was a significant association found between laser speckle contrast imaging and laser Doppler flowmetry when measuring resting blood flux on the plantar aspect of the 3rd MPJ in both the group as a whole, and the subjects with Type 2 diabetes and control subjects.

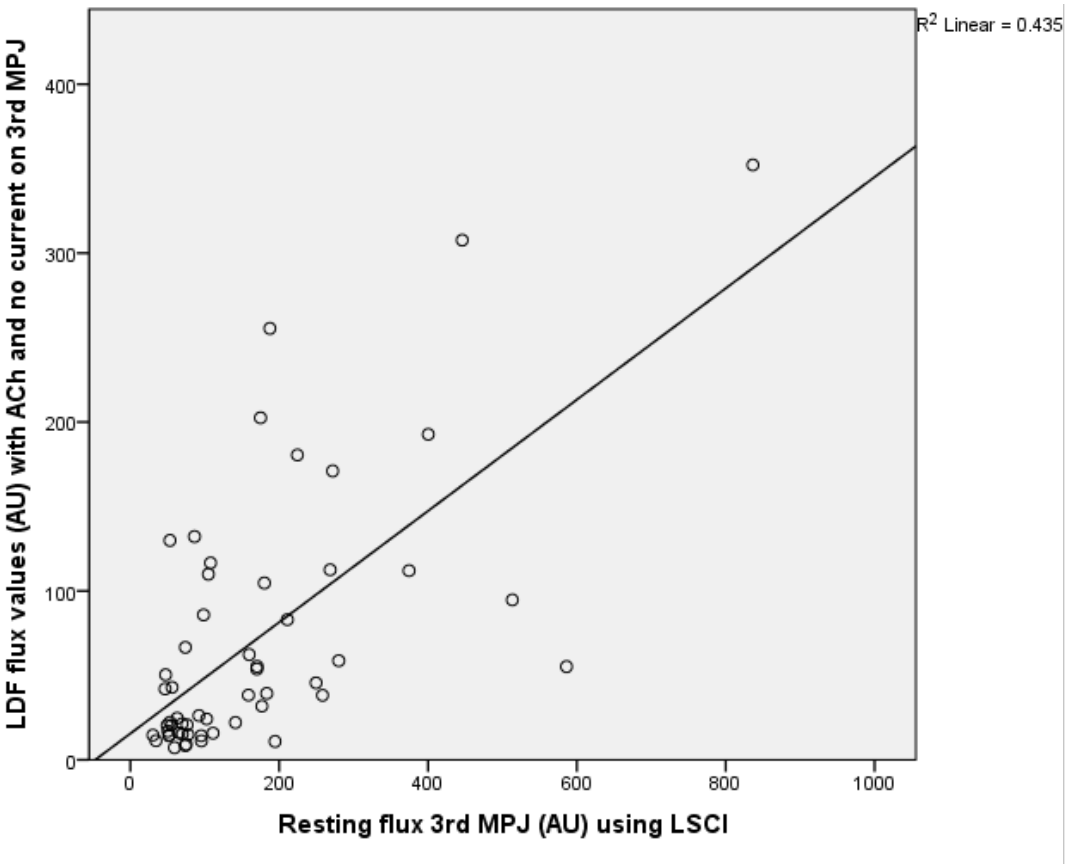

Figure 4.31 Scatterplot of the association between LSCI and LDF with ACh at the 3rd MPJ across the group with Type 2 diabetes plus the control subjects n=60

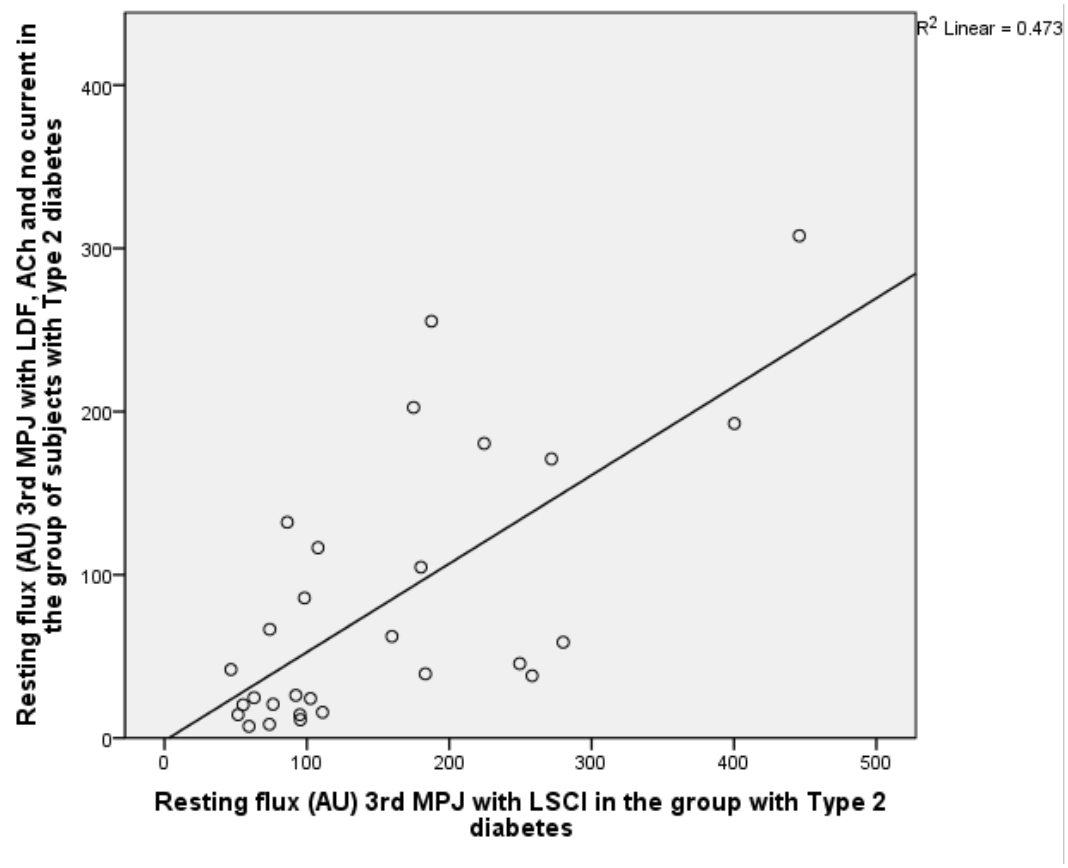

Figure 4.32 Scatterplot of the association between LSCI and LDF with ACh in the group with Type 2 diabetes

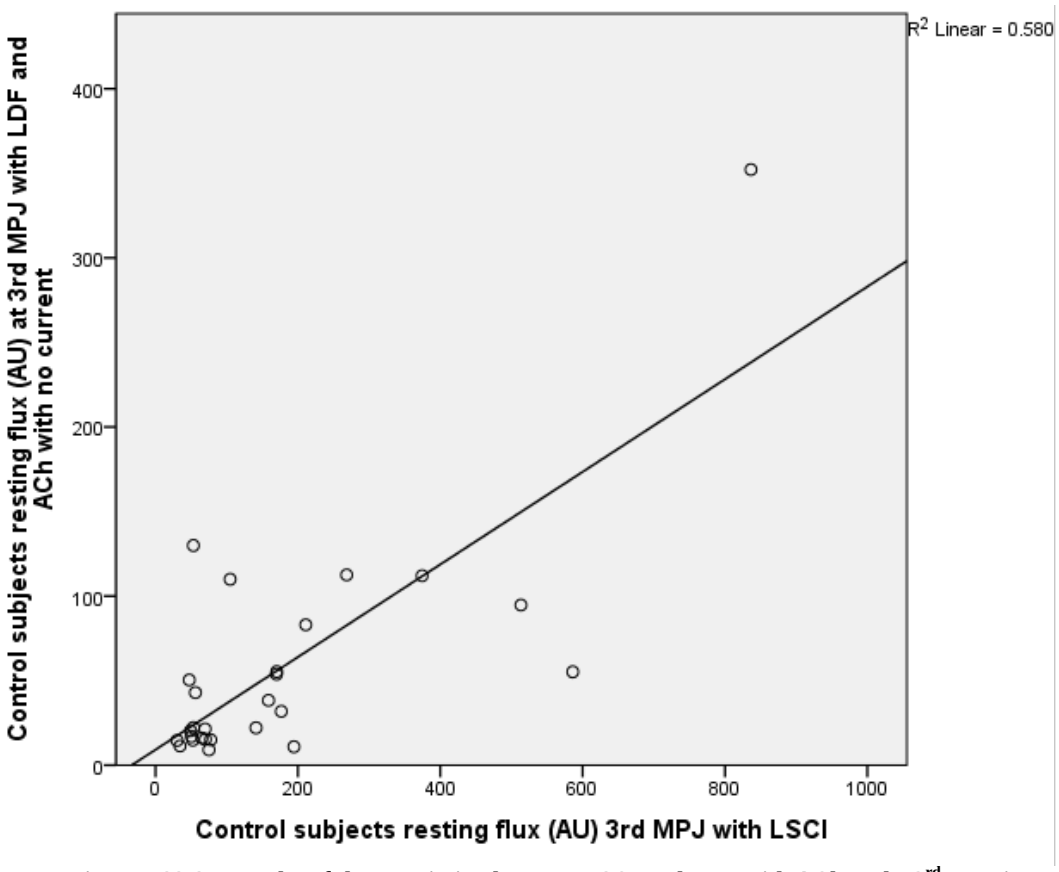

Figure 4.33 Scatterplot of the association between LSCI and LDF with ACh at the 3rd MPJ in the control group

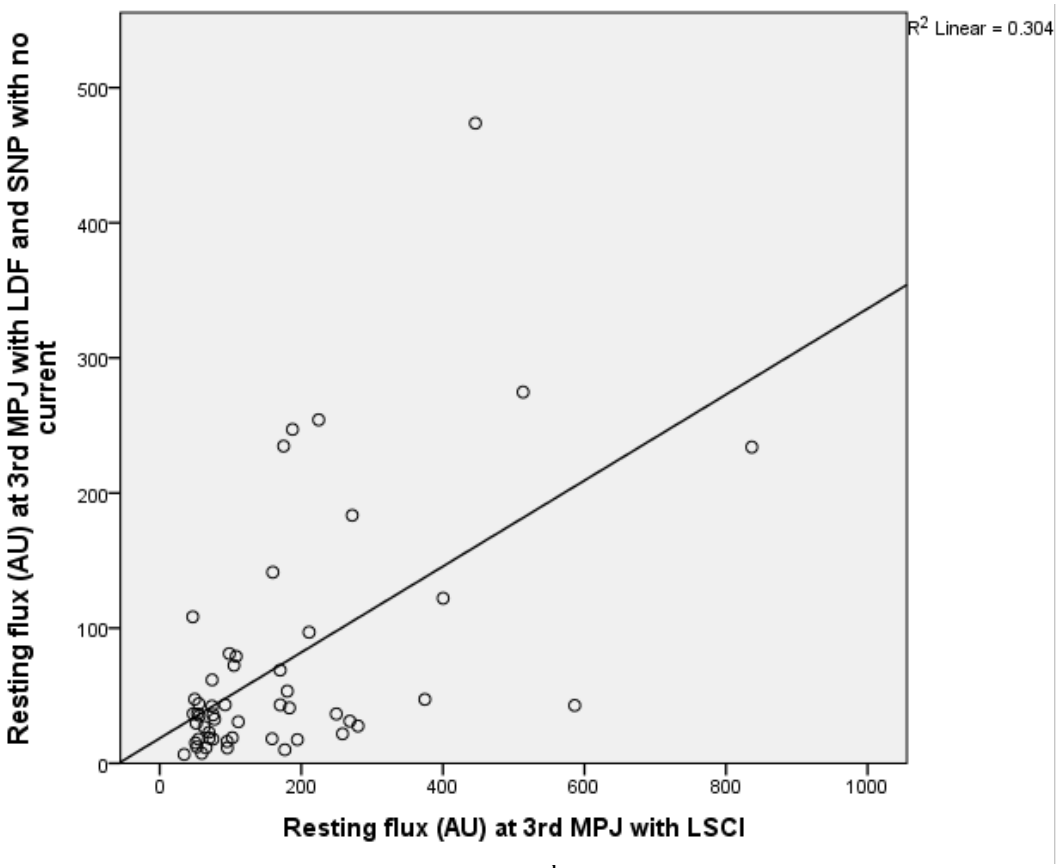

Figure 4.34 Association between the resting flux at 3rd MPJ with LSCI and LDF with SNP and no current across the group with Type 2 diabetes and the control group n = 60

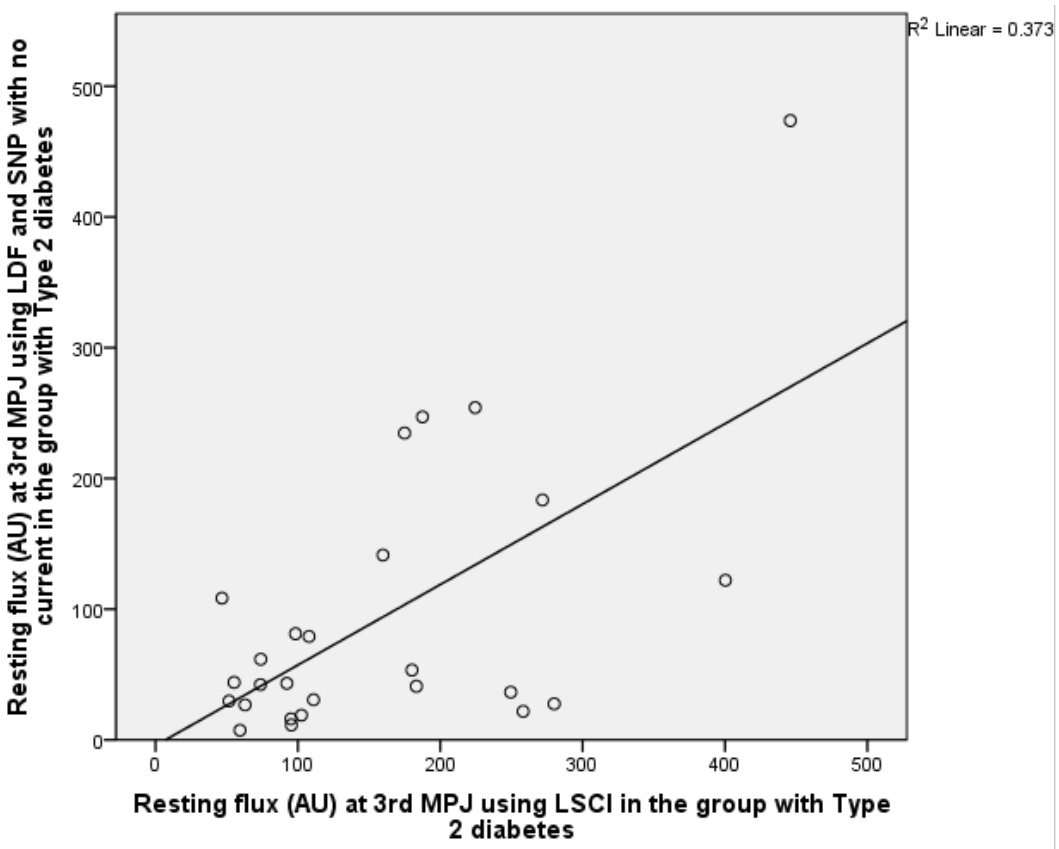

Figure 4.35 Association between the resting flux at 3rd MPJ between the LSCI and LDF with SNP and no current in the group with Type 2 diabetes

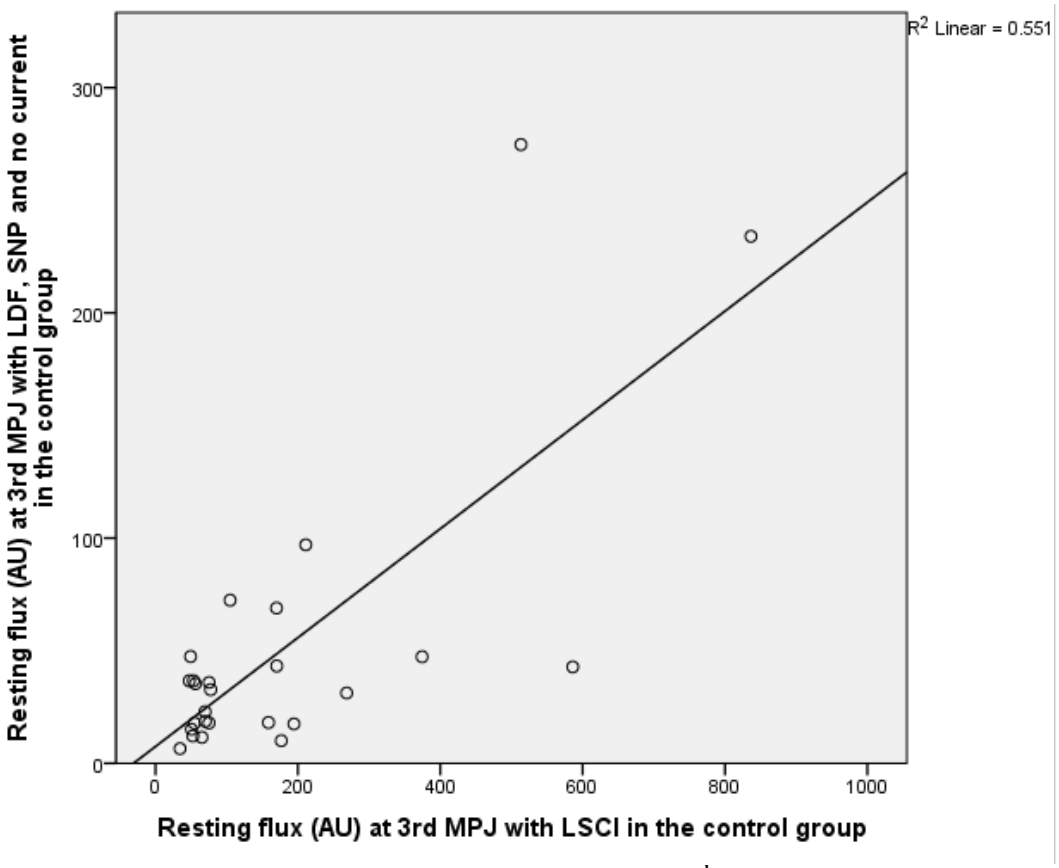

Figure 4.36 Association between the measurement of resting flux at 3rd MPJ between the LSCI and LDF with SNP and no current in the group of control subjects

4.7.4 Results of the impact of pressure on endothelial function as measured with laser Doppler flowmetry (LDF)

4.7.4.1 Introduction

In this component of the study, adding a known pressure and investigating the impact of the pressure on endothelial function was measured. Of the 60 subjects who took part in this aspect of the study, laser Doppler flowmetry data was available for 91.5%, for the group with Type 2 diabetes, 90.65% of the data was available and for the control group 92.35%. A small number of participants requested to stop the study prior to completion, in the case of two individuals a tingling sensation was felt at the site of the iontophoresis, and with the other subjects time was given as the reason to stop. As found with the assessment of reactive hyperaemia, examination of the data revealed that it did not follow a normal distribution pattern, when the z value was calculated for the data using a skewness value of 1.96 or greater to indicate significance at $p < 0.05$.The majority of z values obtained were in excess of 1.96, as noted in Table 4.15 thus non-parametric statistical analysis was utilised for this data again with the exception of repeated measures ANOVA where the F value was thought to be robust (Field 2013).

Table 4.15 Descriptive statistics for the LDF data with ACh (n=60)

ACh Current + pressure	Mean Flux (AU)	Standard Dev.	Skewness Statistic	St. error	Z value	Kurtosis Statistic	St. error	Z value
0µa +0p	68.50	75.80	1.998	0.316	6.32	4.084	0.623	6.56
20µa +0p	82.14	83.24	1.686	0.316	5.34	2.733	0.623	4.39
40µa +0p	101.42	94.50	1.29	0.316	4.08	0.852	0.623	1.37
50µa +0p	129.39	119.22	1.103	0.316	3.49	-0.057	0.623	0.09
75µa +0p	154.36	124.25	1.205	0.316	3.81	0.873	0.623	1.40
100µa +0p	177.38	124.13	0.844	0.316	2.67	-0.141	0.623	0.23
0µa +50%p	58.99	68.53	3.306	0.311	10.63	14.738	0.613	24.04
20µa +50%p	63.84	71.70	2.884	0.311	9.27	10.851	0.613	17.7
40µa +50%p	75.03	79.19	2.216	0.311	7.13	6.308	0.613	10.29
50µa +50%p	87.93	93.98	2.110	0.311	6.78	4.857	0.613	7.92
75µa +50%p	112.48	112.45	1.787	0.311	5.75	3.011	0.613	4.91
100µa +50%p	126.09	114.91	1.283	0.311	4.13	0.727	0.613	1.19
0µa +100%p	53.30	54.09	2.616	0.319	8.20	8.039	0.628	12.80
20µa +100%p	66.09	65.54	2.061	0.319	6.46	4.431	0.628	7.06
40µa +100%p	75.19	75.27	1.803	0.319	5.65	2.991	0.628	4.76
50µa +100%p	85.48	78.26	1.332	0.319	4.18	1.036	0.628	1.65
75µa +100%p	103.52	96.73	1.379	0.319	4.32	1.094	0.628	1.74
100µa +100%p	129.60	106.46	1.095	0.319	3.43	0.712	0.628	1.13

With the majority of z values for skewness in excess of 1.96 utilisation of non-parametric analysis of the data is indicated.

Table 4.16 Descriptive statistics for the LDF data with SNP (n=60)

SNP Current + pressure	Mean Flux (AU)	Standard Dev.	Skewness Statistic	St. error	Zvalue	Kurtosis Statistic	St. error	Zvalue
0µa +0p	72.29	89.29	2.503	0.325	7.70	7.280	0.639	11.39
20µa +0p	77.52	92.98	2.287	0.325	7.04	5.460	0.639	8.54
40µa +0p	77.42	94.56	2.218	0.325	6.82	4.903	0.639	7.67
50µa +0p	89.93	98.39	2.045	0.325	6.29	4.318	0.639	6.76
75µa +0p	107.04	97.31	1.486	0.325	4.57	2.139	0.639	3.35
100µa +0p	123.40	103.78	1.216	0.325	3.74	1.004	0.639	1.57
0µa +50%p	58.76	61.45	1.878	0.325	5.78	3.121	0.639	4.88
20µa +50%p	63.64	65.51	1.767	0.325	5.44	2.469	0.639	3.86
40µa +50%p	63.41	66.25	1.93	0.325	5.94	3.274	0.639	5.12
50µa +50%p	65.08	65.71	2.009	0.325	6.18	4.123	0.639	6.45
75µa +50%p	67.37	61.22	1.583	0.325	4.87	2.640	0.639	4.13
100µa +50%p	66.07	58.40	1.757	0.327	5.37	4.197	0.644	6.52
0µa +100%p	39.66	37.08	1.893	0.340	5.57	3.206	0.668	4.80
20µa +100%p	40.71	40.27	2.079	0.340	6.11	3.730	0.668	5.58
40µa +100%p	41.84	43.20	2.333	0.340	6.86	5.396	0.668	8.08
50µa +100%p	44.94	46.71	2.026	0.340	5.96	4.350	0.668	6.51
75µa +100%p	49.02	47.05	1.246	0.340	3.66	0.634	0.668	0.95
100µa +100%p	53.52	51.61	1.433	0.340	4.21	1.388	0.668	2.08

The majority of z values for skewness are in excess of 1.96 thus would indicate the utilisation of non-parametric analysis of the data.

4.7.4.2 Actual pressure values as measured with Emed-x/E pressure analysis system across the two groups

The actual pressure exerted on the forefoot during normal walking was measured for each subject utilising the Emed-x/E pressure analysis system. There was no significant difference found between the group with Type 2 diabetes and the control group for the mean pressures exerted on the 1st toes, 3rd toes, and 5th toes, 1st MPJs, 3rd MPJs and 5th MPJs. The descriptive statistics and significance values utilising Mann Whitney U analysis are presented in Table 4.17. The pressure values obtained under the 3rd MPJ areas were those used to obtain 50% and 100% of the individual walking pressures added to the laser Doppler probe.

The plantar pressures recorded across the 6 areas of the forefoot under study were compared for both groups, there was a significant association found in the group with Type 2 diabetes between the 5th and 3rd toes, 5th and 1st toes and 3rd and 1st toes as well as the 5th MPJ with 1st, 3rd and 5th toes. In the group of control subjects there was a significant correlation between the 5th toe and 1st MPJ, the 3rd toe and 5th MPJ and the 1st toe and 1st MPJ with no other significant associations found as presented in Table 4.18. and illustrated in Figures 4.37 to 4.45.

The plantar pressures found across the 6 areas of the forefoot were also compared with the baseline flux measured with LDF found in each of the areas, and no significant association was found as can be noted in Table 4.19.

Table 4.17 Descriptive statistics for normal walking pressures of the group with Type 2 diabetes and the control group, units for pressure measurements are kilopascals (kPa)

	Group with Type 2 diabetes					Control Group					p value
Area of the foot	Range	Minimum	Maximum	Mean	St. Dev.	Range	Minimum	Maximum	Mean	St. Dev.	p value
1st toe	**460.00**	**95.00**	**555.00**	**285.25**	**131.66**	**885.00**	**100.00**	**985.00**	**372.80**	**183.50**	**0.65**
3rd toe	**218.30**	**21.70**	**240.00**	**124.93**	**65.43**	**310.00**	**20.00**	**330.00**	**141.09**	**83.21**	**0.643**
5th toe	**166.70**	**0.00**	**166.70**	**59.40**	**48.24**	**251.70**	**0.00**	**251.70**	**76.42**	**50.29**	**0.159**
1st MPJ	**326.70**	**190.00**	**516.70**	**302.36**	**94.12**	**365.80**	**152.50**	**518.30**	**280.55**	**102.72**	**0.300**
3rd MPJ	**645.00**	**230.00**	**875.00**	**434.46**	**125.73**	**390.00**	**290.00**	**680.00**	**474.37**	**121.67**	**0.341**
5th MPJ	**313.40**	**58.30**	**371.70**	**143.70**	**64.01**	**439.20**	**67.50**	**506.70**	**165.67**	**102.85**	**0.755**

Utilising Mann-Whitney U test, there was no significant difference found between the group of subjects with Type 2 diabetes and the control group at any of the areas of the forefoot under study.

Table 4.18 Correlation of pressure found between the areas of the plantar aspect of the forefoot in the group with Type 2 diabetes (n=28) and control subjects (n=27)

Areas	Group with Type 2 diabetes		Control group	
	r	p	r	p
5th toe v 3rd toe	0.375	**0.049 (s)**	0.354	0.070
5th toe v 1st toe	0.516	**0.005 (s)**	0.286	0.148
5th toe v 5th MPJ	-0.596	**0.001 (s)**	-0.142	0.480
5th toe v 3rd MPJ	0.028	0.889	0.238	0.233
5th toe v 1st MPJ	-0.085	0.669	0.533	**0.004 (s)**
3rd toe v 1st toe	0.395	**0.037 (s)**	0.151	0.452
3rd toe v 5th MPJ	-0.457	**0.015 (s)**	0.550	**0.003 (s)**
3rd toe v 3rd MPJ	-0.042	0.830	0.018	0.930
3rd toe v 1st MPJ	-0.068	0.730	0.321	0.103
1st toe v 5th MPJ	0.516	**0.005 (s)**	-0.005	0.981
1st toe v 3rd MPJ	0.272	0.161	0.305	0.122
1st toe v 1st MPJ	-0.060	0.761	0.613	**0.001 (s)**
5th MPJ v 3rd MPJ	0.033	0.869	-0.293	0.138
5th MPJ v1st MPJ	0.154	0.434	0.014	0.943
3rd MPJ v1st MPJ	-0.094	0.636	0.116	0.565

In Table 4.18 toe = toe, MPJ= metatarsophalangeal joint, r = Spearman's correlation coefficient, p=significance and (s) = significant correlation
There was a significant association found in the group with Type 2 diabetes between the 5th and 3rd toes, 5th and 1st toes and 3rd and 1st toes as well as the 5th MPJ with 1st, 3rd and 5th toes.
In the group of control subjects there was a significant correlation between the 5th toe and 1st MPJ, the 3rd toe and 5th MPJ and the 1st toe and 1st MPJ with no other significant correlations found.

Table 4.19 Correlation found between the pressure exerted on the forefoot areas of the plantar aspect v baseline flux measured in the areas n=60

	Baseline flux at 5th toe	Baseline flux at 3rd toe	Baseline flux at 1st toe	Baseline flux at 5th MPJ	Baseline flux at 3rd MPJ	Baseline flux at 1st MPJ
Pressure exerted on 5th toe	**r -0.116**	**r -0.111**	**r -0.181**	**r -0.106**	**r -0.086**	**r -0.109**
	p=0.413	**p= 0.427**	**p=0.195**	**p=0.473**	**p=0.539**	**p=0.438**
Pressure exerted on 3rd toe	**r -0.206**	**r -0.132**	**r -0.126**	**r -0.007**	**r -0.054**	**r -0.094**
	p=0.143	**p=0.347**	**p=0.370**	**p=0.963**	**p=0.704**	**p=0.503**
Pressure exerted on 1st toe	**r -0.182**	**r -0.192**	**r -0.113**	**r -0.115**	**r -0.096**	**r-0.158**
	p=0.200	**p=0.168**	**p=0.420**	**p=0.437**	**p=0.494**	**p=0.259**
Pressure exerted on 5th MPJ	**r -0.041**	**r -0.053**	**r 0.083**	**r 0.141**	**r 0.029**	**r 0.044**
	p=0.775	**p=0.704**	**p=0.555**	**p=0.339**	**p=0.838**	**p=0.756**
Pressure exerted on 3rd MPJ	**r -0.111**	**r -0.179**	**r -0.138**	**r -0.217**	**r -0.101**	**r -0.095**
	p=0.432	**p=0.200**	**p=0.324**	**p=0.139**	**p=0.472**	**p=0.500**
Pressure exerted on 1st MPJ	**r -0.076**	**r 0.050**	**r 0.048**	**r 0.006**	**r -0.028**	**r 0.005**
	p=0.594	**p=0.723**	**p=0.735**	**p=0.966**	**p=0.844**	**p=0.974**

In Table 4.19 r is the Correlation Coefficient and the p value indicates significance. There was no significant association found between any of the areas under study and baseline flux as measured in the locality of the pressure delivered across the whole group n=60.

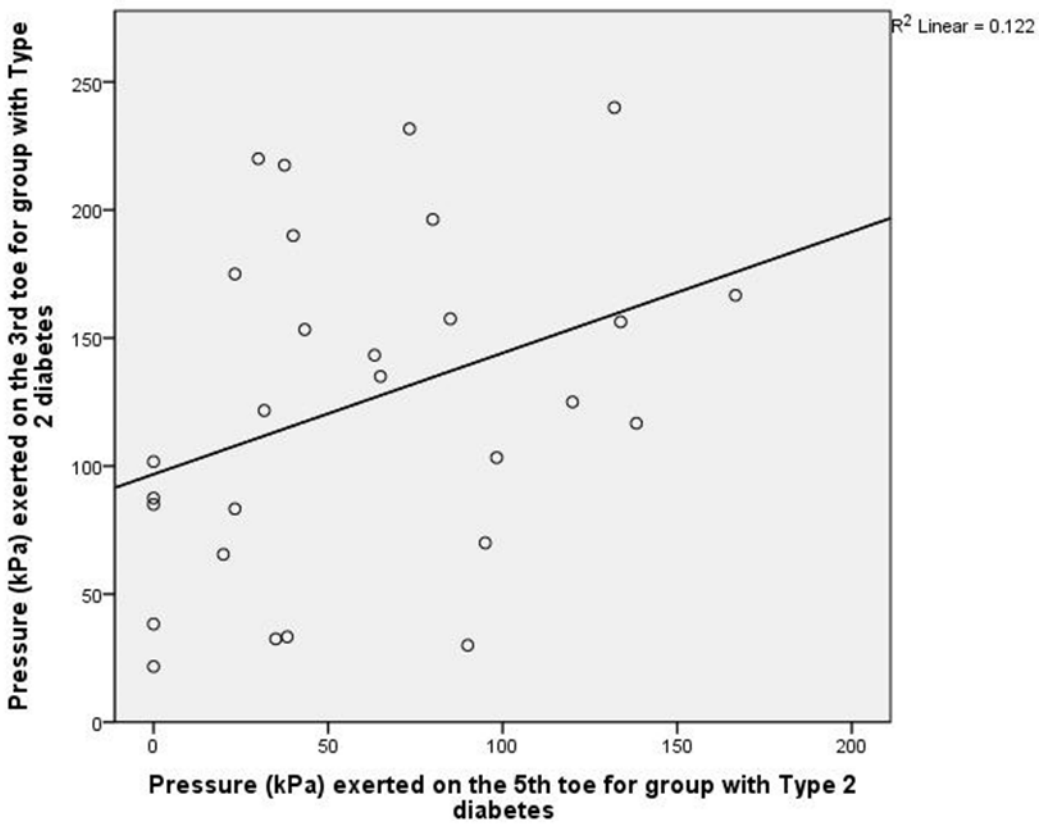

Figure 4.37 Scatterplot of the association between the pressures (kPa) exerted on 5th and 3rd toes for the group with Type 2 diabetes

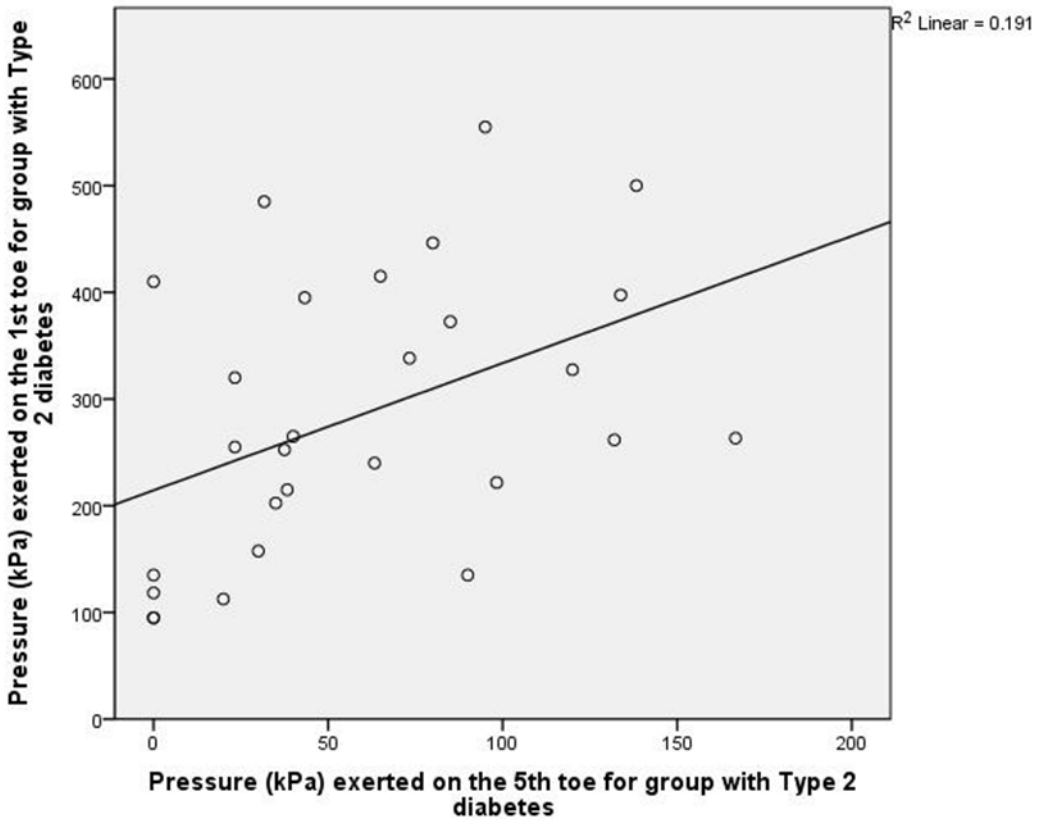

Figure 4.38 Scatterplot of the association between the pressures (kPa) exerted on the 5th and 1st toes for the group with Type 2 diabetes

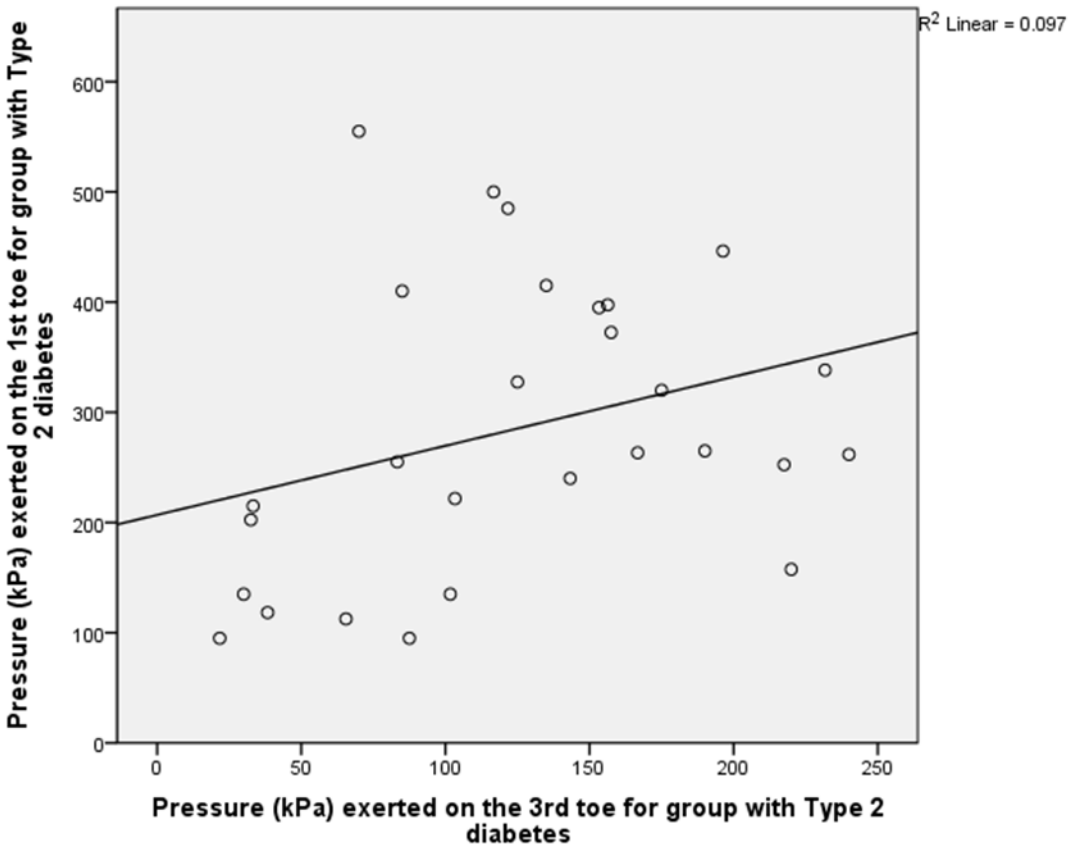

Figure 4.39 Scatterplot of the association between the pressures (kPa) exerted on the 3rd and 1st toes for the group with Type 2 diabetes

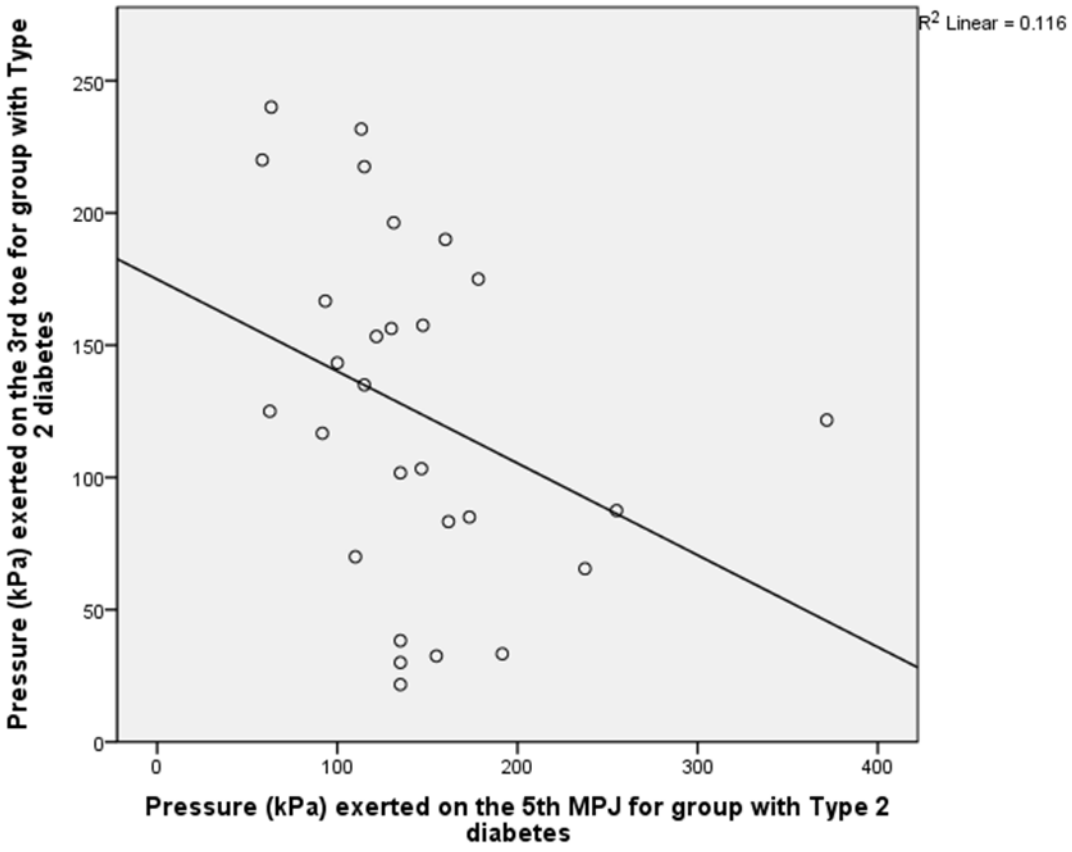

Figure 4.40 Scatterplot of the association between the pressures (kPa) exerted on the 3rd toe and 5th MPJ for the group with Type 2 diabetes

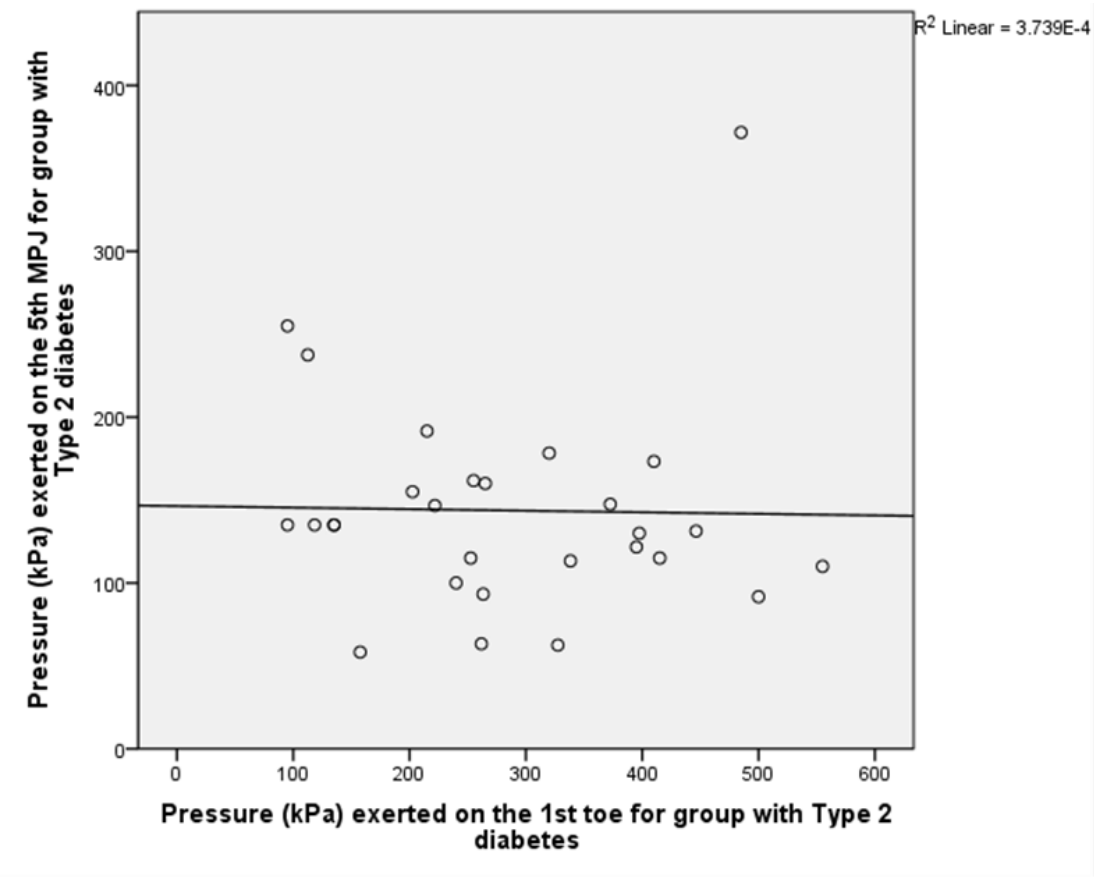

Figure 4.41 Scatterplot of the association between the pressures (kPa) exerted on the 1st toe and 5th MPJ for the group with Type 2 diabetes

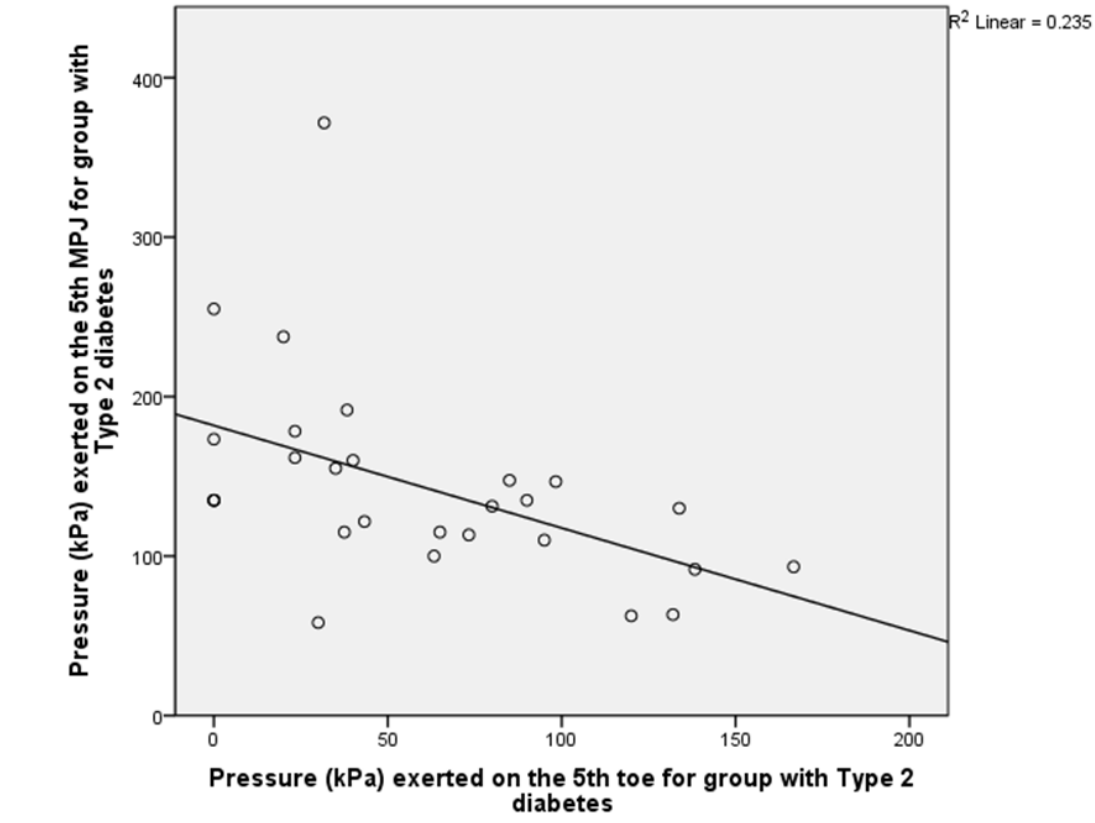

Figure 4.42 Scatterplot of the association between the pressures (kPa) exerted on the 5th toe and 5th MPJ for the group with Type 2 diabetes

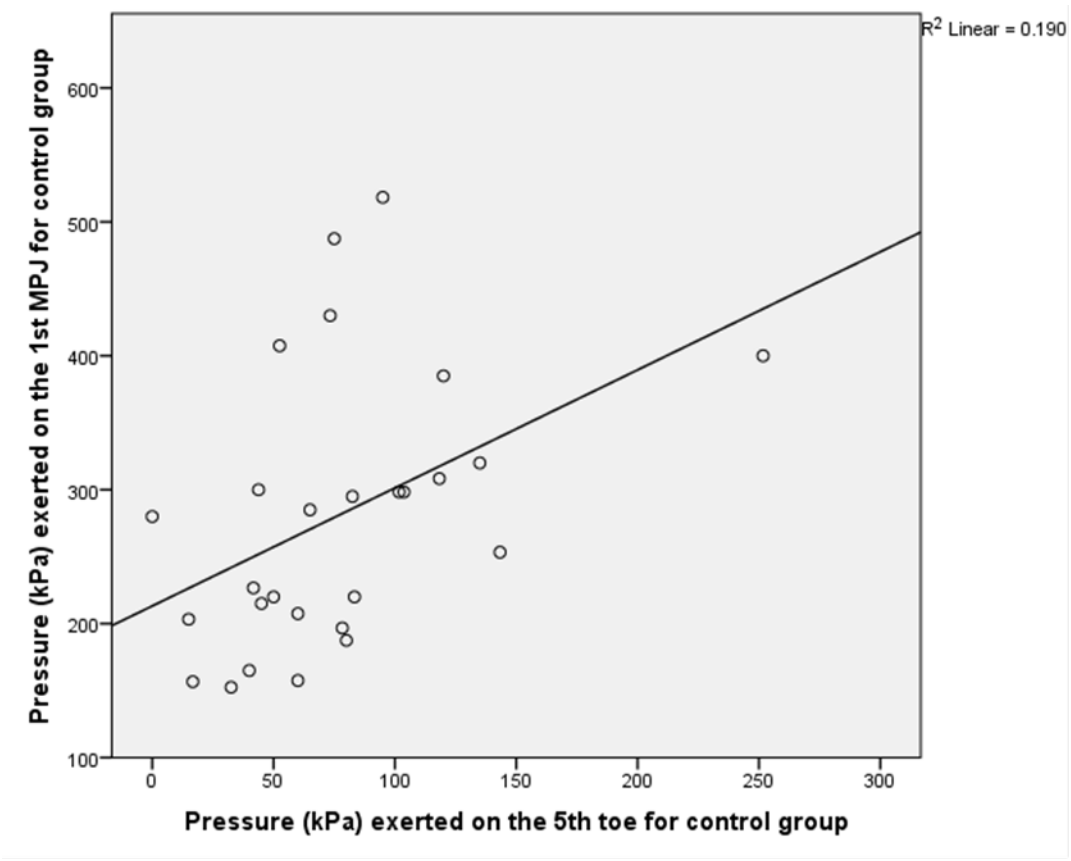

Figure 4.43 Scatterplot of the association between the pressures (kPa) exerted on the 5th toe and 1st MPJ for the control group

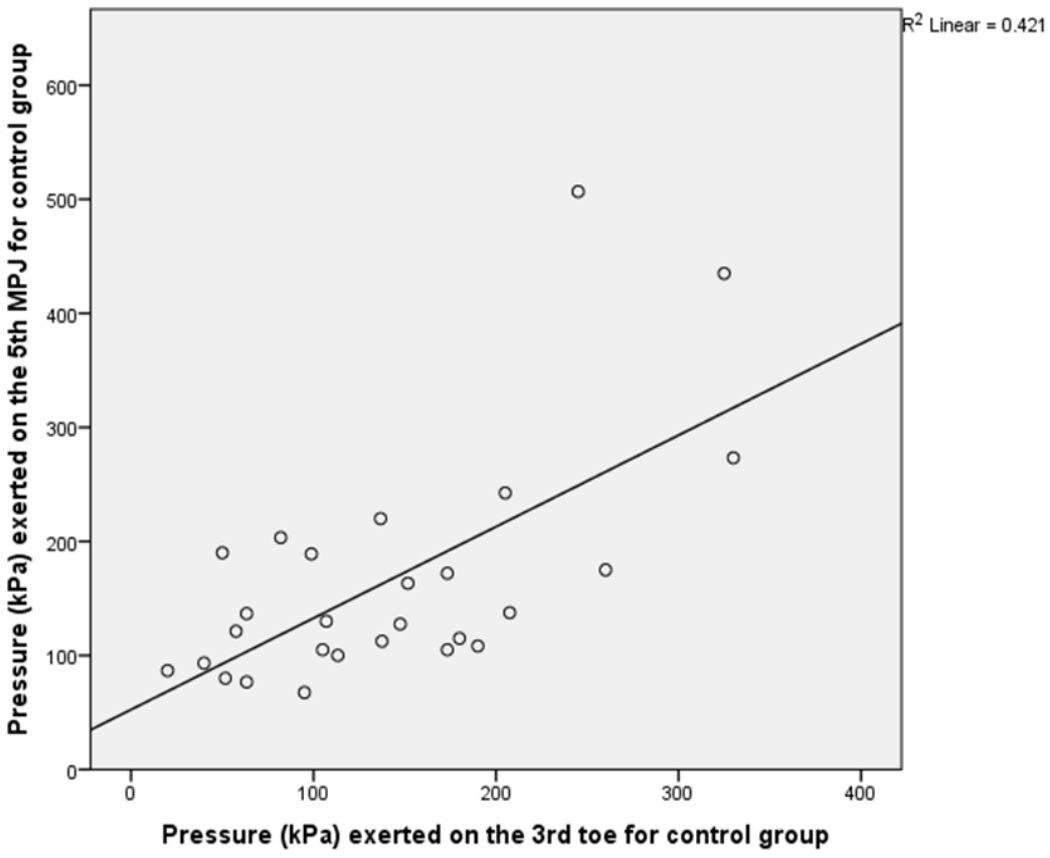

Figure 4.44 Scatterplot of the association between the pressures (kPa) exerted on the 3rd toe and 5th MPJ for the control group

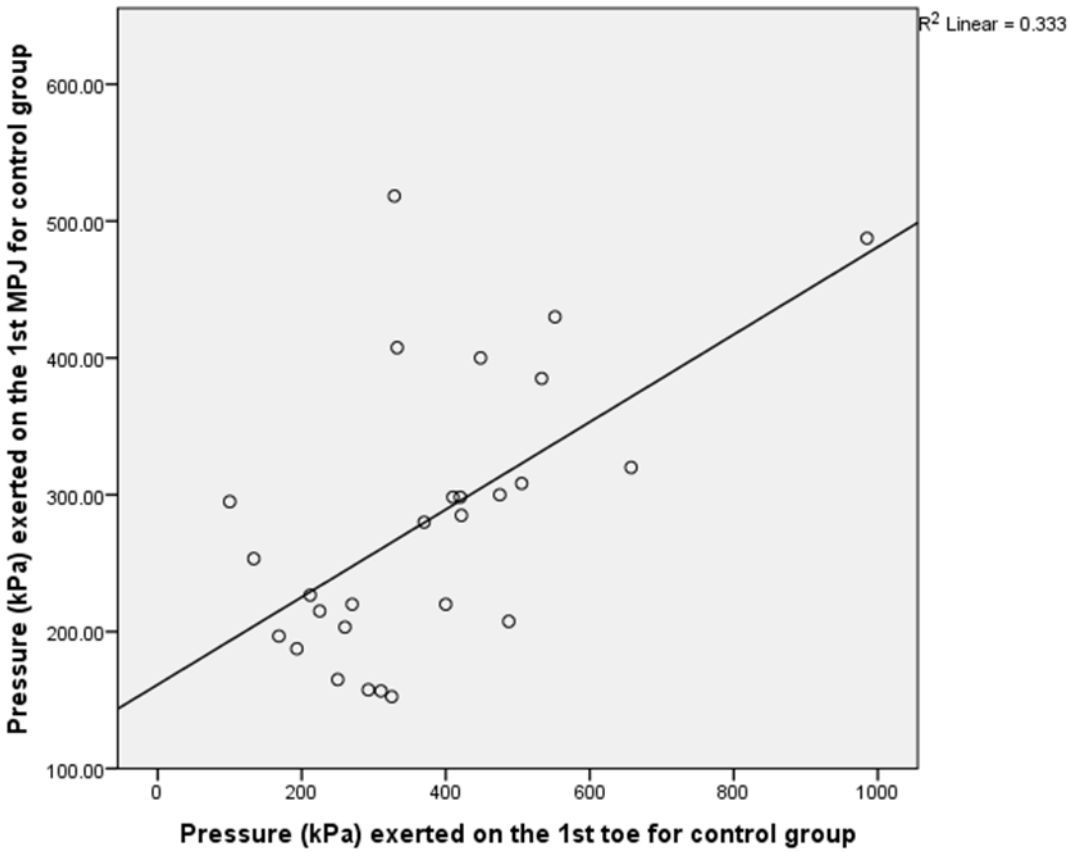

Figure 4.45 Scatterplot of the association between the pressures (kPa) exerted on the 1st toe and 1st MPJ for the control group

4.7.4.3 Results of the impact of pressure on endothelial function when using ACh and iontophoresis for the whole group n=60

At baseline flux with all subjects (both groups) prior to addition of any current across the skin surface, the addition of both 50% and 100% pressure added to the area impacted upon the flux values obtained. The mean baseline flux without pressure was 68.50 ± 75.80 (AU), with 50% simulated walking pressure added was 58.99 ± 68.53(AU) and with the application of 100% pressure, flux at baseline was 53.30 ± 54.09 (AU). The application of ACh with iontophoresis without pressure produced a steady incline in blood flux as the vasodilator effect was apparent, rising to a maximum flux of 177.38 ± 124.13 (AU). With the addition of 50% and 100% pressure blood flux responses were similar with little difference found in the maximum flux values obtained (maximum flux with 50% pressure was 126.09 ± 114.91(AU) and for 100% pressure addition maximum flux achieved was 129.60 ± 106.46 (AU) as can be seen in Figure

4.46. Thus, the major reduction in flux caused by the addition of pressure with ACh and iontophoresis on the plantar aspect of the foot with the whole group occurred at 50% application of the normal walking pressure of the subjects in the study, and the further addition of pressure to 100% had little further impact on flux. Repeated measures ANOVA with post hoc Bonferroni analysis was used to assess the impact of pressure and increase in dose of ACh on flux. Mauchly's test indicated that the assumption of sphericity had been violated therefore degrees of freedom were corrected with Huynh-Feldt and there was a significant difference within subjects from pressure ($F = 8.017$ and $p = 0.001$). There was also a significant effect within subjects of the dose of ACh administered on the pressure ($F = 51.719$ and $p < 0.0001$) and there was a significant interaction effect between the pressure applied and dose of ACh ($F = 2.732$ and $p = 0.033$). Follow up analysis of the individual pressure effect was performed with Mann-Whitney U test and a significant difference was found at maximum flux between no pressure and 50% pressure ($p = 0.006$) and between no pressure and 100% pressure ($p = 0.002$). However as illustrated in Figure 4.46 there was no significant difference found between maximum flux with 50% and 100% pressure delivery ($p = 0.738$), these findings are clearly illustrated in Figure 4.47 with the percentage change from baseline flux displayed against the increase in ACh dose with current strength.

Table 4.20 Descriptive statistics for pressure and increased dose of ACh with iontophoresis

Pressure applied	Resting flux(AU) Mean ± S.D.	Max. flux(AU) Mean ± S.D.	Sig. p value	Sig. p value	Sig. p value
0 pressure	68.50 ± 75.80	177.38 ± 124.13	0.006	0.002	
50% pressure	58.99 ± 68.53	126.09 ± 114.91	0.006		0.738
100% pressure	53.30 ± 54.09	129.60 ± 106.46		0.002	0.738

There was a significant difference found within subjects with pressure ($p = 0.001$, ANOVA) and for increase in dose ($p < 0.0001$, ANOVA) also for the interaction effect of pressure with increase in ACh dose ($p = 0.033$, ANOVA). There was a significant difference between 0 pressure and 50% pressure application ($p = 0.006$, M.W.U) and 0 pressure and 100% pressure ($p = 0.002$, M.W.U) but no significant difference between 50% and 100% pressure ($p = 0.738$, M.W.U).

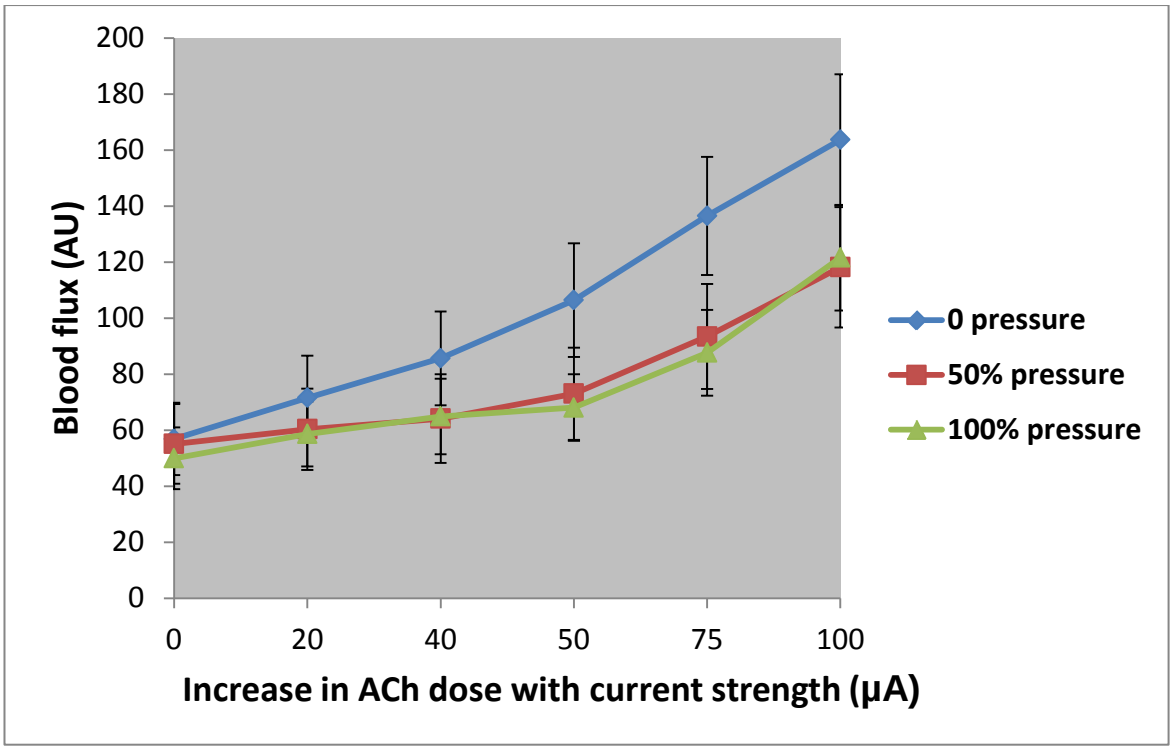

Figure 4.46 Plot of the three levels of pressure against the increase in current strength on application of iontophoresis with ACh for the subjects with Type 2 diabetes and the control group n=60

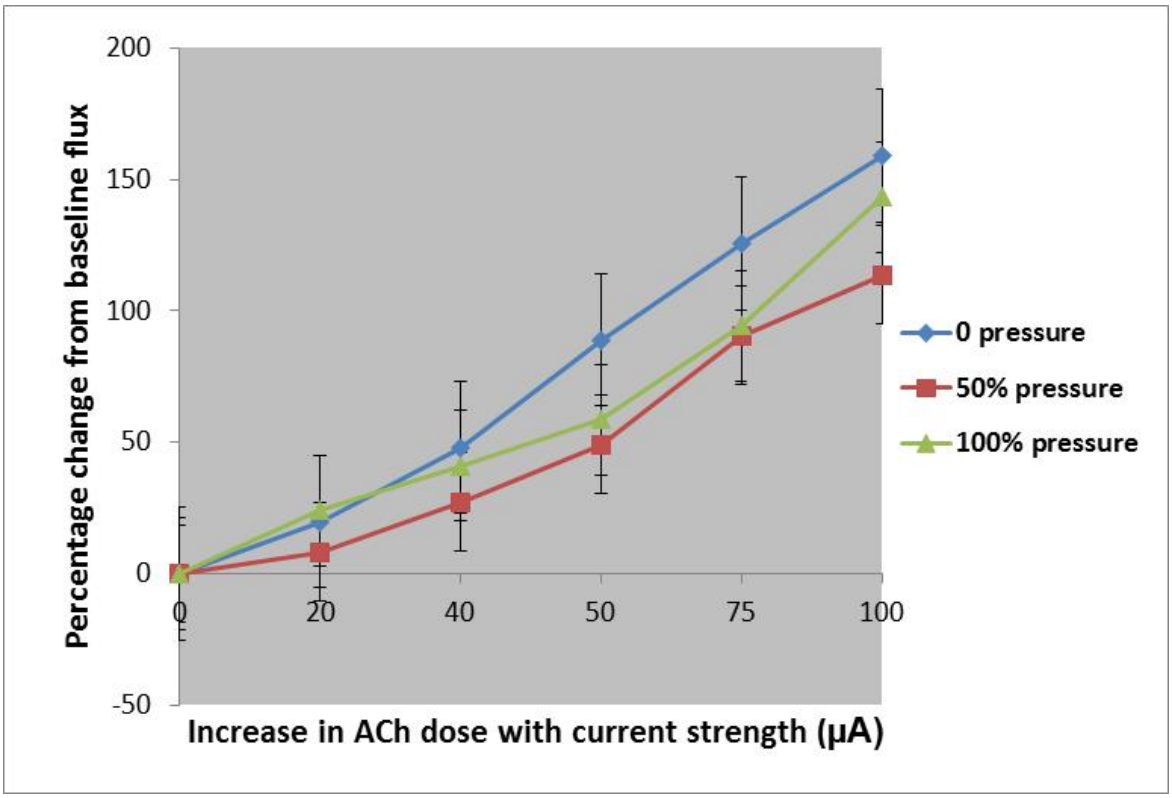

Figure 4.47 Plot of the percentage change from baseline flux against the increase in ACh dose with current for the group with Type 2 diabetes plus the control group n = 60

4.7.4.4 Results of the impact of pressure on endothelial function when using ACh and iontophoresis for the group with Type 2 diabetes

The group with Type 2 diabetes also displayed differences in the baseline flux with the addition of the different pressures prior to starting the iontophoresis current. When no pressure was added a mean and standard deviation for flux of 79.72 ± 81.87(AU) was noted, and with the addition of 50% pressure 63.01 ± 58.49 (AU) and with 100% pressure, flux values were 56.90 ± 48.67(AU). At the higher levels of current it was noticeable that with 50% pressure the peak flux achieved with this group tailed off, whereas the 100% pressure plot actually followed a similar increase to the plot for no pressure additions, although at a lower flux value. The peak flux achieved without pressure for the group with Type 2 diabetes was 190.66 ± 124.72 (AU), with 50% pressure this reduced to 134.28 ± 113.27 (AU), and with the application of 100% pressure 138.18 ± 112.91(AU), so as in the combined groups of those with Type 2 diabetes and the control subjects, 50% addition of pressure had a marked effect on flux values, however increasing this pressure to 100% of the simulated normal walking pressure for the group had little further impact. Repeated measures ANOVA with Huynh-Feldt correction and post hoc Bonferroni test indicated a significant difference for pressure ($F = 5.209$, $p = 0.009$) and for dose of ACh administered with pressure ($F = 24.274$, $p < 0.0001$). In the group with Type 2 diabetes there was no significant interaction effect found between the pressure applied and dose of ACh ($F = 1.084$, $p = 0.369$). Follow up analysis of the individual effects of pressure was carried out and there was no significant difference found between the maximum flux achieved with no pressure and with 50% pressure added, although this result was marginal ($p = 0.058$ M.W.U). There was a significant difference found between the maximum flux achieved with no pressure and with the application of 100% pressure ($p = 0.037$ M.W.U) and as illustrated in Figure 4.48 there was no significant difference found between the addition of 50% and 100% pressure application to maximum flux achieved ($p = 0.614$ M.W.U).

The findings are clearly illustrated in Figure 4.49 with the percentage change from baseline flux against increase in dose of ACh.

Table 4.21 Descriptive statistics for pressure and increased dose of ACh with iontophoresis for the group with Type 2 diabetes

Pressure applied	Resting flux(AU) Mean ± S.D.	Max. flux(AU) Mean ± S.D.	Sig. p value	Sig. p value	Sig. p value
0 pressure	79.72 ± 81.87	190.66 ± 124.72	0.058	0.037	
50% pressure	63.01 ± 58.49	134.28 ± 113.27	0.058		0.614
100% pressure	56.90 ± 48.67	138.18 ± 112.91		0.037	0.614

There was a significant difference found within subjects with pressure (p=0.009, ANOVA) and for increase in dose (p < 0.0001, ANOVA) but no significance was found for the interaction effect of pressure with increase in ACh dose (p = 0.369, ANOVA). Although marginal, there was no significant difference between 0 pressure and 50% pressure application (p = 0.058, M.W.U) there was a significant difference found between 0 pressure and 100% pressure delivery (p = 0.037, M.W.U) and no significant difference between 50 and 100% pressure (p = 0.614, M.W.U).

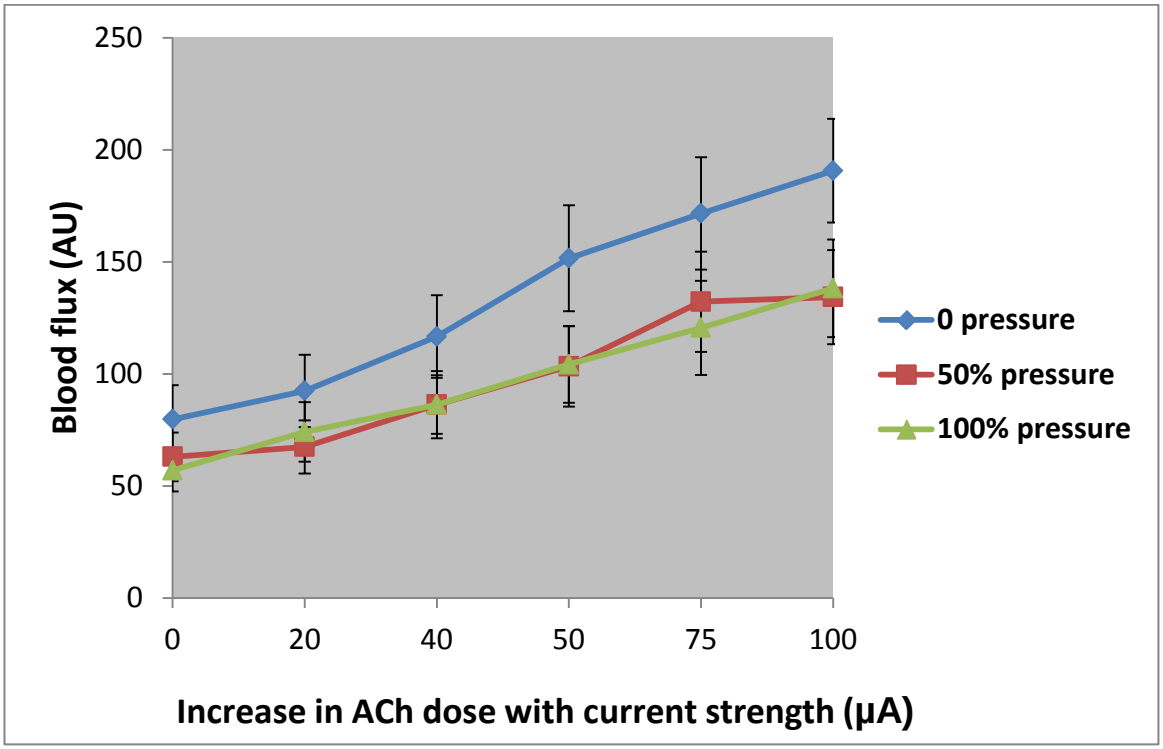

Figure 4.48 Plot of the three levels of pressure against the increase in current on application of iontophoresis with ACh for the group with Type 2 diabetes

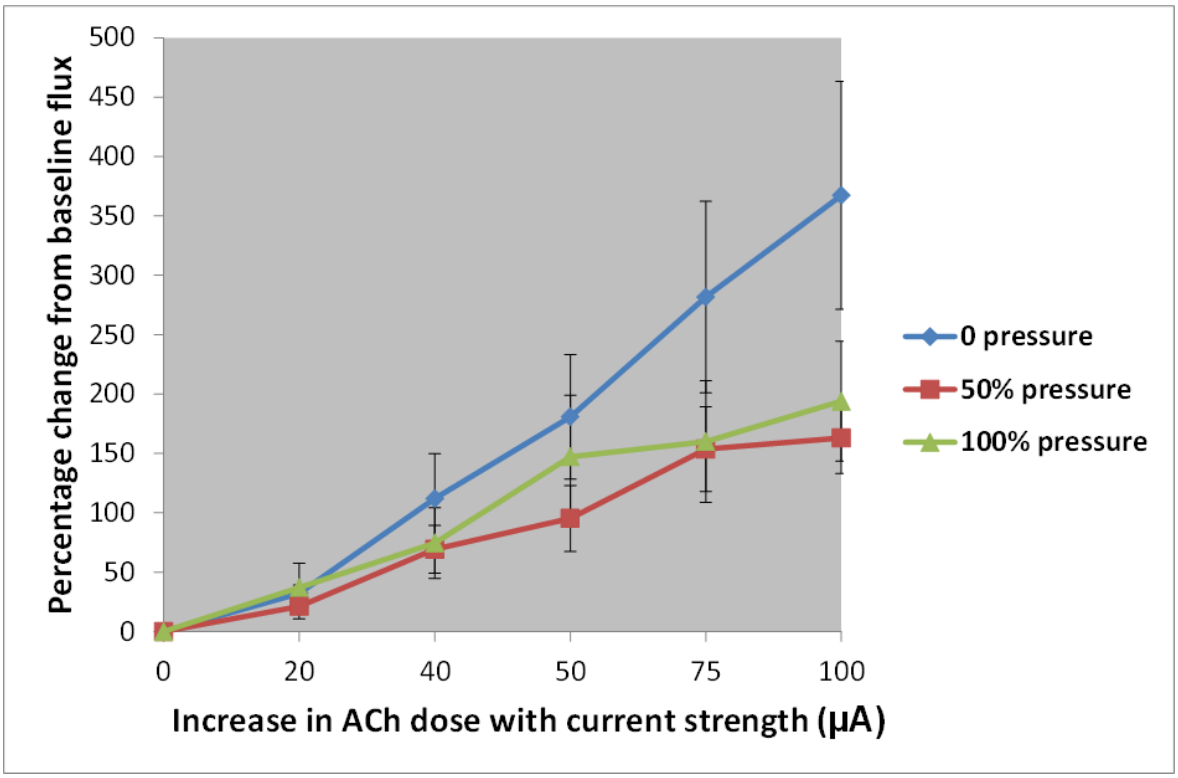

Figure 4.49 Percentage change in blood flux from baseline against increase in ACh dose with current for the group with Type 2 diabetes

4.7.4.5 Results of the impact of pressure on endothelial function when using ACh and iontophoresis for the group of healthy control subjects

For the group of control subjects, the baseline flux with ACh prior to the initiation of current for 50% pressure was very similar to the baseline flux without any pressure (baseline flux with no pressure 56.88 ± 68.48 AU and with 50% pressure 55.11 ± 77.83 AU) thus adding 50% pressure had no impact on the baseline flux, whereas application of 100% pressure reduced the baseline flux to 49.94 ± 59.36 (AU). Once again the maximum flux achieved without pressure was higher (163.64 ± 124.25 AU) than achieved with 50% pressure at 118.17 ± 117.86 (AU), however as with the combined group with both subjects with Type 2 diabetes and control subjects n = 60, and the group with Type 2 diabetes n = 30, increasing the pressure to 100% of natural walking pressure did not have a great impact on the maximum flux achieved over that for 50% pressure, achieving a mean value with standard deviation of 121.60 ± 101.42 (AU). For the control group there was no significant difference found for the effect of pressure

(F = 2.778, p = 0.091 within subject repeated measures ANOVA with Huynh-Feldt correction and post hoc Bonferroni). For dose administered there was a significant difference (F = 31.910, p < 0.0001) and no significant interaction effect found between the pressure applied and the dose of ACh administered (F = 1.872, p = 0.132).

Follow up analysis for the individual elements of pressure noted significant differences between 0 pressure and 50% pressure (p = 0.034 M.W.U) and 0 pressure and 100% pressure (p = 0.013 M.W.U), however no significant difference was found between the maximum flux values for 50% and 100% added pressure (p = 0.991 M.W.U) as illustrated in Figure 4.50. These results are also illustrated in Figure 4.51 which displays the percentage change from baseline flux against increase in dose of ACh.

Table 4.22 Descriptive statistics for pressure and increased dose of ACh with iontophoresis for the group of healthy control subjects

Pressure applied	Resting flux(AU) Mean ± S.D.	Max. flux(AU) Mean ± S.D.	Sig. p values	Sig. p values	Sig. p values
0 pressure	56.88 ± 68.48	163.64 ± 124.25	0.034	0.013	
50% pressure	55.11 ± 77.83	118.17 ± 117.86	0.034		0.991
100% pressure	49.94 ± 59.36	121.60 ± 101.42		0.013	0.991

There was no significant difference found within subjects with pressure (p = 0.091, ANOVA) for the control group. A significant difference was found for increase in dose (p < 0.0001, ANOVA) but no significance was found for the interaction effect of pressure with increase in ACh dose (p = 0.132, ANOVA). There was a significant difference found between 0 pressure and 50% pressure application (p = 0.034, M.W.U) and between 0 pressure and 100% pressure delivery (p = 0.013, M.W.U) however, no significant difference between 50 and 100% pressure was found (p = 0.991, M.W.U).

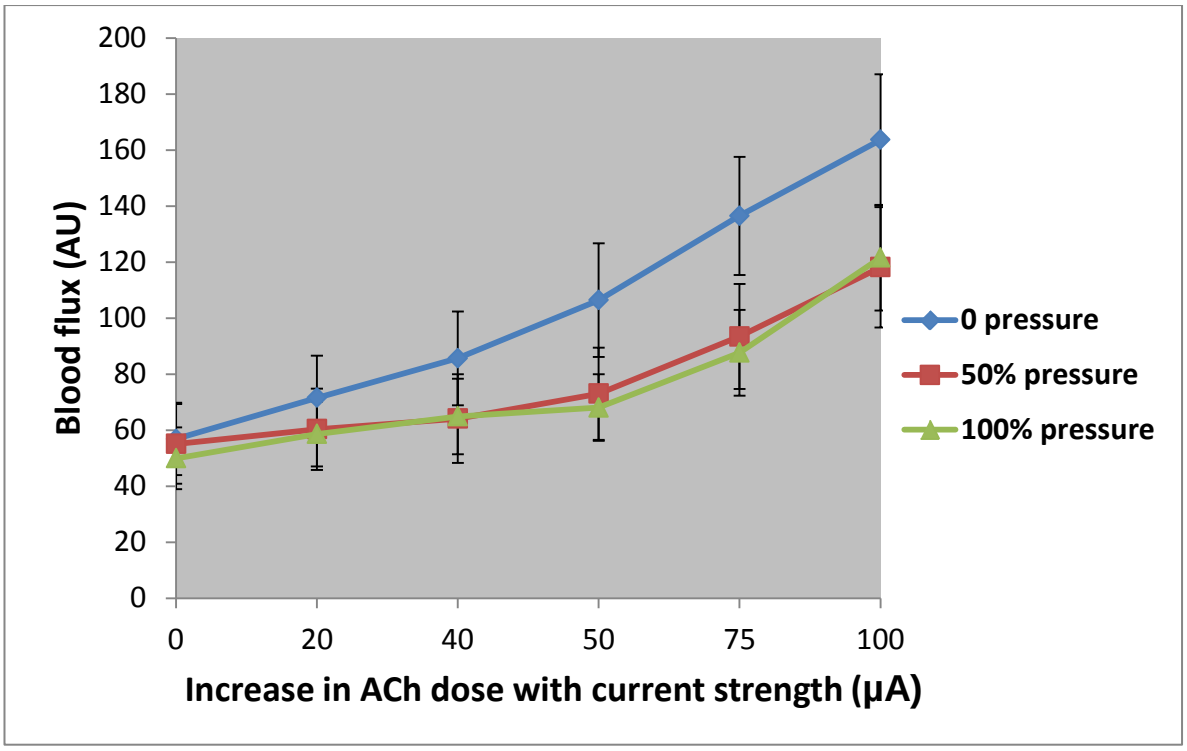

Figure 4.50 Plot of the three levels of pressure against the increase in current on application of iontophoresis with ACh for the healthy control group

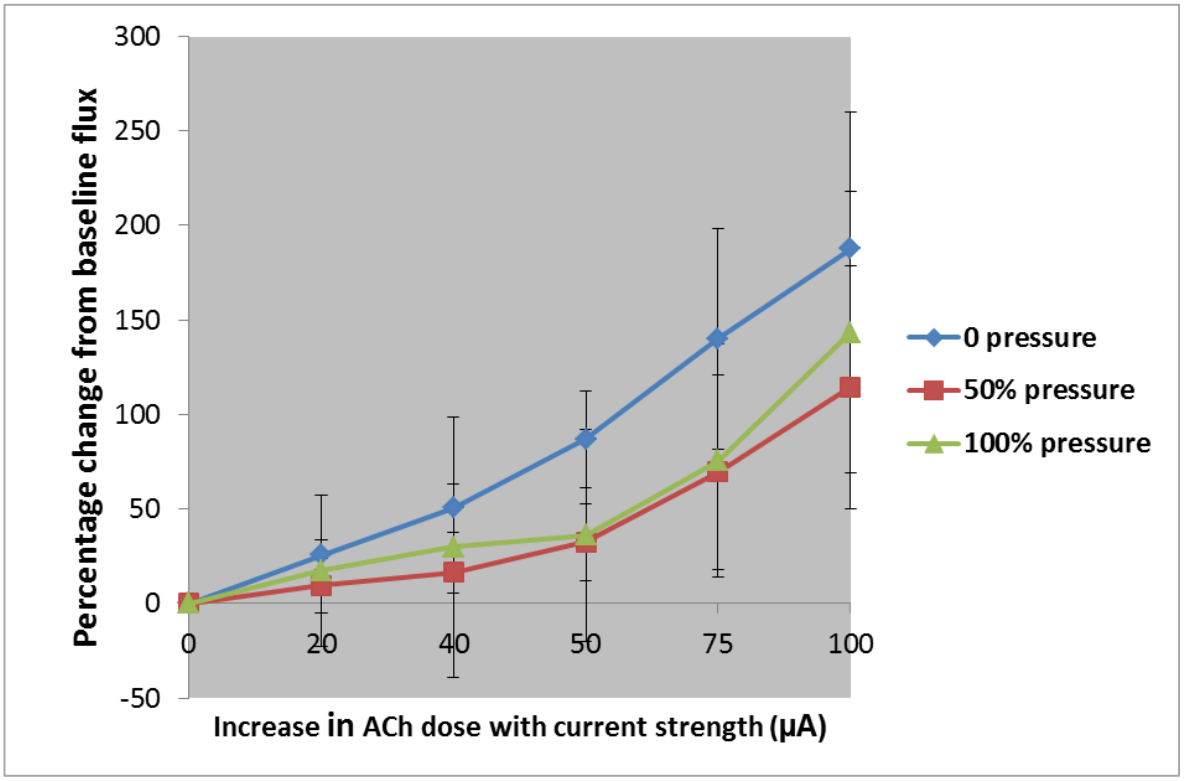

Figure 4.51 Percentage change from baseline flux against increase in ACh dose with current strength for the control group

4.7.4.6 Differences found between the group with Type 2 diabetes and the healthy control group in blood flux response to pressure addition under iontophoresis with ACh

When iontophoresis with ACh was carried out without any added pressure, both groups displayed a similar curve for increase in flux with dose of ACh as can be seen in Figure 4.52. The most noticeable difference was that the group with Type 2 diabetes display higher flux values throughout, with baseline flux prior to current application mean and standard deviation for subjects with diabetes being 79.73 ± 81.87 (AU) and for the control subjects was 56.88 ± 68.48 (AU) rising to a maximum flux for the group with Type 2 diabetes of 190.66 ± 124.72 (AU) and for the control group maximum flux was 163.63 ± 124.25 (AU). Utilising repeated measures ANOVA with Huynh-Feldt correction and post hoc Bonferroni test within subjects there was no significant difference found for the group measured with increase in dose ($F = 2.328$, $p = 0.139$). There was a significant difference for dose administered ($F = 33.042$, $p < 0.0001$) with no significant interaction between the group and dose of ACh ($F = 0.437$, $p = 0.634$). Follow up analysis of the maximum flux achieved in both groups was not significant ($p = 0.527$ M.W.U). The findings are displayed in Figure 4.52, and with the percentage change from baseline flux in Figure 4.53.

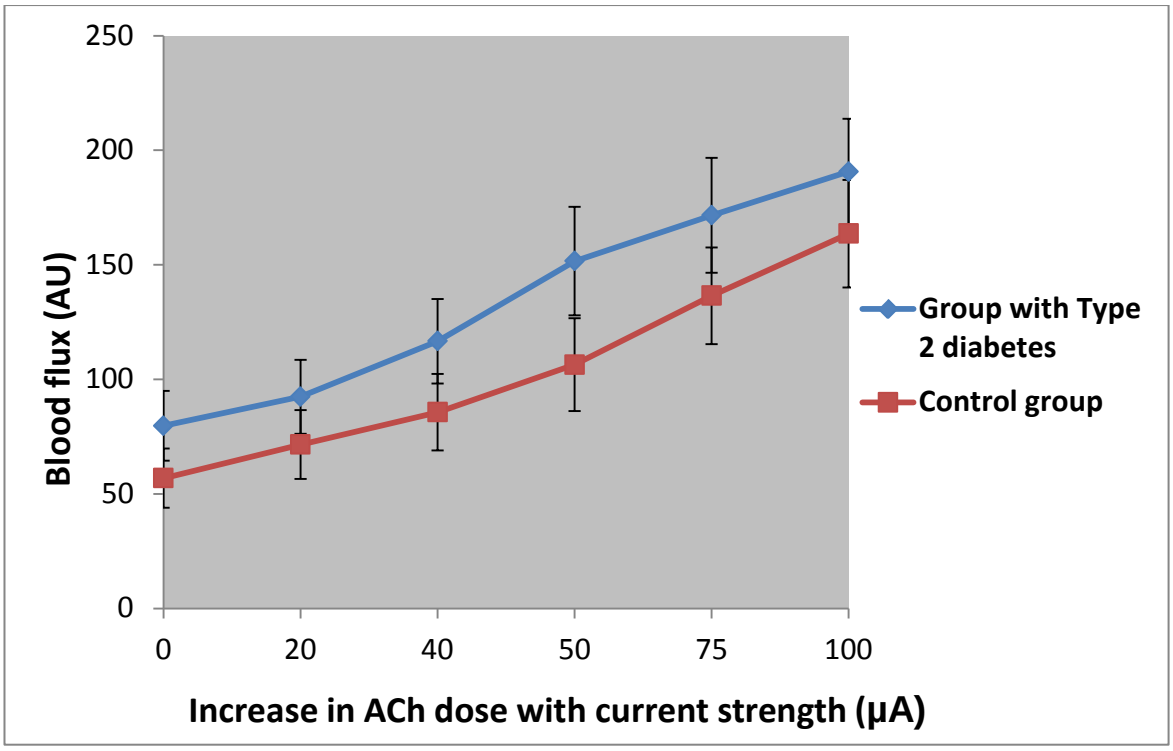

Figure 4.52 Plots of the groups with Type 2 diabetes and controls with increase in flux and no added pressure

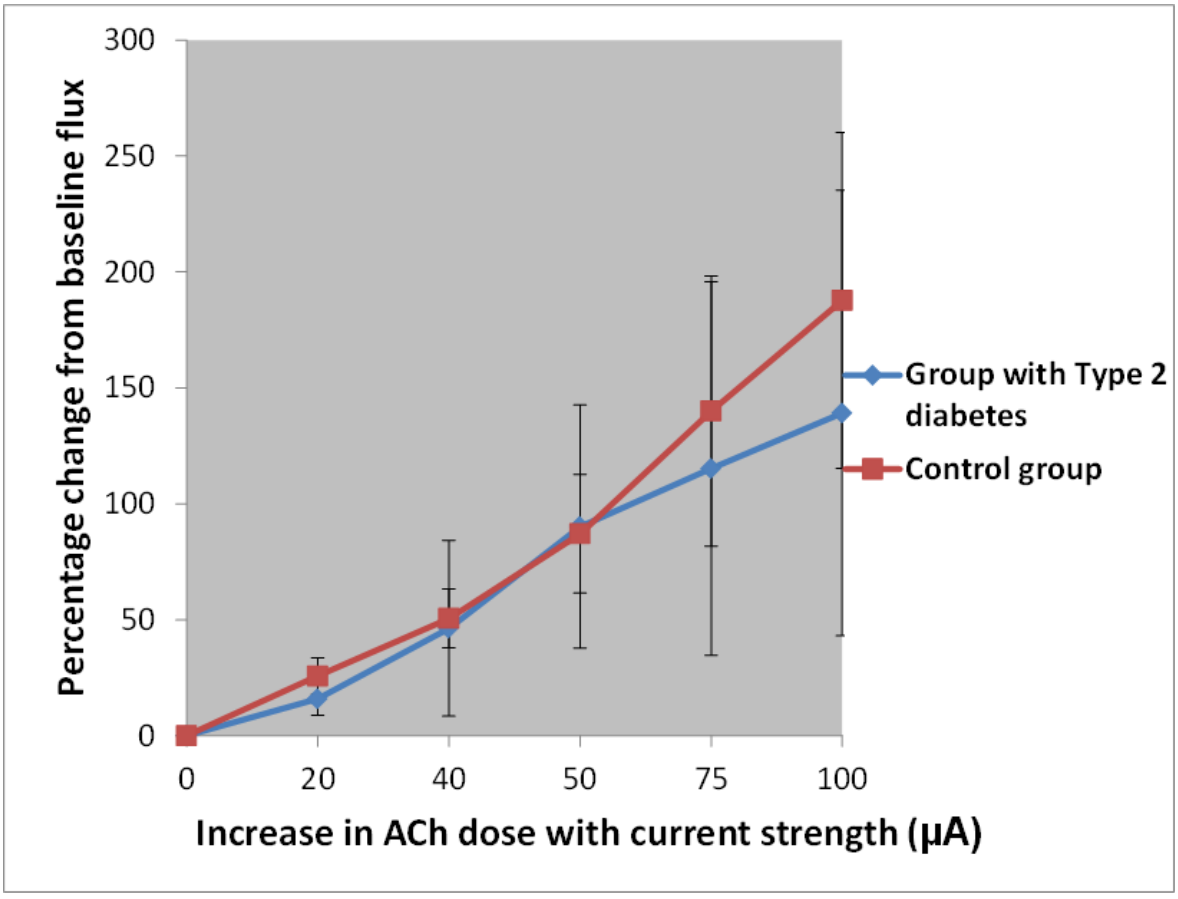

Figure 4.53 Percentage change from baseline flux for the groups with Type 2 diabetes and controls with increase in flux and no added pressure

4.7.4.7 Differences across the groups with the addition of 50% pressure

With the addition of 50% pressure both groups follow a similar flux increase curve as ACh levels were increased across the skin surface, with the group with Type 2 diabetes levelling out at the maximum flux achieved as illustrated in Figures 4.54 and 4.55. As with no pressure application, again the group with Type 2 diabetes both started and ended with higher flux values than the control group displayed. The baseline flux mean and standard deviation values for the group with Type 2 diabetes were 63.01 ± 58.49 (AU) and for the control group were 55.11 ± 77.83 (AU) and the maximum flux values achieved with the group with Type 2 diabetes were 134.28 ± 113.27 (AU) and for the control group were 118.17 ± 117.86 (AU). There was no significant difference found for the main effect of group with increase in ACh dose ($F = 3.072$, $p = 0.91$). There was a significant difference for the dose administered ($F = 22.093$, $p < 0.0001$) with no significant interaction found between the group and dose ($F = 1.002$, $p = 0.362$). Mann-Whitney U test applied to the maximum flux achieved across the groups was not significant ($p = 0.358$).

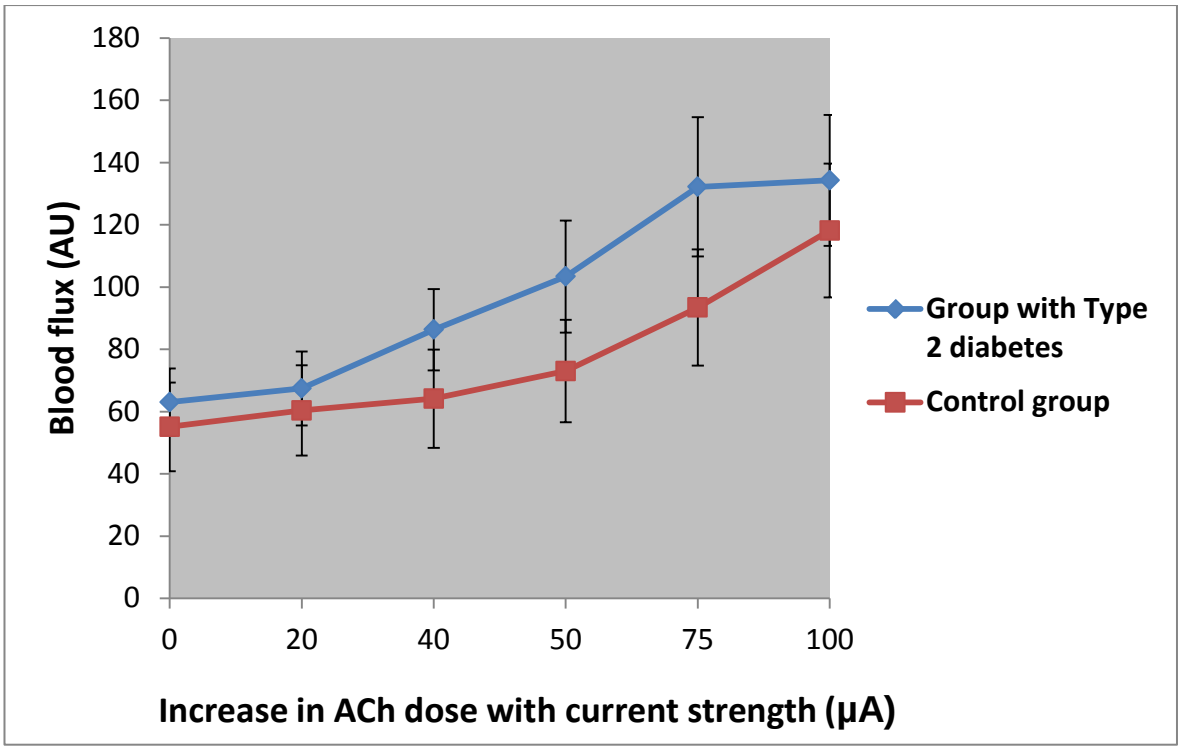

Figure 4.54 Plots of the groups with Type 2 diabetes and control subjects with increase in flux and 50% added pressure

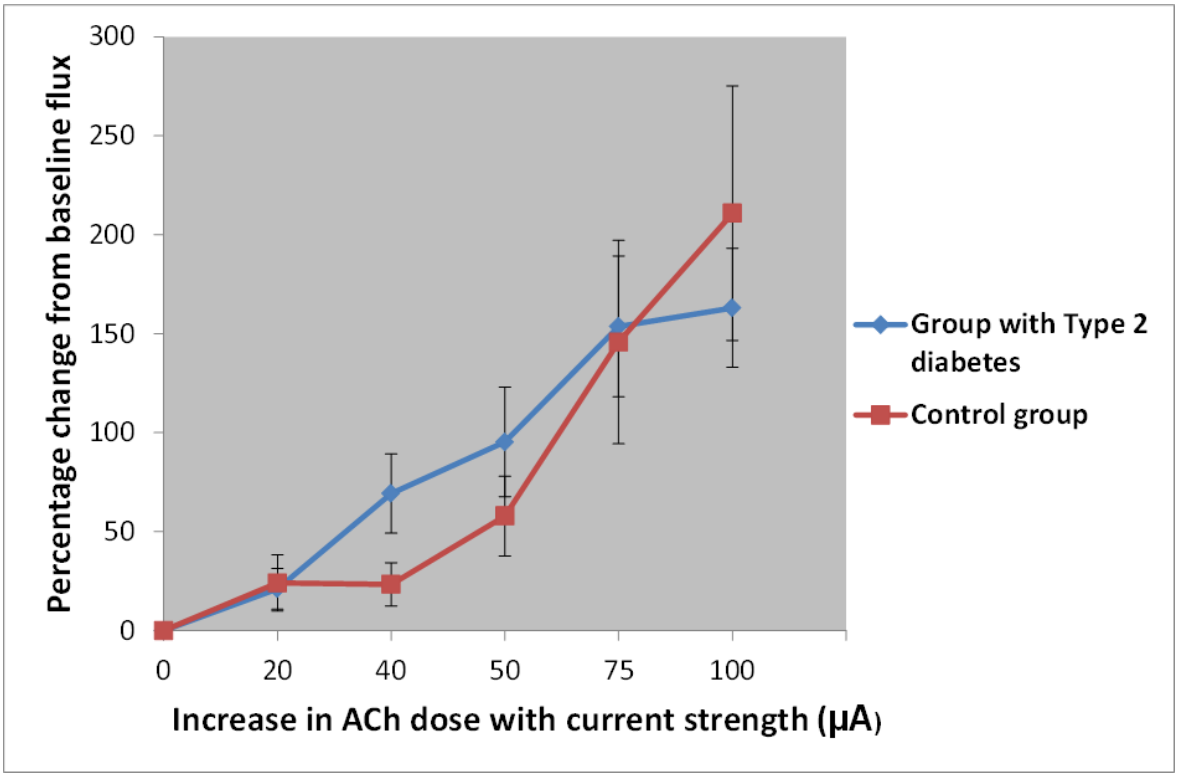

Figure 4.55 Percentage change from baseline flux for the groups with Type 2 diabetes and control subjects with increase in flux and 50% added pressure

4.7.4.8 Differences across the groups with the addition of 100% pressure

The increase in flux with dose of ACh for the group with Type 2 diabetes climbed steadily to a maximum flux whereas with the control group there was a less steep increase (especially noted at lower doses) as can be seen in Figures 4.56 and 4.57. The flux values achieved by the group with Type 2 diabetes were again higher generally than the control group with a baseline flux of 56.90 ± 48.67 (AU) and for the control group 49.94 ± 59.35 (AU) and a maximum flux of 138.18 ± 112.91(AU) and for the controls 121.60 ± 101.42 (AU). A significant difference was found for the dose administered ($F = 14.891$, $p < 0.0001$) however, no significant interaction was found between the group and dose with 100% pressure added ($F = 1.411$, $p = 0.251$) and follow up analysis did not indicate a significant difference between the maximum flux levels achieved by the groups ($p = 0.292$ M.W.U).

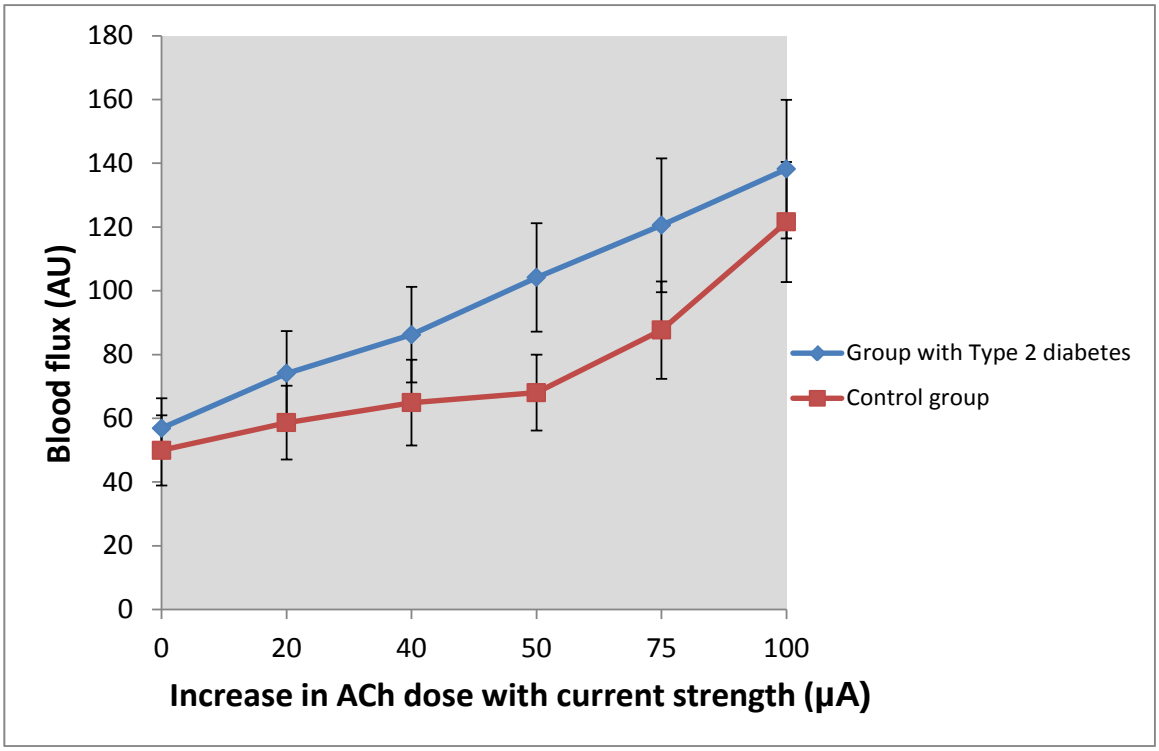

Figure 4.56 Plots of the groups with Type 2 diabetes and controls with increase in flux and 100% added pressure

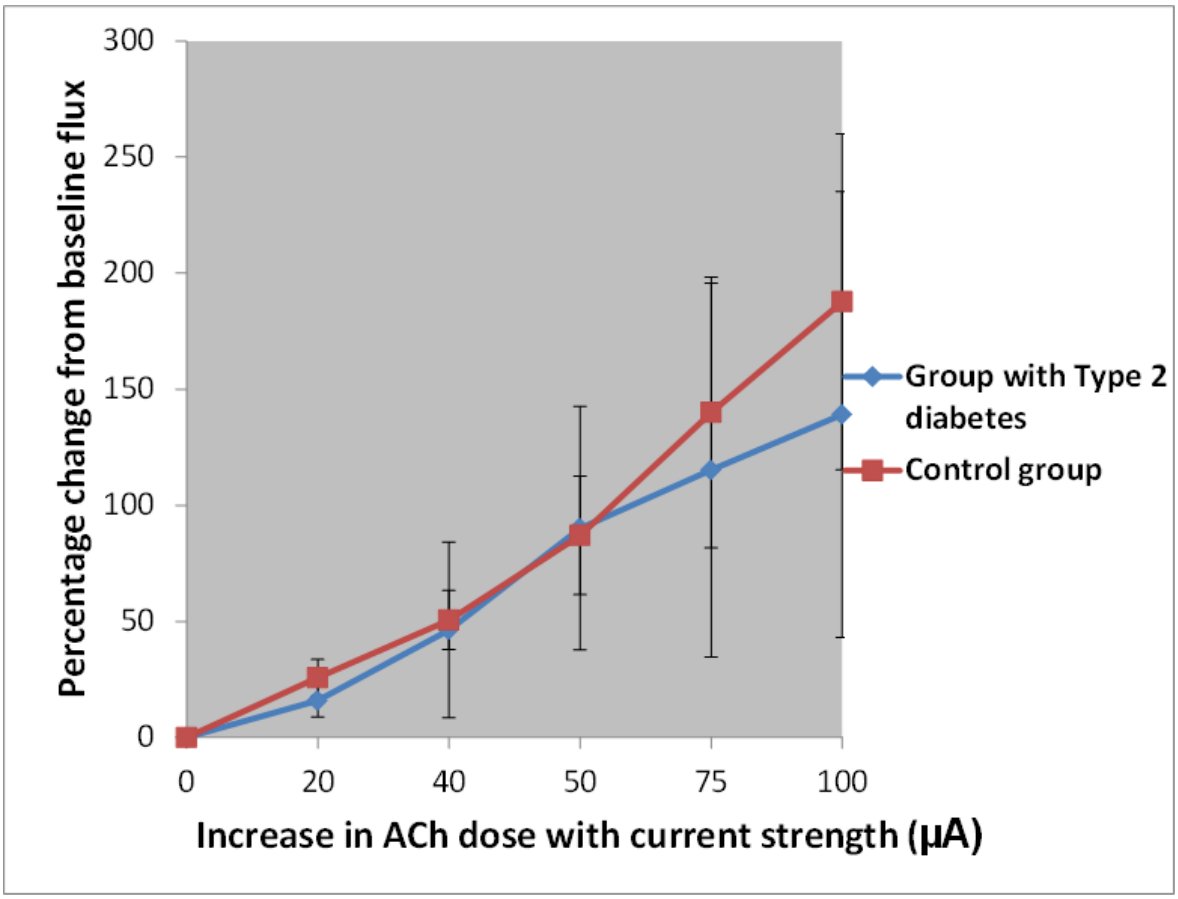

Figure 4.57 Percentage change from baseline flux against increase in dose of ACh with current strength for the groups with Type 2 diabetes and controls with increase in flux and 100% added pressure

4.7.4.9 The impact of pressure on the blood flux values obtained during the process of iontophoresis with SNP

For baseline flux prior to application of current, for the whole group n = 60 inclusive of subjects with Type 2 diabetes and the control group, the addition of both 50% and 100% of the simulated walking pressure had an impact on the flux values obtained with SNP. The mean and standard deviation for SNP delivery (for baseline flux) with no pressure added was 72.29 ± 89.29 (AU), with 50% pressure was 58.76 ± 61.45 (AU) and for 100% of added pressure flux was 39.66 ± 37.08 (AU). With SNP delivery via iontophoresis and no pressure the response to increasing the dose took longer than with ACh to produce an increase in flux, but once a response was achieved, there was a steady increase to maximum flux. However, 50% and 100% pressure delivery impacted greatly in that the increase in flux was very much impeded with both, and the rise from baseline flux to the maximum flux achieved was small as illustrated in Figure 4.58. The

findings can be clearly seen in Figure 4.59 with the percentage change from baseline flux against increase on dose. The maximum flux mean and standard deviation for no pressure delivered being 123.40 ± 103.78 (AU), for 50% pressure was 66.07 ± 58.40 (AU) and for 100% pressure was 53.52 ± 51.61(AU). There was a significant main effect within the participants for pressure ($F = 16.752$, $p < 0.0001$) and a significant effect of the dose of SNP administered ($F = 9.223$, $p < 0.0001$). There was also a significant interaction between pressure and dose of SNP delivered ($F = 7.762$, $p < 0.0001$). ANOVA (repeated measures with post hoc Bonferroni) was used to analysis the data. Once again Mauchly's test indicated violation of assumption of sphericity so Huynh-Feldt correction was used. Follow up analysis was carried out using the non-parametric Mann-Whitney U test and a significant difference was noted for maximum flux achieved between no pressure and 50% pressure ($p < 0.0001$) and no pressure and 100% pressure ($p < 0.0001$). There was no significant difference found in maximum flux achieved with 50% and 100% pressure added, however this result was marginal ($p = 0.058$).

Table 4.23 Descriptive statistics for pressure and increased dose of SNP with iontophoresis

Pressure applied	Baseline flux(AU) Mean ± S.D.	Max. flux(AU) Mean ± S.D.	Sig. p values	Sig. p values	Sig. p values
0 pressure	72.29 ± 89.29	123.40 ± 103.78	<0.0001	<0.0001	
50% pressure	58.76 ± 61.45	66.07 ± 58.40	<0.0001		0.058
100% pressure	39.66 ± 37.08	53.52 ± 51.61		<0.0001	0.058

There was a significant difference found within subjects with pressure ($p < 0.0001$, ANOVA) for the group. A significant difference was found for increase in dose ($p < 0.0001$, ANOVA) and for the interaction effect of pressure with increase in SNP dose ($p < 0.0001$, ANOVA). There was a significant difference found between 0 pressure and 50% pressure application ($p < 0.0001$, M.W.U) and between 0 pressure and 100% pressure delivery ($p < 0.0001$, M.W.U) however although marginal, no significant difference was found between 50 and 100% pressure ($p = 0.058$, M.W.U).

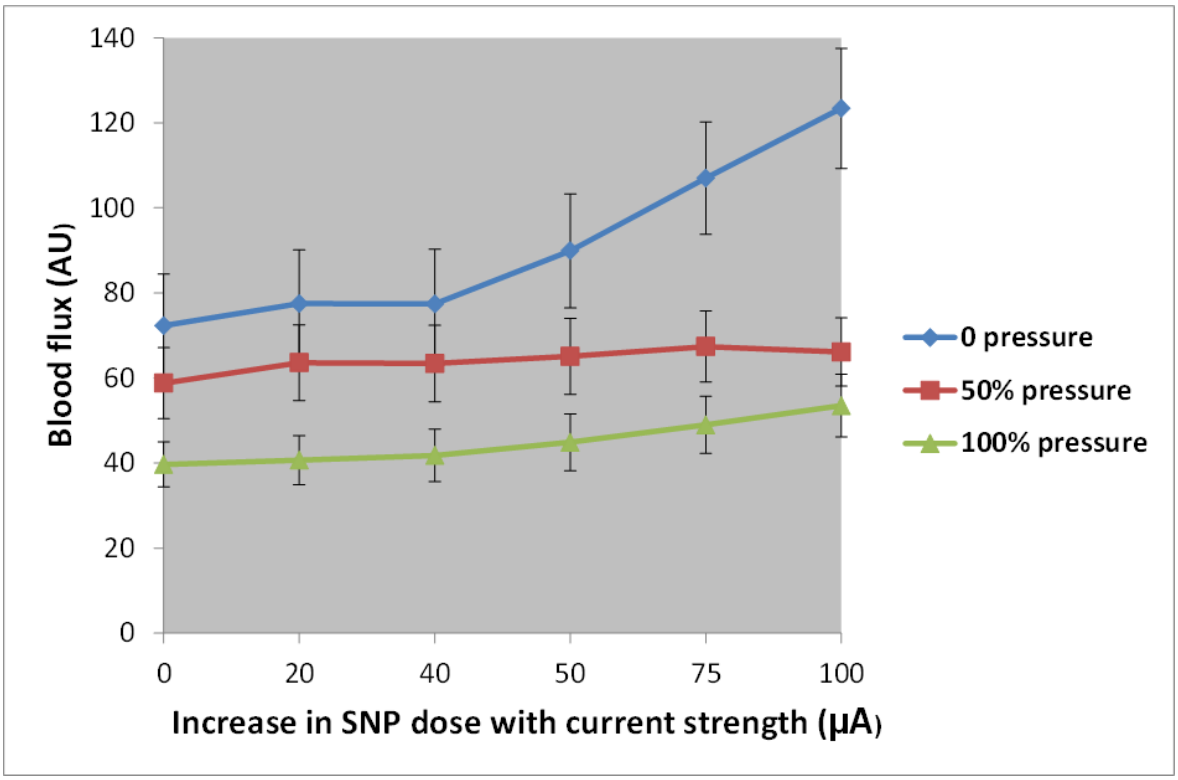

Figure 4.58 Plots of the whole group with no pressure, 50% and 100% pressure delivery with increasing SNP dose against blood flux

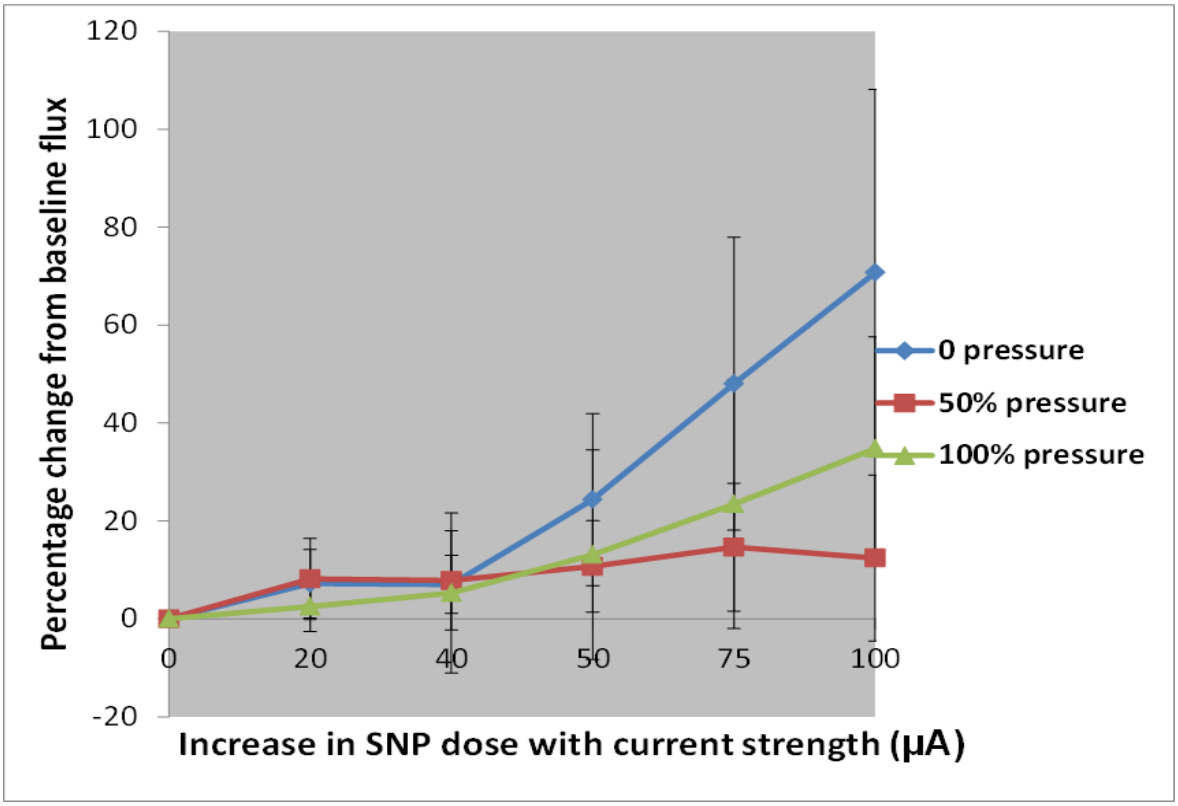

Figure 4.59 Percentage change from baseline flux against increase in dose of SNP with current strength for the whole group with no pressure, 50% and 100% pressure delivery

4.7.4.10 The impact of pressure on the blood flux values obtained during the process of iontophoresis with SNP in the group with Type 2 diabetes

With the group with Type 2 diabetes, on the application of SNP the baseline flux for pressure additions impacted on the flux values obtained with baseline flux mean and standard deviation when no pressure was added (91.32 ± 106.30 AU), with 50% pressure added 74.38 ± 76.11(AU) and with 100% pressure added 53.11 ± 46.56 (AU). Without any pressure added the flux increased to a maximum mean value of 128.51 ± 110.86 (AU), however the impact of 50% pressure delivery in the group with Type 2 diabetes was marked in that the final flux value on completion of the iontophoresis delivery of SNP resulted in a lower mean value than the starting flux at 72.98 ± 65.01(AU). 100% pressure also impacted greatly in that there was a very small difference between the baseline flux and the maximum flux value achieved on completion of iontophoresis with a mean value of 59.61 $\pm$ 57.71(AU). There was a significant main effect found within subjects for pressure ($F = 8.532$, $p = 0.006$ ANOVA). No significant difference was found for the effect of dose administered ($F = 0.608$, $p = 0.535$ ANOVA) however there was a significant interaction between pressure and the dose of SNP administered ($F = 2.424$, $p = 0.044$ ANOVA). Follow up analysis indicated a significant difference between the final flux values between no pressure and the addition of 50% pressure ($p = 002$ M.W.U) and between no pressure and 100% pressure ($p = 0.001$ M.W.U). No significant difference was found between 50% and 100% added pressure at the final flux value ($p = 0.115$ M.W.U). The impact of pressure addition to flux levels can be noted in Table 4.24 and is illustrated in Figure 4.60 and the percentage change from baseline flux is clearly indicated in Figure 4.61.

Table 4.24 Descriptive statistics for pressure and increased dose of SNP with iontophoresis in the group of subjects with Type 2 diabetes

Pressure applied	Baseline flux(AU) Mean ± S.D.	Max. flux(AU) Mean ± S.D.	**Sig. p values**	**Sig. p values**	**Sig. p values**
0 pressure	91.32 ± 106.30	128.51±110.86	0.002	0.001	
50% pressure	74.38 ± 76.11	72.98 ± 65.01	0.002		0.115
100% pressure	53.11 ± 46.56	59.61 ± 57.71		0.001	0.115

There was a significant difference found in the group with Type 2 diabetes within subjects with pressure (p = 0.006, ANOVA) for the group. There was no significant difference found for increase in dose (p = 0.535, ANOVA) and for the interaction effect of pressure with increase in SNP dose there was a significant difference found (p = 0.044, ANOVA). There was a significant difference found between 0 pressure and 50% pressure application (p = 0.002, M.W.U) and between 0 pressure and 100% pressure delivery (p = 0.001, M.W.U) however no significant difference was found between 50 and 100% pressure (p = 0.115, M.W.U).

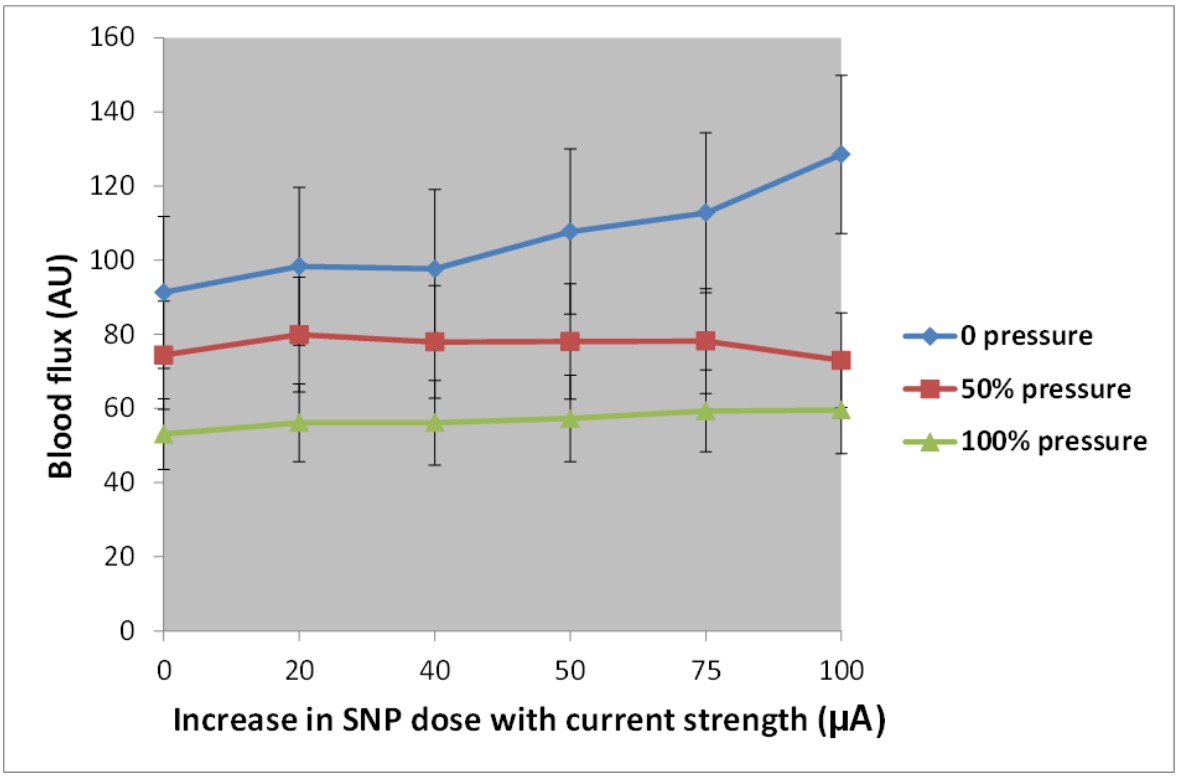

Figure 4.60 Plots of the group with Type 2 diabetes with no pressure, 50% and 100% pressure delivery with increasing SNP dose against blood flux (AU)

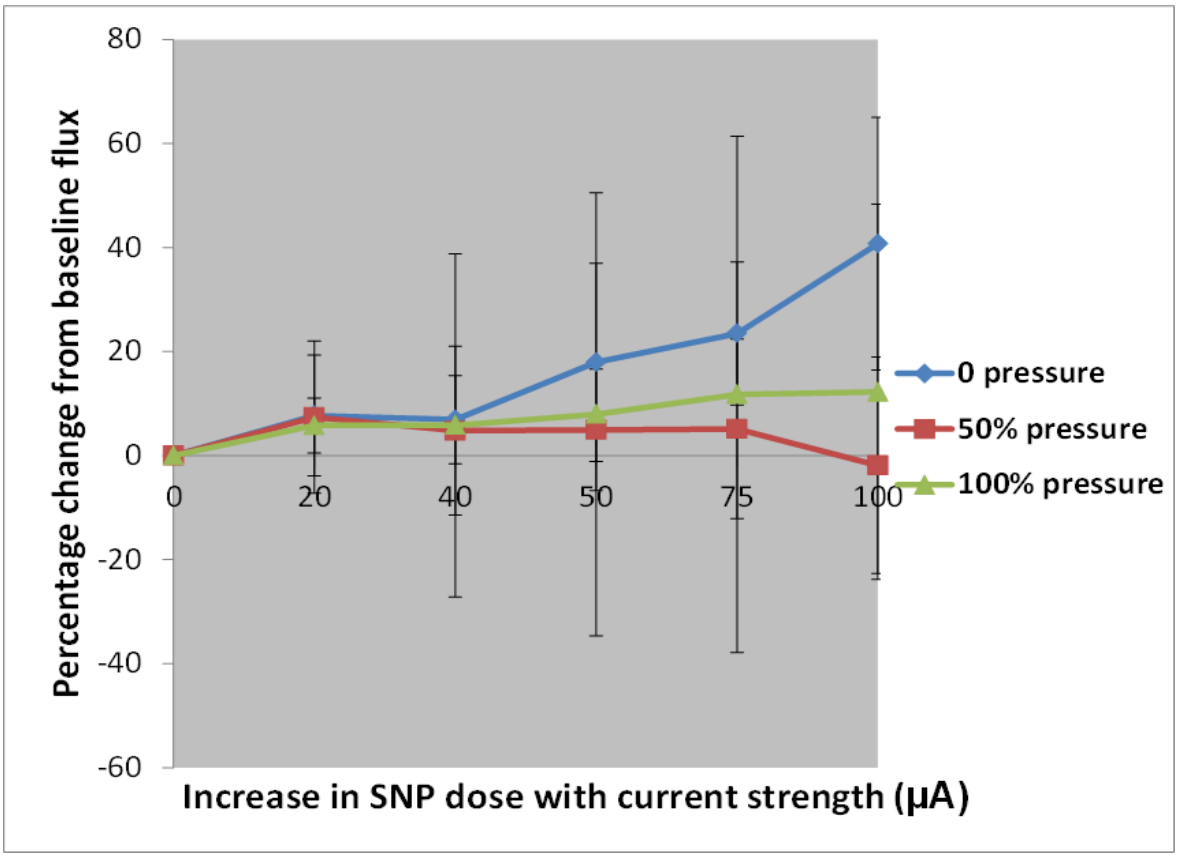

Figure 4.61 Percentage change form baseline flux against increase in dose of SNP with current strength in the group with Type 2 diabetes with no pressure, 50% and 100% pressure delivery

4.7.4.11 The impact of pressure on the blood flux values obtained during the process of iontophoresis with SNP in the group of control subjects

The addition of pressure at 50 and 100% of simulated walking pressure did impact upon the baseline flux in this group as it did in the group with Type 2 diabetes. Baseline flux without pressure addition mean and standard deviations were 53.27 ± 64.82 (AU) with 50% pressure were 43.13 ± 37.39 (AU) and with the addition of 100% pressure were 26.74 ± 17.81(AU). With the control subjects and the iontophoresis of SNP it did take some time before the flux began to rise with no pressure however, once it started to increase it climbed steeply to a maximum value of 118.28 ± 98.02 (AU). There was an impact with both 50 and 100% pressure addition, with 50% maximum mean 59.41 ± 51.62 (AU) and 100% pressure achieving 47.68 ± 45.42 (AU), however the control group did deal better with the pressure than the group with Type 2 diabetes in that an increase in flux values was recorded at the end of the iontophoresis process. A

significant difference was found for the main effect of pressure (F = 7.986, p = 0.004 ANOVA), and for the effect of dose of SNP (F = 15.139, p < 0.0001 ANOVA) as well as for the interaction between pressure and dose of SNP (F = 5.911, p = 0.001 ANOVA). There was a significant difference found between the maximum flux value for no pressure when compared with 50% pressure (p = 0.002 M.W.U) and between no pressure and 100% pressure (p = 0.0001 M.W.U), however no significant difference was found between the maximum flux obtained with 50% and 100% pressure added (p = 0.253 M.W.U). The impact of pressure can be seen in Table 4.25 and is illustrated in Figures 4.62 and 4.63.

Table 4.25 Descriptive statistics for pressure and increased dose of SNP with iontophoresis in the control group

Pressure applied	Baseline flux(AU) Mean ± S.D.	Max. flux (AU) Mean ± S.D.	**Sig. p values**	**Sig. p values**	**Sig. p values**
0 pressure	53.27 ± 64.82	118.28 ± 98.02	0.002	0.0001	
50% pressure	43.13 ± 37.39	59.41 ± 51.62	0.002		0.253
100% pressure	26.74 ± 17.81	47.68 ± 45.42		0.0001	0.253

There was a significant difference found in the control group within subjects with pressure (p = 0.004, ANOVA) for the group. There was a significant difference found for increase in dose (p < 0.0001, ANOVA) and for the interaction effect of pressure with increase in SNP dose (p = 0.001, ANOVA). There was a significant difference found between 0 pressure and 50% pressure application (p = 0.002, M.W.U) and between 0 pressure and 100% pressure delivery (p = 0.0001, M.W.U) however no significant difference was found between 50 and 100% pressure (p = 0.253, M.W.U).

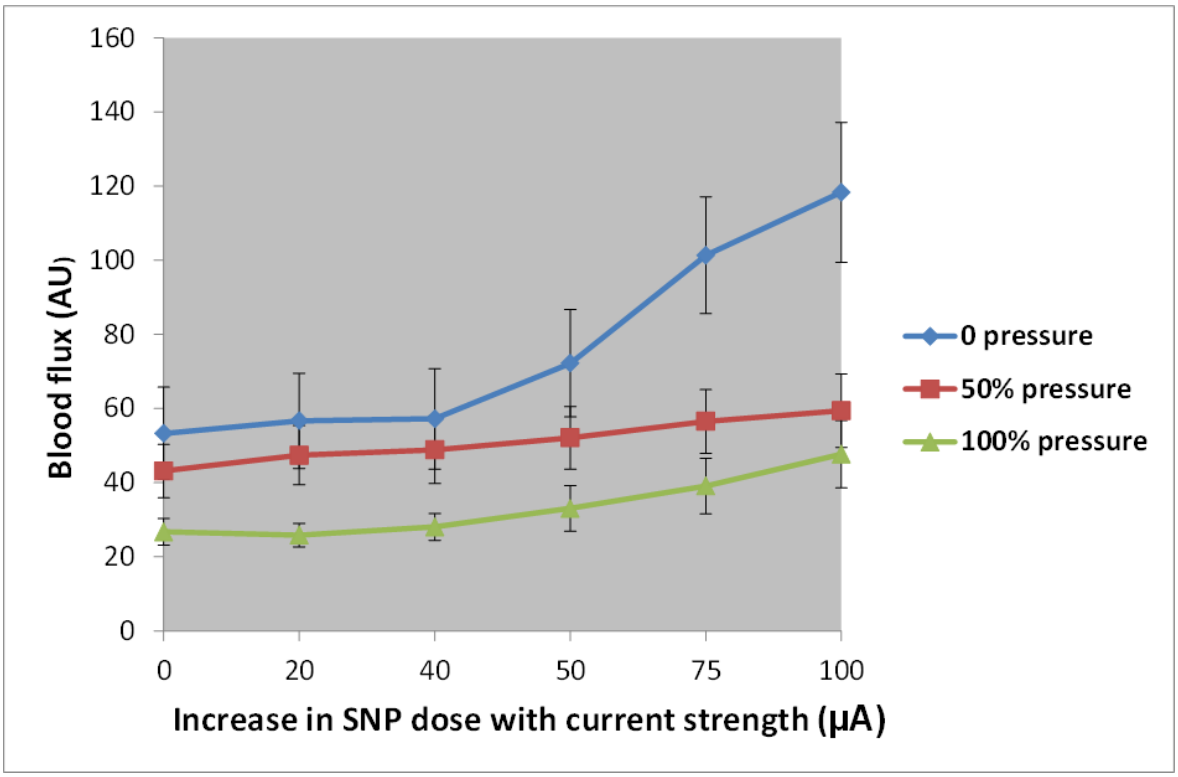

Figure 4.62 Plots of the control group with no pressure, 50% and 100% pressure delivery with increasing SNP dose against blood flux

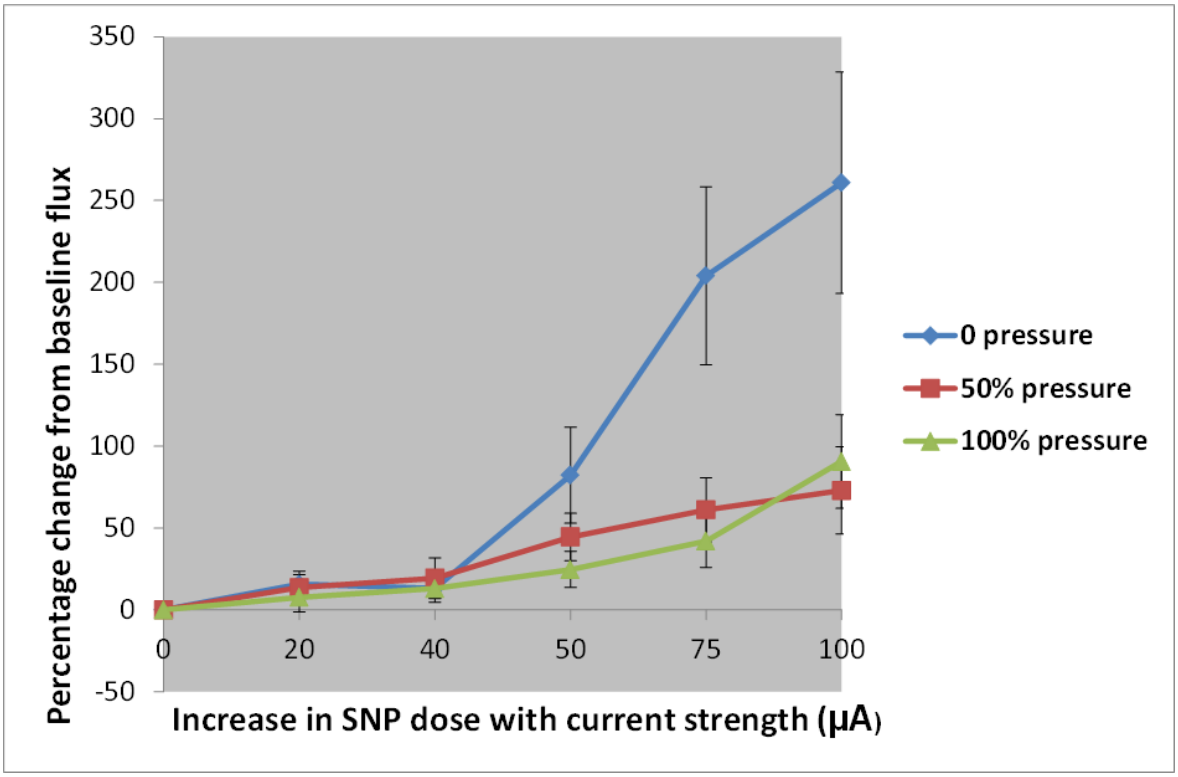

Figure 4.63 Percentage change from baseline flux with increase in dose of SNP for the control group with no pressure, 50% and 100% pressure delivery

4.7.4.12 Differences found between the two groups in reaction to the pressure delivered under iontophoresis with SNP

Without the addition of any pressure, there was a marked difference in the baseline flux found between the groups, with the group with Type 2 diabetes displaying a higher baseline flux (91.32 ± 106.30 AU) than the age matched control group (53.27 ± 64.82 AU). However, the maximum value achieved by the control subjects (118.28 ± 98.02 AU) was quite close to that of the subjects with Type 2 diabetes (128.51 ± 110.86 AU), thus displaying a greater overall response and this was achieved by a much steeper curve of the response as can be seen in Figure 4.64. No significant difference was found for the main effect of group measured with the increase in SNP dose ($F = 0.855$, $p = 0.365$ ANOVA), however there was a significant difference found for dose administered ($F = 18.138$, $p < 0.0001$ ANOVA). No significant interaction between group and the dose of SNP was found ($F = 1.468$, $p = 0.241$ ANOVA). Follow up analysis of the resting flux ($p = 0.130$ M.W.U) and the maximum flux ($p = 0.909$ M.W.U) did not indicate a significant difference between the 2 groups which can be noted in Figure 4.64 and indicated in the percentage change from baseline flux illustrated in Figure 4.65.

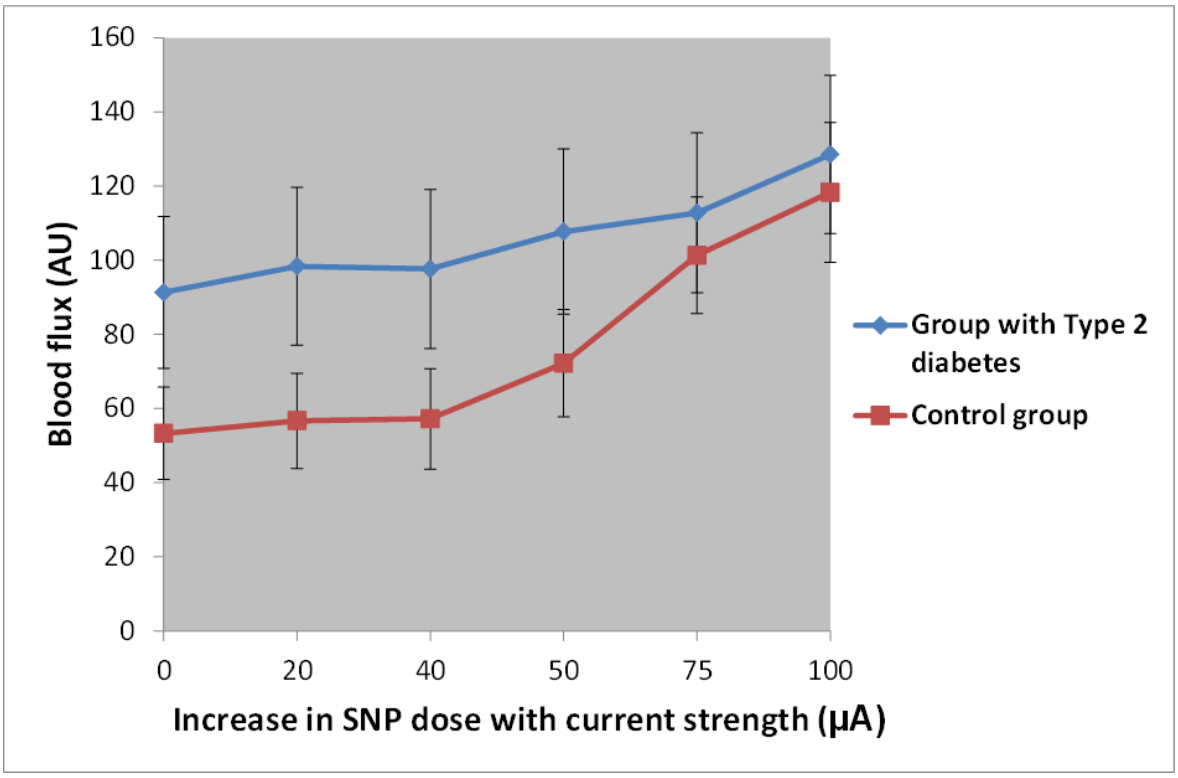

Figure 4.64 Differences found between the 2 groups with delivery of SNP with no added pressure

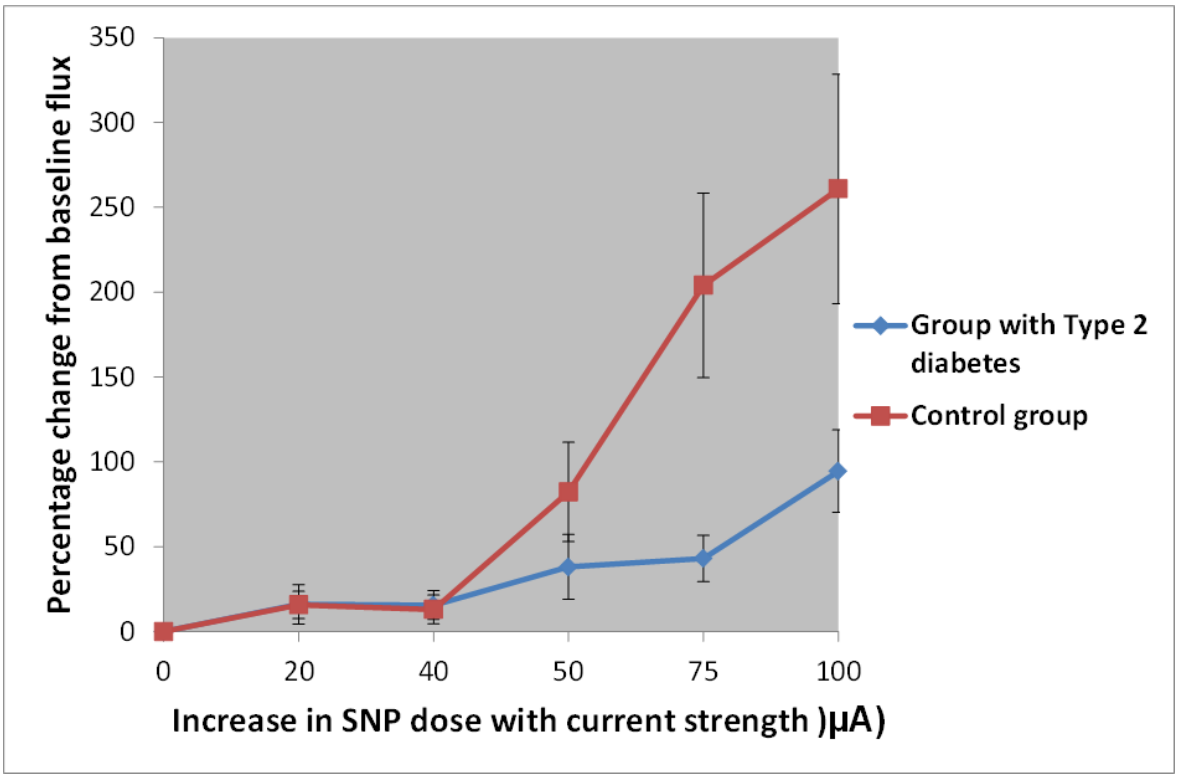

Figure 4.65 The percentage change from baseline flux with increase in SNP dose between the groups with no added pressure

4.7.4.13 The impact of 50% pressure addition on the 2 groups with SNP delivery under iontophoresis

Both groups reacted differently to the addition of 50% pressure. The group of control subjects started with a baseline flux lower than the group with Type 2 diabetes (43.13 ± 37.39 vs 74.38 ± 76.11 AU), but then a gradual increase in flux was found up to the maximum flux of 59.41 ± 51.62 (AU), whereas the group with Type 2 diabetes while starting with a higher baseline flux actually achieved a slight lowering of flux to 72.98 ± 65.01(AU), thus the impact of 50% pressure delivery was greater on the group with Type 2 diabetes than the control group as displayed in Figure 4.66. Despite the differences noted between the two groups as illustrated in Figure 4.67, no significant difference was found for the main effect of group on the dose of SNP ($F = 1.194$, $p = 0.286$ ANOVA) or for the dose administered ($F = 1.860$, $p = 0.161$ ANOVA) or in fact for the interaction between group and the dose of SNP ($F = 1.092$, $p = 0.354$ ANOVA). Between participants did display a significant difference ($F = 74.627$, $p < 0.0001$ ANOVA). There was no significant difference found either with resting flux ($p = 0.189$ M.W.U) or with final flux values ($p = 0.784$ M.W.U) between the two groups.

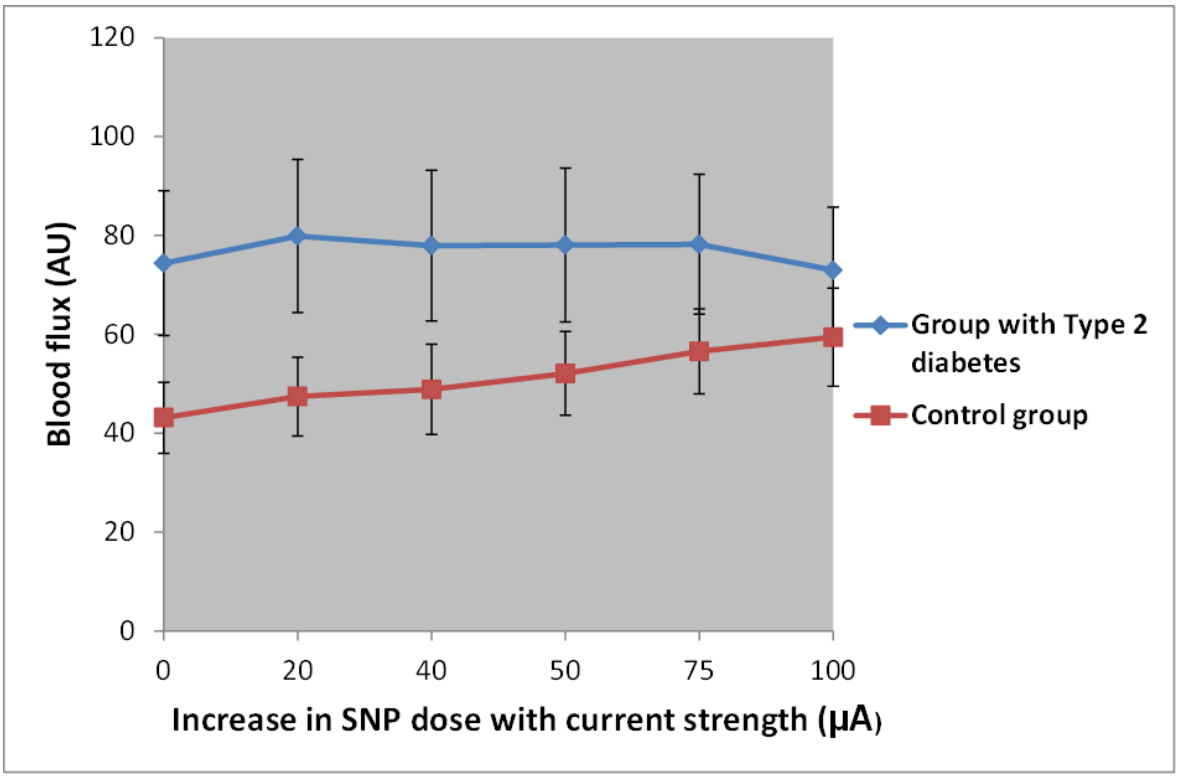

Figure 4.66 Differences found between the 2 groups with delivery of SNP and 50% added pressure

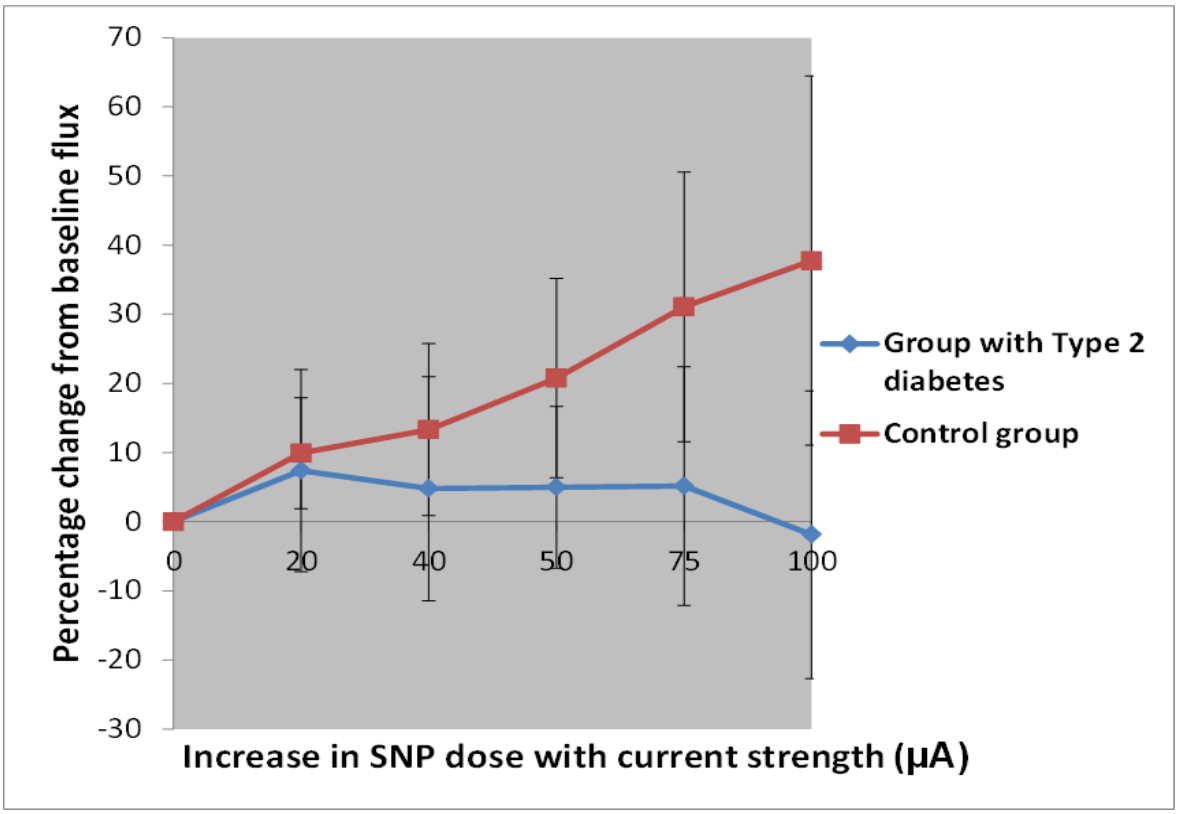

Figure 4.67 Percentage change from baseline flux with increase in dose of SNP displaying the differences found between the 2 groups with delivery of SNP and 50% added pressure

4.7.4.14 The impact of 100% pressure addition to the two groups with SNP delivery under iontophoresis

Again a notable difference was found between the baseline flux of the two groups with the group with Type 2 diabetes having a higher baseline flux than the control group when 100% pressure was added (53.11 ± 46.56 versus 26.74 ± 17.81 AU). The group with Type 2 diabetes had a marginal increase in flux from baseline to maximum flux (53.11 ± 46.56 to 59.61 ± 57.71 AU) whereas the control group displayed a rise from baseline flux at 26.74 ± 17.81 (AU) to 47.68 ± 45.42 (AU) as displayed in Figure 4.68 and 4.69. There was a significant difference found for the main effect of the group ($F = 4.589$, $p = 0.045$ ANOVA) but no significant difference for the dose administered ($F = 0.942$, $p = 0.389$ ANOVA). There was a significant difference for the interaction between the group and dose administered ($F = 2.918$, $p = 0.047$ ANOVA) and between the subjects ($F = 46.096$, $p < 0.0001$ ANOVA). On follow up analysis there was a significant difference found between the resting flux of the two groups ($p = 0.017$ M.W.U) but not between the maximum flux ($p = 0.575$ M.W.U).

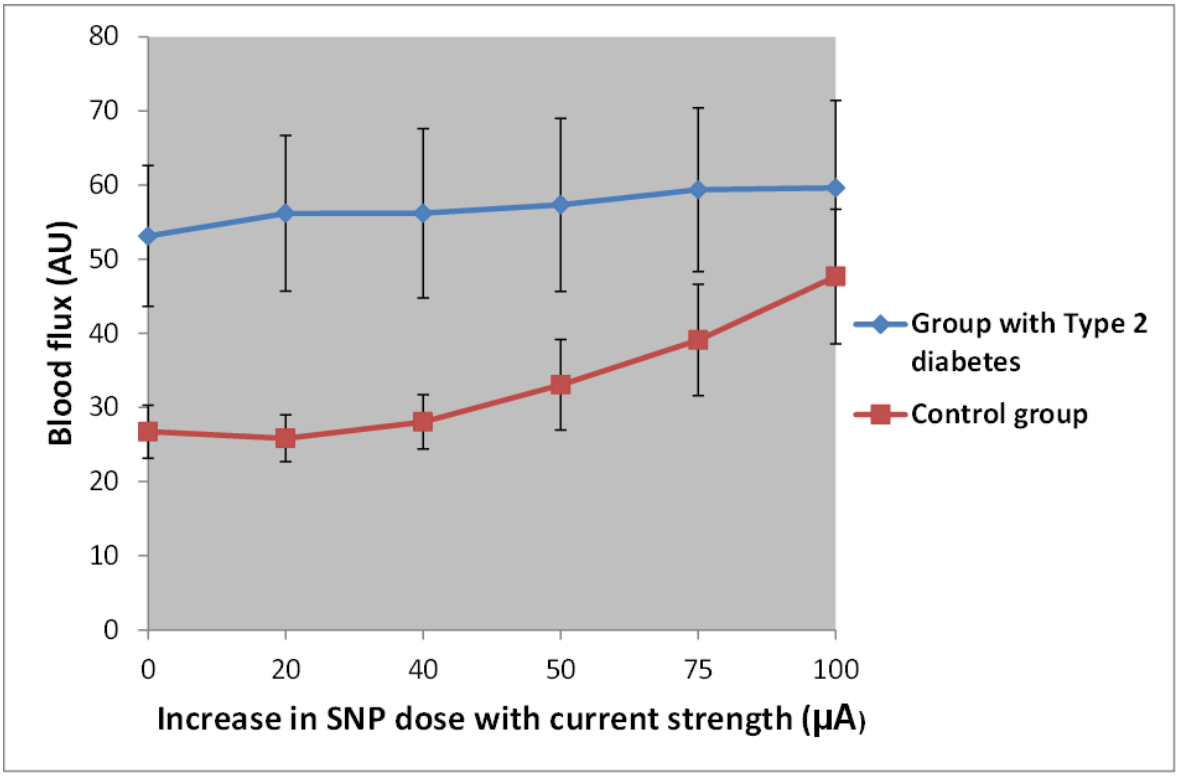

Figure 4.68 Differences found between the 2 groups with delivery of SNP and 100% added pressure

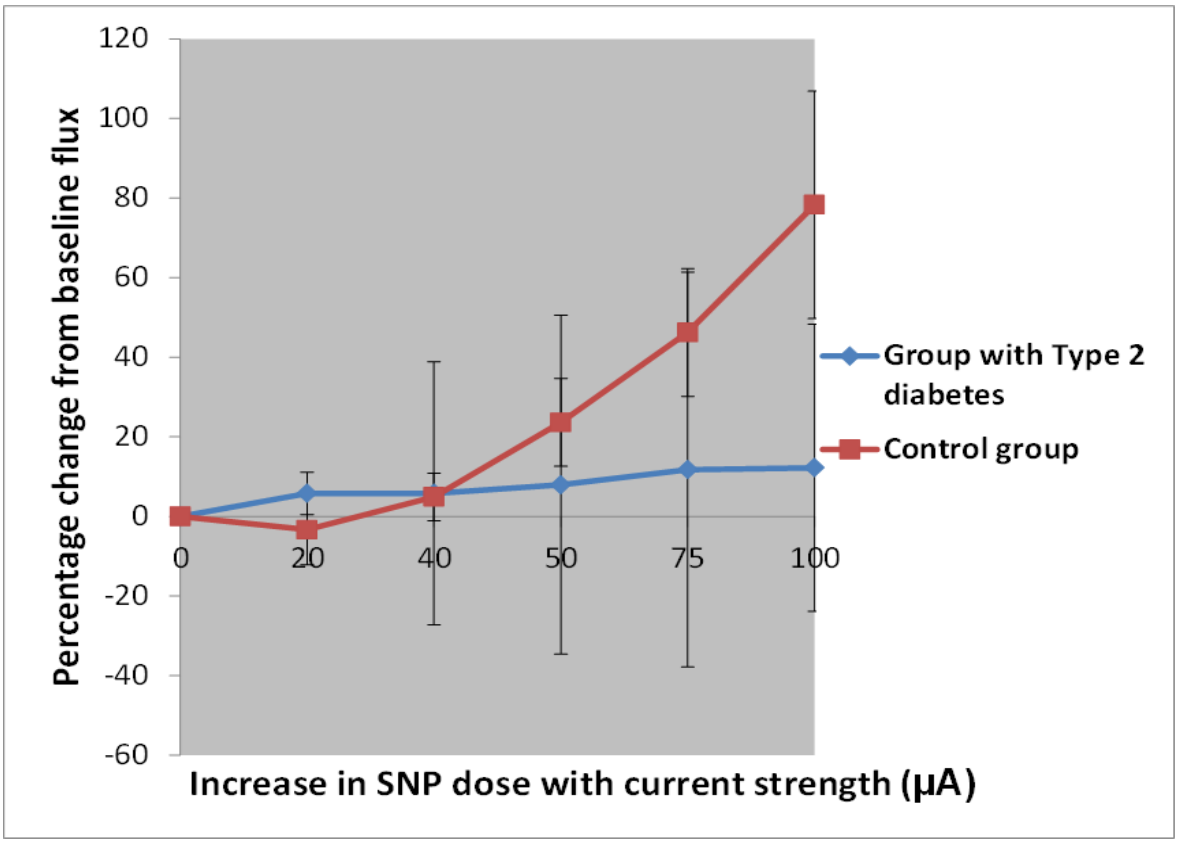

Figure 4.69 Percentage change from baseline flux with increase in SNP dose for the 2 groups with delivery of SNP and 100% added pressure

4.8 SUMMARY

Investigation of the reaction to PORH in the two groups indicated that there was no significant difference found for baseline flux or the groups, and no significant interaction was found between baseline flux and the groups. On further analysis, there was no significant difference found between the groups for baseline flux at any of the six areas under study, being the plantar aspect of the 5th toe, 3rd toe, 1st toe, 5th MPJ, 3rd MPJ and the 1st MPJ areas. This was also the finding for the occlusive stage of reactive hyperaemia, for the time taken on release of the occlusion to achieve peak flux, and for 2 minutes post peak flux measurements. One element of the PORH that did display a difference was the peak flux values achieved on release of occlusion. Generally values for the group with Type 2 diabetes were higher than the control group, and the main effect of peak flux produced a significant difference, as did the group and a significant

interaction was found between the peak flux and the groups. On follow up analysis, all areas under study with the exception of the 1st toe displayed a significant difference between the groups. On investigating 5 minutes post peak flux values; although the main effects were not significant, on follow up analysis it was found that the 1st toe and 5th MPJ areas were significantly different between the group with Type 2 diabetes and the control group.

The actual pressure measured across the forefoot displayed no significant difference between the group with Type 2 diabetes and the control group. A significant correlation between the 6 areas under study was found for both groups with regard to pressure, but no association was noted between the plantar pressures and the baseline flux across the forefoot areas.

There was an association for perfusion found between the six forefoot areas under study with the LSCI equipment, and also an association between the values obtained at baseline flux between the LSCI flux measurements and the LDF blood flux values obtained at the plantar aspect of the 3rd MPJ.

Laser Doppler flowmetry was utilised in conjunction with iontophoresis with ACh and SNP to investigate endothelial function when placed under a known pressure. Findings with the analysis of blood flux using ACh as the endothelium-derived vasodilator indicated a significant difference within the whole group with pressure, increase in dose of ACh and a significant interaction effect of pressure with increase in ACh dose. Further analysis indicated a significant difference between the addition of no pressure and 50% added pressure, no pressure and 100% added pressure, but no significant difference between 50% and 100% added pressure. For the group with Type 2 diabetes as with the group as a whole, a significant difference was found for the effect of pressure and increases in dose, but there was no significant interactive effect of pressure

and dose. On further analysis, there was no significant difference with the group with Type 2 diabetes between no pressure and 50% pressure, or between 50% and 100% pressure, but there was a significant difference between no pressure and 100% pressure. The control group displayed no significant difference within subjects for pressure. There was a significant difference for increase in dose, but no significant interaction between the two elements of pressure and dose. There was a significant difference within the group between no pressure and 50% pressure as well as no pressure and 100% pressure, but again no significant difference between 50% and 100% pressure. Thus, the main impact on blood flux of pressure when exerted with ACh and iontophoresis occurred when 50% of normal walking pressure was exerted.

When SNP was utilised as the endothelium-independent vasodilator with iontophoresis a significant difference was found for the group as a whole with pressure and for increase in dose as well as the interaction effect of both dose and pressure. There was also a significant difference found between no pressure and 50% pressure, no pressure and 100% addition but no significant difference was noted between 50% and 100% pressure. For the group with Type 2 diabetes, there was a significant difference found with pressure but not for increase in dose. The interaction effect of pressure and dose was significant and again on further analysis a significant difference occurred between no pressure and 50%, no pressure and 100% pressure but no significant difference was found between 50% and 100% pressure. The control group also displayed a significant difference for pressure, as well as for increase in dose and for the interaction between pressure and dose. Again a significant difference was found on further analysis between no pressure and 50%, no pressure and 100% but not between 50% and 100% added pressure. Increase in pressure with SNP was found to have a greater impact on the subjects with Type 2 diabetes than the control group.

Chapter Five

Discussion

5 DISCUSSION

5.1 INTRODUCTION

The key findings for this study relate to the design and development of equipment, and its utilisation to assess endothelial function by measuring blood flux during the process of iontophoresis on the plantar aspect of the forefoot simultaneously, with the addition of pressure. A pressure delivery system was specifically developed for this study, and repeatability of the system was found to be good during both in vitro and in vivo testing, thus enabling utilisation in conjunction with LDF on the 3rd MPJ during the process of iontophoresis using the developed and tested protocol.

Inter-day repeatability was found to be good for the LSCI equipment when tested on the lower limb for assessment of PORH. Investigation of the 6 plantar areas under study (5th,3rd and 1st toes, 5th, 3rd and 1st MPJs), during PORH found no significant difference in blood flux between the group with Type 2 diabetes and the matched control group for any of the stages of PORH with the exception of the peak flux found on release of the occlusive stage. The group with Type 2 diabetes were found to have a significantly higher peak flux than the control group. A strong correlation was found amongst the 6 plantar areas under study, and this association was stronger in the group with Type 2 diabetes than in the control group. A strong correlation was also found between LDF and LSCI when used to measure baseline blood flux on the plantar aspect of 3rd MPJ.

Actual plantar pressure values across the forefoot were not significantly different between the groups, and no association was found between plantar pressures and baseline flux in the forefoot. However, a significant association was found in both groups for the actual plantar pressure measured amongst the 6 forefoot areas.

Findings indicated that the main impact of pressure delivery on blood flux measurements during the process of iontophoresis with ACh occurred when 50% of simulated, normal walking pressure was exerted, and no significant difference was found between the groups. With the endothelium-independent vasodilator SNP, again the main impact of pressure was found at 50% of simulated normal walking pressure, however there was also a significant difference found between the groups with the delivery of 100% pressure. Thus, when the endothelium was stimulated to dilate with an endothelium-derived vasodilator there was no significant difference found between the subjects with Type 2 diabetes and the control group, whereas when the stimulation was delivered via a none-endothelium derived vasodilator, significant differences were found in the blood flux between the groups perhaps indicative of structural changes within the vessel or by a reduction in NO activity (Turner *et al.* 2008).

5.2 EQUIPMENT DESIGN AND TESTING

5.2.1 The pressure delivery system

The pressure delivery system was specifically developed for this study as no system was available that could deliver pressure to the forefoot whilst investigating endothelial function simultaneously. Some preliminary experimental work had been carried out by several groups to investigate blood flow simultaneously with pressure application to the plantar aspect of the foot, but none of the systems developed enabled endothelial function assessment to be carried out (Abu-Own 1995; Meinders *et al.* 1996; Fromy *et al.* 2000; Cobb & Claremont 2001 and 2002; Santos *et al.* 2003; Hahn *et al.* 2007). For this current study, the pressure delivery system developed had to be robust, and able to produce repeatable results; as well as feasibly working in conjunction with the other

equipment necessary for endothelium function assessment such as an iontophoresis chamber and LDF probe. A stainless steel spring was decided upon to actually deliver the pressure, but much effort was given to how the pressure could be delivered to the same area as blood flux measurements were being undertaken. Eventually the DP2 probe was utilised as the end point for pressure delivery and a housing case designed to firmly hold the probe in position while delivering the known pressure. The security of the probe within the housing was paramount as movement of the probe during use could have led to errors in the pressure actually delivered. During testing of the equipment, and throughout the data collection the probe housing proved highly successful with no movement of the probe observed. Repeatability testing with a universal testing machine over 14 sessions with known pressures as displayed in Table 3.1, and in vivo analysis carried out on the equipment (Table 3.3) found the system to display good repeatability with high ICCs obtained.

However, the system was held in place with a modified post-surgical boot which had an adjustable metal baseplate to ensure that the system could be held in the correct position for all foot sizes. Whilst this modified boot held the equipment securely, there was some possibility for slight flexion of the foot to occur at the ankle joint which could have reduced the actual pressure being delivered. In hindsight perhaps a firmer based boot could have been utilised which would have held the foot in a fully secured position thus preventing any reduction in pressure delivery. However, this could have compromised the comfort for the subjects, and as such the mental stimulation caused by discomfort may have impacted on blood flux, or the subject might have asked for the process to stop.

5.2.2 Measurement of blood flux under pressure in a supine position

Although perhaps not fully representative of the blood flux when the limb is dependent and loaded, as dependency of the limb has been found to lead to a decrease in cutaneous blood flow (Eun 1995; Santos *et al.* 2003), in this current study the decision was made to carry out measurements with the subject in a supine position. This allowed the collection of data to be carried out with minimal movement artefacts and pressure loading of the area to occur simultaneously with the process of iontophoresis and blood flux measurement. The possibility of carrying out simultaneous blood flux measurements during ambulation has proven impossible to date. Laser Doppler flowmetry was considered the most suitable method of assessing blood flow, and a prototype sensor was developed which could be housed within a 'shoe' and sit flush with the insole, thus allowing the individual to walk with the sensor in situ (Cobb & Claremont 2001). The shoe was based upon an orthopaedic insole made of plastazote foam with a cut out at the area of the 1st metatarsal head to house the sensor. A custom made shoe had to be manufactured for each subject, but the main problems were with movement artefacts which were found on movement of the probe in relation to the plantar tissue. There was an attempt made to overcome this by taping the probe to the skin surface as well as restraining it within the recess. However, a second type of artefact occurred with compression and decompression of the tissues when the foot was loading during the stance phase of gait and unloading during the swing phase. Eventually in a pilot study it was only possible to measure flux during the unloaded swing phase of gait, thus not truly measuring the blood flow during loading of the foot (Cobb & Claremont 2002). They also had to heat the foot prior to gathering data, as it was not possible to maintain temperature required for the analysis of blood flow in an open shoe during walking. Meinders *et al.* (1996) also found that signal disturbances relating to the displacement of tissue cells with loading and unloading made it

impossible to observe blood flow during dynamic loading. They also found movement artefacts with subjects in a standing position, due to the small natural movements of the individual which would also cause a disruption to the LDF signal.

5.2.3 Choice of LDF

The requirement of a system which would allow a method of applying local pressure to the area under assessment of blood flux, coupled with the non-invasive methodology of LDF made the system an obvious choice for this study. It is a system which has been extensively validated for superficial blood flux assessment with the use of standardised protocols. It comes with a large variety of probes, and the DP2 probe was found to be durable enough to simultaneously measure flux as well as acting as the final component of the pressure delivery system. One of the issues generally noted about the use of LDF is that it is not possible to register measurements in absolute perfusion values which would be the blood flow in ml/min. relative to the tissue volume (Cracowski *et al.*2006). Most studies using LDF use arbitrary units (AU) or perfusion units (PU) and the term flux which is the product of average speed and concentration of the moving erythrocytes in the tissue sample volume rather than actual blood flow (Moor Instruments Ltd 1997), and this has been utilised in this study.

5.2.4 Standardisation of PORH and iontophoresis protocols

In order to ensure that this study was testing the aims and objectives set out, development work was carried out to obtain robust protocols which can be found in Chapter 3. Previous work has been carried out to ensure standardisation. Bircher *et al.* (1994) published a report with guidelines for cutaneous blood flow by LDF which most

studies adhere to regarding the environment and food, drugs and nicotine intake prior to assessment. Work carried out by Morris & Shore (1996) and Droog *et al.* (2004) suggested that protocols should be devised for iontophoresis which would minimise the nonspecific vasodilatory effects found. A protocol for PORH which suggested the parameters for analysis of baseline resting flux, occlusive flux, peak flux, time taken to reach peak flux and recovery times was devised by Morales *et al.* (2005), and was used in this current study. Cracowski *et al.*(2008) noted that there was no concensus around the parameters selected for analysis with PORH which is a complex microvascular response following a period of occlusion. It has been noted in a review article that while LDF in conjunction with iontophoresis has proven to be an easy to administer, validated and reproducible method of assessing endothelial function, standardisation of protocols would reduce controversy around the area (Turner *et al.* 2008). Thus, a lack of standardisation of protocols generally for both POHR and iontophoresis has posed difficulties in comparisons being made between this study and others in the areas of study.

5.2.5 Spatial variation

For the pressure and blood flux elements of this study the LDF probe had a dual purpose of measuring blood flux and acting as the final component with skin contact of the pressure delivery system. While this reduced any error in loading and measuring localities being in the same position for the individual, the size of the probe was fairly small and did require to be situated in the same place for each participant. The location of the probe and instrumentation was determined by one person (the researcher) on each occasion and location of the 3rd MPJ was carried out and carefully marked for each case; but it does mean that human error could have altered the positioning and site

slightly for some individuals. However, at baseline flux measurement, good correlations were found amongst the 6 different forefoot sites thus indicating that the single point measurements were representative of other areas in the forefoot.

As the probe has a small surface area, it is possible for the addition of high pressures to provoke pain or discomfort for the individual. While during this study, no subjects complained about pain or reported discomfort, pain does stimulate the sympathetic nervous system which also controls AVA blood flow so could have produced error in the flux readings obtained (Flynn & Tooke 1990).

5.2.6 Iontophoresis chamber size

As discussed in Section 3.2.3 the chamber size used for the process of iontophoresis of the vasodilators was small enough to be located in the area of the 3rd MPJ to ensure total contact and to house the LDF probe as well as holding enough solution for iontophoresis. Small chambers can elicit the 'galvanic response' i.e. a greater electrically induced hyperaemic response, which could have led to errors in the flux values measured, so the process was started off with relatively low currents to avoid any possible galvanic effect (Ferrell *et al.* 2002; Turner *et al.* 2008).

5.2.7 Choice of vehicle for iontophoresis

In this study 2% solution of methylcellulose was found to be a suitable vehicle being comparable with deionised water, and was used mainly because it provided a thickened solution which was found to be retained by the iontophoresis chamber in the vertical position on the foot, and was useful for ensuring contact on contoured sites. The impact of the vehicle for iontophoresis has been debated by several authors in relation to also

causing the ‘galvanic response’ (Morris & Shore 1996; Noon *et al.* 1998; Ferrell *et al.* 2002; Droog *et al.* 2004; Turner *et al.* 2008). Several vehicles have been investigated with commonly deionised water, saline and methylcellulose being advocated. Deionised water was found to be a better vehicle for iontophoresis than NaCl (Khan *et al.* 2004) however, 2% methylcellulose, an inert gel, was found not to induce vasodilation so was a suitable vehicle for iontophoresis (Noon *et al.* 1998). Ferrell *et al.* (2002) could not confirm these findings when using 2% methylcellulose as the hyperaemic response still occurred, so the choice of vehicle in this study could be a source of error. Thickening the solution could also have led to the development of air pockets and bubbling which would prevent the uptake of the solution by the chamber which would also have an impact on the delivery of the vasodilator.

5.2.8 Vehicle impact in this study

During the iontophoresis protocol development work carried out prior to commencing this study, comparisons were made between deionised water and 2% methylcellulose as the vehicle, and methylcellulose was found to be a suitable vehicle in comparison to deionised water as can be found in Section 3.2.5. However this work was carried out with the vasodilators included in the solutions and in hindsight it could have been useful to also have investigated the impact of the vehicles alone by carry out iontophoresis with just the vehicles. Morris & Shore (1996) found that when utilising 3% mannitol in water as the vehicle for ACh and deionised water as the vehicle for SNP alone, without the addition of the vasodilators, a response was elicited which was thought to be caused by local nerve stimulation. This finding was established as when causing a local block with EMLA cream the vehicle induced vasodilation was inhibited. The impact was thought to be greater in response to the cathode current, and could be responsible for

inducing vasodilation with endothelium-independent SNP. Droog *et al.* (2004) noted that the way to eliminate nonspecific vasodilation during iontophoresis was to subtract the response to the vehicle alone from the overall response to the vasodilator in the vehicle. This did not change the outcome of their results as such, and there is some debate around the response actually being purely additive, so in this study the response to the vehicle was not subtracted. Again, like Morris & Shore (1996), Droog *et al.* (2004) found that stimulation of the local sensory nerves could be responsible for the impact of the deionised water vehicles, and used EMLA cream as a local topical blocking agent. They did note that other mechanisms could also be involved, but did not specify.

5.2.9 Utilisation of LSCI

PORH is a non-invasive, but sensitive indicator for microvascular function, which requires a reliable method of detecting and recording parameters of the reactive hyperaemia. Parameters of PORH have been noted as possibly providing early detection of vascular changes before clinical manifestations are detectable in diabetes mellitus, thus it is a useful investigative test for possible early microvascular manifestations (Humeau *et al.* 2002; Morales *et al.* 2005; Rossi *et al.* 2005). LSCI is a fairly recent development which can capture the perfusion from larger areas of tissue than LDF in near real time. On use in the upper limb it has proven to compare favourably with methods such as LDF for PORH assessment (Roustit *et al.* 2010; Tew *et al.* 2011), however it had not been used to measure perfusion in conjunction with PORH on the plantar aspect of the foot. In this study a strong correlation was found between LDF and LSCI when used to measure baseline flux on the plantar aspect of the 3rd MPJ, thus validating its utilisation on the lower limb for PORH assessment.

It proved to be easy to use with good inter-day repeatability (ICC = 0.982). On occasion following deflation of the leg cuff providing the occlusion, the subject slightly moved which negated the available data.

5.3 POST OCCLUSIVE REACTIVE HYPERAEMIA ASSESSMENT

In this study there was no significant difference found in any areas of the forefoot with PORH using LSCI for resting or baseline flux, occlusive flux values, time to reach peak flux following release of occlusion, or flux values when measured two minutes post peak flux. There was a significant difference found between the group with Type 2 diabetes and the healthy control group for peak flux, the group with Type 2 diabetes displaying higher peak flux values than the control group. Although generally in this current study flux values at 5 minutes post peak were not significantly different across all areas of the forefoot under study, both the hallux and 5^{th} MPJ areas did display significant differences between the group with Type 2 diabetes and the controls in that the subjects with Type 2 diabetes displayed higher flux values, which may indicate a trend towards an extension of the recovery phase of the hyperaemia. As can be seen from the variety of findings noted below, a definitive pattern utilising PORH has not been established and generally conflicting findings have been reported. As yet no studies have been conducted utilising PORH on the lower limb with LSCI, thus a direct comparison with this current study is difficult. However, LSCI has generally been shown to display good comparative results with LDF when utilised in the forearm (Roustit *et al.* 2010; Tew *et al.* 2011), and in this current study was found to have a strong correlation with LDF baseline flux values in the forefoot, and as such some comparisons can be drawn between the utilisation of both systems in the lower limb.

The finding of a significant increase in peak flux values with Type 2 diabetes is in agreement with Schlager *et al.* (2012) who found peak perfusion to be higher in a group of 58 children with Type 1 diabetes in comparison with age and sex matched healthy controls when measuring PORH with LDF in the upper limb. They also found a significant difference between the group with Type 1 diabetes and the controls for the occlusive stage, but no significant differences were found for baseline perfusion, time to peak or recovery time. Yamamoto-Suganuma & Aso (2009) investigated the relationship between post occlusive forearm skin reactive hyperaemia and vascular disease in Type 2 diabetes when used in conjunction with LDF, postulating that this method could be a sensitive indicator of microvascular dysfunction. They found no significant difference in peak flux values between a group with Type 2 diabetes and a healthy control group, however while the group of subjects with Type 2 diabetes was made up of 104 subjects, the control group was a much smaller sample of 20 volunteers so limiting the power of the study. They also found no significant difference at 3 minutes and 5 minutes post peak flux, but developed an index for the late phase prolonged total hyperaemia which was significantly longer in the group with Type 2 diabetes. Pazos-Moura *et al.* (2008) noted a significantly lower peak flux in a group of 16 subjects with Type 2 diabetes when compared with 15 healthy control subjects and also found the time to reach peak flux significantly longer with the group with diabetes. Rossi *et al.* (2005) utilised PORH with LDF for the assessment of patients with peripheral arterial obstructive disease (PAOD). They found no significant difference between the group and healthy controls at baseline flux, although the group with PAOD did display higher flux values, peak flux displayed no significant difference between the groups, however time to reach peak flux and recovery were significantly different being longer in the group with PAOD. There are several reasons why conflicting results have been reported, including subject numbers recruited and sample sizes, different

equipment utilisation, but also a lack of standardisation of protocols used. A protocol for standardisation of PORH was developed by Morales *et al.* (2005) which included subject preparation i.e. subject positioned in a supine position and a 30 minutes acclimatisation period in a room of 25 ± 1^0 C, a recording session of 15minutes for referencing flux, 3 minutes occlusion and post occlusion recording for a further 15 minutes with the LDF probe positioning on the dorsum of the foot between the 2nd and 3rd MPJs. They investigated its use with 24 patients with PAOD and 30 healthy subjects in a 3 centred study. They found resting flux, as well as peak flux, were not significantly different between the groups, however the time elements such as time to reach resting flux, peak flux and recovery time were generally significantly longer for the PAOD group than in the healthy control subjects.

5.3.1 Phases of PORH reaction-implications for this current study

Although none of the subjects in this study presented with known neuropathy, it may be possible that early, clinically undetectable neurological alterations were developing, and as such could have impacted on the significantly higher peak flux detected in the group with Type 2 diabetes. The neuropathic changes in the lower limb are insidious in onset and can be present for some time prior to clinical manifestations, or symptoms occurring. Small muscle atrophy leading to altered toe position was found in subjects with Type 2 diabetes before any standard techniques noted the presence of clinical peripheral neuropathy (Greenman *et al.* 2005).

Although PORH is a well-recognised method of assessing vascular reactivity following a period of occlusion, there is no real consensus regarding how the phases of the reaction occur other than a link with myogenic, neural and metabolic factors in addition

to endothelial function (Humeau *et al.* 2002; Koller & Bagi 2002; Cracowski *et al.* 2006; Rossi *et al.* 2007; Yamamato-Suganuma & Aso 2009; Schlager *et al.* 2012). The debate concerning when and if NO activity is involved in the reaction is very pertinent, particularly for the study of those with diabetes. It would appear that the majority of studies have found that NO does not play a particular role in the early peak flow, but does have an involvement in the later extended hyperaemic phase (Larkin & Williams 1993; Paniagua *et al.* 2001; Zhao *et al.* 2004; Cracowski *et al.* 2006; Joannides *et al.* 2006; Yamamoto-Suganuma & Aso 2009; Schlager *et al.* 2012). Thus, the high peak perfusion observed in the group with Type 2 diabetes in comparison to the healthy control group in this study may not have been related to NO activity. However, whilst NO is one of the major endothelium-derived dilators, there are other factors which may be altered or deficient with diabetes, and thought to play a role in the early peak flux such as prostaglandin as noted by Larkin & Williams (1993) and Paniagua *e al.*(2001). Vasoconstrictor prostaglandin H_2 acts on smooth muscle and causes vasoconstriction to occur, but is normally released in small amounts and masked by prostacyclin production, NO and EDHF (Mombouli & Vanhoutte 1999). It is possible that the significant difference in peak flux found in this study could be related to reduction in prostaglandin production in the group with Type 2 diabetes i.e. it may be that the increased vasodilation found in this study was related to the impact of diabetes on vasoconstriction ability of the arterioles rather than on the release of dilatory mediators. Oxidative stress has also been implicated in endothelial dysfunction related to diabetes (De Vriese *et al.* 2000) and an increase in oxidative stress as well as raised serum levels of proinflammatory cytokines was thought to affect micro and macrovascular reactivity (Schlager *et al.*2012). It was thought that it may also be linked with regulation of the tone of arteriolar smooth muscle cells ‘upstream’ being compromised thus impairing the regulatory ability of the precapillary arterioles, rather than being related to the release of

vasodilatory mediators. Again both oxidative stress and regulation ability of precapillary arterioles could be implicated in the higher peak flux found in the group with Type 2 diabetes in this present study.

Mayer *et al.* (2003) found that arteriolar vasomotion could be an early index for sympathetic neuropathy in Type 2 diabetes. Impaired cutaneous blood flow in the microcirculation as well as an alteration in sweating, were thought to be amongst the first manifestations of peripheral sympathetic neuropathy and could often precede the detection of other manifestations (Sun *et al.* 2012). Pfutzner *et al.* (2001) also noted an association between small nerve fibre dysfunction and microvascular control in subjects presenting with diabetes.

Given the complexity of the reactive hyperaemic response, and the many conflicting theories concerning how the elements of the reaction are brought about, it would seem quite possible that multiple mechanisms could be responsible for the differences found between the groups in this current study. This possibility is indicative that further work is required in this area.

5.4 COMPARISON OF LDF AND LSCI FOR MEASURING PERFUSION

The results from this study showed a good correlation between LDF and LSCI when used to compare baseline blood flux on the plantar aspect of the 3rd MPJ area of the forefoot, which corroborated the utilisation of LSCI for PORH in the lower limb. Further to this, LSCI offered a much more extensive perfusion map of the forefoot than was possible with the single probe LDF system, and in fact a strong association was found between the 6 forefoot areas under study. Laser Doppler flowmetry has been extensively used to assess skin microvascular function for many years (Briers 2001;

Roustit *et al.* 2010), whereas commercial systems available for laser speckle contrast imaging are relatively new and so far LSCI in conjunction with PORH has not been used on the lower limb, although a small number of studies have utilised the system for assessing forearm perfusion successfully (Roustit *et al.* 2010; Mahe *et al.* 2012). Thus, it was important in this current study to assess the utilisation of the relatively new methodology of LSCI and to compare the system with the well-established LDF.

Both LDF and LSCI are based upon the same fundamental principle, but with two ways of approaching the same phenomenon (Briers 2001). With LDF the light scattered from moving objects has its frequency shifted by Doppler. The light waves are combined with an un-shifted frequency as the frequencies of light waves are high, and a beat frequency is then produced from the difference between the two waves. Laser speckle on the other hand is a random interference effect between waves of the same frequency. A charge couple camera records fluctuations in the speckle pattern which is dependent upon the movement of the image. The level of blurring in the area of interest is then quantified by the speckle contrast. When the objects causing scatter are static the speckle pattern intensity is constant, with movement the pattern fluctuates with related path lengths from the different scatters (Tew *et al.* 2011).

LDF provides a measurement of blood flux which is a product of average speed and concentration of the moving red blood cells within the sample area (Cracowski *et al.* 2006). This measurement of velocity and concentration are linearly related which is preferable for physiological research. However, speckle contrast imaging has focussed on information regarding the velocity of the scattered particles, and this relationship between speckle contrast and velocity is non-linear, although more recent models have claimed to measure actual perfusion. Tew *et al.* (2011) in a study utilising PORH and LSCI on the forearm found that the cutaneous perfusion measured on the forearm was

non-linear and that LSCI was more sensitive to changes in the velocity of the red blood cells than to red cell concentration. LSCI has recently been of interest as it offers a greater area of coverage ie wider area of interest with good resolution than is possible with single point LDF. Roustit *et al.* (2010) compared the inter-day reproducibility of PORH when using single point LDF in comparison to LSCI. They noted that while LDF was acceptable in some locations, because of the spatial variability between sites which was felt to be caused by the small size of the area of assessment, then on areas such as the forearm some problems with reproducibility had been noted. Two similar studies were carried out and both Roustit *et al.* (2010) and Tew *et al.* (2011) found LSCI compared favourably with LDF when used in conjunction with PORH for inter-day reproducibility. Roustit *et al.* (2010) noted that the full-field techniques of LSCI probably produced a better inter-day reproducibility due to lowering the inter-site variability. They also tried to standardise the temperature of the area prior to collecting baseline data which provided only moderate improvement in reproducibility. Briers (2006) noted that laser speckle imaging was effective in real-time when the Doppler scanning with LDF actually took several minutes. The usefulness of LSCI was assessed on pressure induced ischaemic wounds in rats. It was found to more accurately assess the blood flow in that it could differentiate between the high and low loadings whereas the group found thermography could not, thus it could become a useful method of assessing skin blood flow (Nakagami *et al.* 2010). Further to this, as LSCI was known to be based upon the velocity of red blood cells, it was thought that it could have even further benefits in wound assessment as it could provide details about perfusion during the stage of infectious inflammation, providing monitoring of the possible increase in blood flow during this stage in wounds. Some caution must be added to this statement however, as it was not actually tested in the study.

Rousseau *et al.* (2011) investigated the impact of increasing the 'region of interest ROI' and 'time of interest TOI' with LSCI. As part of their study they utilised two skin areas situated 1cm apart. Both intra and inter-subject variability was recorded for the two sites and the intra-subject variability between sites a and b was also evaluated. Coefficients of variation were <35% which was found to be acceptable. Their findings indicated that moderate improvement was found when the TOI increased, but that increasing TOI did not have an impact on results. Their finding that there was an acceptable coefficient of variation between the two sites is in agreement with this current study, where there was a strong association found between the 6 areas under study when assessed across the whole group and also across the group with Type 2 diabetes and the control group.

Also in agreement with work in this thesis, Roustit *et al.* (2010) found a significant correlation between LSCI and LDF data, although in the current study this was carried out on the foot rather than the forearm. In this study, the location for both LSCI and LDF was the plantar aspect of the forefoot under the 3rd MPJ, which is a specific area to palpate and as such reduced the special variability in this study. Royl et al. (2006) also carried out a comparison study with LDF and LSCI when assessing cerebral blood flow in animal studies. They also found LSCI to be a reliable method which compared well with the utilisation of LDF.

Commercial LSCI systems are relatively new and as such there is little evidence available for their use, particularly for the lower limbs. Available evidence in the main concentrates on the upper limbs, and has provided valuable and supportive evidence which generally has found the equipment to compare well with the established LDF (Roustit *et al.* 2010, Tew *et al.* 2011). Further work and much more evidence is required with LSCI to establish the possibilities for accurate utilisation of the equipment.

5.5 THE IMPACT OF PRESSURE

The current study found no significant difference between plantar pressure measured across the two groups, but with a greater range of pressure values found in the group with Type 2 diabetes in comparison with the control subjects. The group of subjects with Type 2 diabetes in this study did not display clinical signs of peripheral neuropathy. There is some debate in the literature regarding the levels of plantar pressure which has been reported in a group not displaying clinical signs of neuropathy, but the association generally found would suggest that unless there is some evidence of neuropathy, then as found in this study, the plantar pressures do not appear to be significantly raised in comparison to matched control subjects.

Acharya *et al.* (2010) measured plantar pressure in subjects with Type 2 diabetes with and without assessed neuropathy, using the F scan in-shoe analysis system. This was an attempt to find a method of early identification of lower limb neuropathy. The group concluded that foot pressure distribution was an indicator for the health of the diabetic subject in relation to lower limb neuropathic involvement. Their study involved 34 patients with Type 2 diabetes, 19 of which displayed indications of neuropathy and in addition 38 healthy control subjects. Findings from their study suggested that plantar pressures were significantly higher in the group with known neuropathy in comparison to the group without neuropathy, and suggested that this increase in plantar pressure could be utilised as an identification marker for the presence of early neuropathy. Tong *et al.* (2011) investigated in-shoe plantar pressures in a group of 35 non-neuropathic subjects with Type 2 diabetes and a control group of 38 control subjects without diabetes. They established that either sensory or motor neuropathic components were required to be present to impact upon plantar pressure, and in agreement with this current study, they also found no significant differences were noted in peak pressures

between the groups, although generally they did find higher pressures were recorded in the group with Type 2 diabetes. In agreement, Malloy *et al.* (1999) noted that limited joint mobility and motor neuropathy played an important pathogenic role in ulcer formation in diabetes by increasing forefoot plantar pressures. They studied a small cohort of 5 males and 5 females with no history of lower limb pathologies and restricted their ankle dorsiflexion. Neutral positioning of the foot without dorsiflexion was found to have no significant effect on plantar pressures, however limitation to 5 and 10 degrees of plantarflexion was found to significantly alter the size, distribution and time frame of plantar pressures from the heel to the forefoot. While the results of their study were extrapolated to imply impact on the foot in diabetes with regard to limited joint mobility, they found significantly higher pressures with limited plantarflexion as well as distribution in a group of only 10 subjects, with none of the subjects actually having diabetes.

In contrast, Bacarin *et al.* (2009) investigated plantar pressure distribution patterns during gait in patients with diabetes and neuropathy with an actual history of foot ulceration. Although they acknowledged that a strong association had been reported in the literature between diabetic neuropathy and high pressures, there was a lack of evidence linking pressure increase and the pathogenesis of ulceration. They studied 10 subjects with diabetic neuropathy and a history of a least one healed ulceration within the last year, 17 subjects with known diabetic neuropathy but with no history of ulceration and 20 control subjects. They found peak pressure was significantly different among all of the groups at midfoot, with the two groups with neuropathy having statistically higher values than the control group, but the group with a history of ulceration presenting with the highest peak pressure values. They also noted a lateral shift of pressure in the neuropathic subjects. Foot pressures were measured in a group of 200 subjects, with 160 patients with diabetes of which 50 did not present with

neuropathy, 90 with neuropathy and 20 with neuropathy and a history of ulceration and 40 control subjects (Plank *et al.* 2000). Their findings indicated that peak plantar pressures were progressively higher in patients with diabetes and in those with neuropathy in comparison to subjects presenting without neuropathy, with the highest peak pressures recorded in the group with a history of ulceration. Although they included a control group in the study, little mention was given to the group in the results section, only an indication that plantar pressures were progressively higher in each of the four groups. It could be assumed from this that the control group displayed significantly lower peak pressures than the diabetic subjects. Whereas Bacarin *et al.* (2009) reported a lateral shift in pressures with neuropathy, Plank *et al.* (2000) found changes in pressures had a medial load shift, which was displayed in the neuropathic groups when retested some 12 months later. As early as 1987, Boulton *et al.*, investigated foot pressures in a group of 44 subjects with diabetes, 20 Type 1 and 24 Type 2 diabetes in a state of 'early' diabetic neuropathy which they defined as subjects having no clinical evidence of neuropathy with no history of neuropathic symptoms, normal clinical sensory examination, no obvious muscle wastage and normal ankle reflexes with no signs of peripheral vascular disease. Of the group 16 displayed abnormally high plantar pressures under the metatarsal heads whereas the other 28 subjects had normal results, thus a mixed picture was presented with the majority of subjects displaying a normal plantar pressure pattern.

Generally the findings suggest that there is a link with neuropathy and plantar pressures, but it should also be noted that other elements will increase plantar pressure such as foot deformity (Bus *et al.* 2005), joint mobility (Fernando *et al.* 1991) and structural changes in keratin in the skin, muscle, cartilage, tendons and ligaments (Abouaesha *et al.*2001; Kogler & Shorten 2001).

5.5.1 Plantar pressure and baseline blood flux values

Additionally, no correlation was found in the present study between the natural walking plantar pressure values across 6 areas of the forefoot, and the baseline blood flux values. It would appear that plantar pressures per se did not influence the baseline resting perfusion, or that areas which experienced higher pressure during walking did not register lower or altered perfusion when unloaded. This is in agreement with an earlier study carried out by Newton *et al.* (2005) where their group of 16 subjects with diabetes, of which 2 had a history of ischaemic heart disease, 2 had a previous myocardial infarction, 2 had peripheral neuropathy and 3 had a history of foot ulceration, did display higher plantar pressures than a control group of 8 healthy matched subjects, but baseline skin blood flow using LDF was not significantly different between the 2 groups, thus pressure did not influence baseline blood flux values when the area was unloaded.

5.5.2 Effect of diabetes on flux values under pressure and vasodilation with both ACh and SNP

One of the main differences found between the two groups in this study was that the group with Type 2 diabetes displayed higher flux values throughout the delivery of pressure, at resting flux, 50% pressure and 100% pressure delivery with both vasodilators. The area under study was the plantar aspect of the 3rd MPJ, which was found to be one of the high pressure areas under the foot, and has commonly been the site for ulceration (Veves *et al.* 1992; Proano *et al.* 1992; Bowering 2001). Areas which displayed chronically raised plantar pressures i.e. over the 1st, 2nd and 3rd metatarsal heads were found to display an increase in blood flux when compared with sites of lower pressure on the same foot in the subjects with diabetes, whereas in the group of control subjects flow was found to be slightly decreased at the high pressure sites

(Newton *et al.* 2005). When investigating the differences between foot and forearm microcirculation with ACh and SNP delivered via iontophoresis, Arora *et al.* (1998) found that the baseline blood flow measurements were consistently higher in the non-neuropathic group with diabetes in comparison to a neuropathic group and a control group. They were also found to be significantly higher in the non-neuropathic group at foot level (measured on the dorsum), and it was noted that this was an 'unexpected finding' and that further work would be needed to examine how this increase in blood flow could be explained in the absence of long term complications. Blood flux values on the plantar aspect of the hallux were also found to be substantially higher in the group with diabetes than for the control subjects (Cobb & Claremont 2002). Belcaro *et al.* (1989) when investigating skin blood flow in the distal foot found subjects with diabetes to have a significantly higher resting blood flow than control subjects. On standing, a higher skin blood flow was found in the group with diabetes which was not significantly different from resting blood flux indicative of an ineffective venoarteriolar response displaying an inability of the microcirculation to adapt to dynamic changes. However it should be noted that all 30 subjects with diabetes had peripheral neuropathy.

The PIV response was found to be missing in the foot of subjects with diabetes (Koitka *et al.* 2004b), and Mayrovitz *et al.*(1997) when investigating heel blood perfusion responses to pressure loading and unloading found during loading a slight progressive increase in flux, but the trend was not significantly higher than when the heel was unloaded.

Peripheral neuropathy present with diabetes was found to involve sympathetic fibres which could undergo damage relatively early in the disease process, thus damage could be sustained prior to clinical evidence of neuropathy (Tooke & Brash 1995). They found that the impact on foot microvascular haemodynamics included increased

arteriovenous shunt flow as well as increased nutritive capillary flow at rest. This could produce an increase in blood flux in a group with Type 2 diabetes in comparison to a control group as found in this current study. Perfusion pressure was noted to rise due to an increase in blood flow, and was thought to be possibly due to a reduction in pre-/post capillary resistance (Flynn & Tooke 1992). Tissue hypoxia which was related to increased demand or to altered oxygen transport was thought also to be a factor in the dilation of vessels. Capillary pressure as an indicator of capillary hypertension measured in nail-fold capillaries was found to be higher in a group of subjects with Type 1 diabetes in comparison to a control group (Sandeman *et al.* 1992). They hypothesised that nail-fold capillary hypertension could develop early in the course of diabetes again before microvascular disease became apparent and was found to be elevated less than one year after diagnosis, which could represent an early functional change. Clinical microvascular complications were found to differ between Type 1 and 2 diabetes, and it was felt that care should be taken when extrapolating finds across the two groups (Shore *et al.* 1994) however, they did note that capillary pressure may have been raised in Type 2 diabetes and could be instrumental at an early stage of the disease process.

The increased blood flux found in areas of high pressure was thought to be a physiological response to repeated tissue trauma from the pressure. With diabetes, this repeated trauma could lead to the development of a local inflammatory response which would produce local oedema and possible hypoxia resulting in tissue breakdown (Newton et al. 2005). Veves *et al.* (1998) noted that there appeared to be an interaction between peripheral neuropathy and endothelial dysfunction which resulted in an inability to increase blood flow in the diabetic foot in conditions of stress leading to the development of ulceration. As the blood flux was already raised in the locality, the

capacity of the microcirculation to respond to any further trauma could be limited as also suggested by Mayrovitz *et al.* (1999).

Fromy *et al.* (2002) reported the opposite effect in that their findings have been indicative of a reduction in blood flow with diabetes, even without neuropathy being present to locally applied pressure on the internal ankle bone. They also found that the skin blood flow decreased significantly from baseline at a much lower pressure with diabetes. However, the internal ankle bone is not an area which should during normal foot function be subjected to pressure so would not have been expected to display higher pressure readings with diabetes.

5.6 THE FLUX RESPONSE TO THE ENDOTHELIUM-DERIVED VASODILATOR ACH WHEN PRESSURE IS APPLIED

The group was initially taken as a whole to investigate possible overall trends, and the main finding for this aspect of the study was that 50% of the normal walking pressure produced a significantly reduced blood flux measurement under the 3rd MPJ when using ACh as the vasodilating agent. When the pressure was increased from 50% to 100%, blood flux reduced slightly, but no significant difference was found between the addition of 50% and 100% normal walking pressure. Thus, the main damaging impact of pressure on blood flux when utilising an endothelium-derived vasodilator on the plantar aspect of the 3rd MPJ was found on application of only half of the measured normal pressure exerted. This finding was generally mirrored when taking the group with Type 2 diabetes and the control subjects as two individual groups thus, the impact of pressure on vasodilation with ACh delivered by iontophoresis in the group with Type 2 diabetes was not significantly different from the control group. However, for the group taken as a whole and the group with Type 2 diabetes, the addition of pressure significantly reduced baseline blood flux, whereas with the control subjects there was

no significant impact from pressure delivery found. In fact with baseline flux measurements under pressure the control group displayed similar flux values with 0 pressure and 50% pressure (56.88 ± 68.48 AU and 55.11 ± 77.83 AU). These findings would indicate that the control subjects could maintain their baseline flux values when placed under 50% of normal walking pressure, whereas those with Type 2 diabetes were unable to do so.

Work has been carried out in the past on pressure and blood flow (Abu-Own 1995; Meinders *et al.* 1996; Fromy *et al.* 2000; Cobb & Claremont 2001, 2002; Santos *et al.* 2003; Hahn *et al.* 2007) and on the effects of endothelium-derived and non-endothelium-derived vasodilators on blood flux (Newton *et al.* 2001, 2005; Ferrell *et al.* 2002; Khan & Newton 2003; Turner *et al.* 2008, Henricson *et al.* 2009), but no studies have been found which have investigated the two elements concurrently so no direct comparison can be made with these findings.

5.6.1 Mechanisms for ACh-mediated vasodilation

Developing an understanding of how endothelium-derived vasodilation is derived is important for many reasons, not least being the inflammatory elements required for wound healing and the ability of the body to deal with local infection which can in turn lead to ulceration and gangrene. However there is much debate concerning the mechanisms involved (Morris & Shore 1996; Berghoff *et al.* 2002; Durand *et al.* 2004). The vascular response elicited from the administration of ACh may be dependent on several mechanisms the initial one being the release of NO, prostacyclin and endothelium-derived hyperpolarising factor, and the constrictor prostanoids (Morris & Shore 1996; Aso *e al.* 1997; Khan *et al.* 1997; Noon *et al.* 1998; Berghoff *et al.* 2002;

Durand *et al.* 2004; Newton *et al.* 2013). As well as the endothelium-dependent effects ACh has in the past been found to stimulate local sensory nerves leading to vasodilation near to the site of administration and inhibit the release of noradrenaline from nerves with high charge density (Walmsley &Wiles 1990; Morris &Shore 1996). This can be overcome by utilisation of lower currents and/or larger chambers for iontophoresis (Ferrell *et al.* 2002; Turner *et al.* 2008).

5.6.2 Evidence of the impact of pressure on vasodilation with ACh

In a sample of 16 patients, 5 with Type 1 diabetes and 11 with Type 2 diabetes and 8 healthy matched control subjects, Newton *et al.* (2005) investigated the impact of pressure on blood flow to the plantar aspect of the foot. For both groups, the areas registering the highest pressures were 1st, 2nd and 3rd MPJ areas which are common sites for plantar ulceration in diabetes. They found that the subjects with diabetes had significantly higher pressures than the control group in the location of highest pressure (without the simultaneous application of pressure). This finding would be expected in a mixed group of subjects with both Type 1 and Type 2 diabetes, as several of the group presented with known complications such as peripheral neuropathy and ischaemic heart disease. There was a trend towards a reduction in ACh induced vasodilation at the sites which would generally be under high pressure in comparison with low pressure sites, this reached significance only in the sub group of those with diabetes who were recorded as ‘high risk’ due to the pressures noted during walking being higher than 6kg/cm^2, however the decision was made to avoid heavily callused areas. This could have impacted on the results in that plantar callosities are found in areas of high dynamic pressures, thus would be locate over the areas of high pressure (Young *et*

*al.*1992) and could have been reduced prior to iontophoresis to allow penetration of the solution through the epidermis.

Using their developed system, Abu-Own *et al.*(1995) studied the blood flow in 30 subjects, 10 elderly patients at risk of developing decubitus ulcers with a Norton scale score of 14 or less, and 10 sex and age matched healthy volunteers plus another group of 10 younger healthy volunteers. Resting flux in the 'at risk' group was significantly lower than both the age matched and younger control groups and no significant difference was found between the younger and older control groups. This was an interesting finding in that the 'at risk' group would have been expected to display lower values of resting flux, but it would appear that in this study, age did not impact on the flux measured in the other two groups. However, the addition of pressure caused similar results in all 3 groups; very low levels of compression caused no significant change in LDF values and higher levels of compression lead to a progressive decrease in flux with compression of 50mmHg or greater reducing the signal to a minimal value in all of the groups. Again this finding was interesting in that the 'at risk' group might have had a different response to the healthy volunteers to the addition of pressure, although the authors claimed that this was not surprising due to the higher pressures being delivered over a small area of the heel.

Meinders *et al.* (1996) developed a system to investigate the effect of pressure delivery on the microcirculation of the sole of the foot in 11 healthy subjects. They noted that the area across the metatarsal heads and the heel was an area of high pressure which experienced compression of the skin and underlying tissues during walking, and the pressure would have an impact on the two parallel functioning microcirculation systems i.e. the surface capillary loops for nutrition as well as the deeper arteriovenous anastomoses for body temperature regulation. Although they did not include subjects

with diabetes in this study (using 11 healthy volunteers), they did note that a disturbed interaction between externally applied pressure and the microcirculation of the skin appeared to be important in diabetes, and that an understanding of this interaction even in healthy subjects was required to contribute to the understanding of the pathophysiology of diabetic foot problems. Each pressure load was applied for a period of 5 minutes with post pressure flux measured for a follow up period of 5 minutes. The 11 subjects were divided into two groups; one group of 5 subjects underwent application of relatively low pressures, and the second group of 9 relatively high pressures (3 subjects participated in both). The numbers and grouping are somewhat puzzling, for example, if it was possible for 3 subjects to take part in both protocols why not all of the subjects. During loading there was an exponential decrease found in blood flux until pressure reached 40kPa. At levels higher than this there was minimal further effect found and release of the pressure resulted in a reactive hyperaemic response.

In 2000, Fromy *et al.* developed a progressive calibrated pressure device which was used in a further study in 2002 where the group investigated skin blood flow in response to pressure in subjects with diabetes. They included 4 groups of subjects in the study, subjects with diabetes (no specification re Type of diabetes was included) and with clinical neuropathy, with subclinical neuropathy, diabetes without neuropathy and a control group without diabetes. The grouping of subclinical neuropathy was for those subjects who presented with a neuropathy symptom score and neuropathy disability score of >0 but <5 (where severe neuropathy would score between 17 and 28). The area under study was the middle of the internal ankle bone and an increase in pressure of 5.0mmHg/min rate. The results were that the skin blood flow in the locality of the ankle bone decreased significantly from baseline with much lower pressure application in all 3 of the groups with diabetes, regardless of their level of neuropathy, and even the group

of subjects with diabetes and no neuropathy displayed that a significantly lower pressure application caused reduction in skin blood flow than in the control group.

When Cobb & Claremont (2001) developed a prototype of an in-shoe laser Doppler probe for assessing plantar blood flow in diabetes, their system was developed to actually measure the flux on the plantar surface during walking, and in 2002 they carried out a pilot study utilising the equipment with 9 subjects, 3 subjects with Type 2 diabetes and known vascular complications, 3 with Type 2 diabetes and known neurological complications and 3 controls. Results were limited in that although both static and dynamic loading was assessed, during dynamic loading i.e. walking, only the swing phase of gait could be assessed rather than the stance phase due to movement artefacts. This was the first attempt to measure blood flow during loading in the dynamic state of walking rather than by simulation, however did prove to present difficulties. Subject numbers were so low in this pilot that no statistical analysis was possible; however the inference was that those subjects with Type 2 diabetes did have a reduced vascular response in comparison with the control subjects.

5.6.3 Pressure application and vasodilation with ACh delivered via iontophoresis summarised

In this current study, the impact of pressure delivered at 50% of normal walking pressure was enough to significantly reduce the vasodilator effect of ACh delivered with iontophoresis in both groups, and further addition to full walking pressure did not significantly reduce the blood flux from 50% pressure, in agreement with the findings from Meinders *et al.* (1996). There was no significant difference found between the subjects with Type 2 diabetes and the control group with the exception that at baseline flux, 50% pressure addition did not significantly reduce the flux measured in the control

subjects, but did for the group with Type 2 diabetes. This could indicate that in agreement with Veves *et al.* (1998), Hamdy *et al.* (2001) and Brooks *et al.* (2008) that endothelium-derived vasodilation using ACh delivered via iontophoresis is not significantly altered in comparison with control subjects in a group with Type 2 diabetes, without peripheral neuropathy.

5.7 *EFFECT OF PRESSURE ON FLUX USING THE ENDOTHELIUM-INDEPENDENT VASODILATOR SNP AND IONTOPHORESIS*

The addition of both 50% and 100% of normal walking pressure had an immediate and significant impact on blood flux with SNP delivered using iontophoresis to the group as a whole as indicated clearly in the percentage change from baseline flux in Figure 4.59. Both 50% and 100% pressure addition markedly reduced resting flux and the rise to maximum flux was reduced greatly with both pressures. However, when investigating the response of the groups, both displayed different results with pressure additions when assessed separately. For the group with Type 2 diabetes, 50% pressure addition actually resulted in a final flux value following iontophoresis with SNP of a lower mean value than the resting flux, and with 100% pressure addition the flux values were markedly lower than with 50% pressure with the final flux value only marginally higher than the resting flux (Figure 4.60). Although the pressure application did impact upon the control subjects resulting in a significant reduction in flux with both 50 and 100% pressure, the group did deal better with the pressure in that an increase in flux was recorded, albeit significantly reduced in comparison to the value achieved without pressure (Figure 4.62). Thus, both groups reacted differently to pressure addition under the vasodilator SNP. From this finding it is possible to state that even without known complications being present, diabetes mellitus has an impact on the ability of the superficial blood vessels to react to the NO donor SNP when the vessels are placed

under pressure of up to 100% of normal walking pressure on the plantar aspect of the foot, which could influence the development of plantar complications such as ulceration.

Sodium nitroprusside is an inorganic, potent peripheral vasodilator (Kaisserlian *et al.* 2005). It reacts with tissue sulfhydryl groups to produce NO, therefore is considered an NO donor. The NO produced stimulates the smooth muscle cells causing relaxation, as SNP works on arterial and venous smooth muscle cells it is used frequently as an endothelium-independent vasodilator. A reduction in response to SNP could be interpreted as structural change within the vessel leading to the reduction in vasodilation with Type 2 diabetes, or by a reduction in activity of NO caused by e.g. a reduction in the release of NO due to oxidative stress (Turner *et al.* 2008). Brooks *et al.* (2008) noted that SNP bypasses the endothelium to directly relax the smooth muscle, thus measuring endothelium-independent vasodilation.

5.7.1 Evidence of the effect of diabetes on SNP delivery with iontophoresis i.e. non-endothelium-derived dysfunction

Although no study has investigated the simultaneous impact of pressure delivery on blood flux, there appears to be a number of studies in agreement with this current study that endothelium-independent vasodilation has been found to be reduced with diabetes per se. (Arora *et al.* 1998; Caballero *et al.* 1999; Khan *et al.* 2000; Gomes *et al.* 2008; Beer *et al.* 2008; Brooks *et al.* 2008). Veves *et al.* (1998) found that there was a reduced dilator effect with both SNP and ACh delivery with diabetes found in conjunction with neuropathy, ischaemia or arthropathy. Vasodilation was more severely reduced with SNP in patients presenting with ischaemia than with iontophoresis and ACh, however no difference was noted between the subjects with diabetes and no complications, and

control subjects. In contrast Beer *et al.* (2008) found the response to SNP was equally blunt in diabetes with or without complications in comparison to control subjects.

A similar response was found with SNP and ACh by Caballero *et al.* (1999) who found that the vasodilation was reduced not only in known cases of diabetes, but also in relatives of those with diabetes and IGT individuals. They noted that it was extremely difficult to discriminate whether the reduction in dilation was a result of decreased production of NO, an increase in destruction, inactivation or a decrease in vascular smooth muscle cell responsiveness from their results. Arora *et al.* (1998) found that vasodilation was reduced at foot level with ACh, however found no difference between the groups of subjects with diabetes with and without neuropathy and controls with SNP delivered via inotophoresis. They postulated in agreement with Hamdy *et al.* (2001) that the difference was probably down to SNP not having a direct stimulating effect on C fibres whereas ACh did. As a result Hamdy *et al.* (2001) went on to state that with SNP there was no significant difference in the percentage contribution of the nerve axon related response. Pitei *et al.* (1996) found that on the dorsum of the foot, vasodilation with ACh was reduced in patients with and without neuropathy, whereas with SNP it was only reduced in the group of 8 subjects with diabetes and peripheral neuropathy. In contrast Kelly *et al.* (2001) found no significant difference with the vasodilator response to SNP on the dorsum of the foot in subjects with diabetes, with or without peripheral neuropathy. However it must be noted that protocols are not standardised for the iontophoresis process, and there are many differences in subject numbers incorporated in the studies mentioned which does make direct comparisons quite difficult.

Brooks *et al.* (2008) found that SNP and ACh both produced a reduced response with diabetes and this appeared to progress with complications. The questioned their own results by noting that if ACh reactivity was to be regarded as an index for endothelial

function, then SNP should not have produced similar results. As it had done so, they postulated that this must indicate an impairment in the ability of the vessels to respond directly to a vasodilator which could be due to a functional defect of the smooth muscle in the response to NO, excessive destruction of NO, or perhaps to changes in the structure of the vessel walls leading to increased stiffness. The process was thought likely to be a complex interaction of several structural and/or functional abnormalities and factors which have been mentioned as possible contributors are advanced glycation end products (AGEs) or increased oxidative stress (Khan *et al.* 2000; Brooks *et al.* 2008). Sampathkumar *et al.* (2005) found serum levels of AGEs were significantly higher in subjects with diabetes than in control subjects, and even higher in those with diabetes and complications. While it was noted that a causal relationship could not be established between AGEs and microvascular complications, it was suggested that they may play a role in accelerating the process with diabetes. Although the formation of AGEs occurred as part of the natural ageing process, it was found to be accelerated with diabetes (De Vriese *et al.* 2000). Advanced glycosylation is known to modify tissue proteins in diabetes which is the precursor for early glycosylation products which in turn become AGEs. Among the outcomes found with an increase or early production of AGEs in animal studies has been a reduced vasodilator response to the NO donor nitroglycerine (SNP and nitroglycerine belong to the same family of drugs), which would suggest that the efficacy and or synthesis of NO may be affected (Tribe & Poston 1996). In addition endothelial vasodilation dysfunction can also occur indirectly through AGR induced oxidation of low density lipoproteins (LDLs) which again results in reduced synthesis of NO (DeVries *et al.* 2000). Alp *et al.* (2003) found endothelial cell superoxide production was higher with impairment of NO mediated endothelial vasodilation in diabetes induced mice when investigating the role of tetrahydrobiopterin (BH4) in eNOS activity. Generally evidence has been increasing to establish a role for

oxidative stress and oxidised lipids in microvascular dysfunction and the endothelium and the derived vasodilators would appear to be central to this (Tribe & Poston 1996; Pacher *et al.* 2002; Kampoli *et al.* 2009).

5.7.2 Pressure application and SNP delivery in combination summarised

There is a lack of agreement between the studies investigating the effect of the delivery of SNP with iontophoresis in conjunction with diabetes. Some in agreement with this current study have found the response to SNP to be reduced regardless of complications being present or not (Khan *et al.* 2000; Beer *et al.* 2008; Gomes et al. 2008), other studies found no difference between subjects with diabetes and control subjects (Arora *et al.* 1998; Kelly *et al.* 2001) and others found that the vasodilator effect was reduced in conjunction with the presence of complications like neuropathy (Pitei *et al.*1996; Veves *et al.* 1998; Brooks *et al.* 2008).

Again it should be emphasised that no other studies have investigated the response to SNP in conjunction with pressure delivery and as such a direct comparison of results found in this current study is not possible. In the current study there were some differences found between the groups caused by the application of pressure in conjunction with SNP and iontophoresis. The group with Type 2 diabetes showed a poor microvascular response with the addition of pressure in conjunction with iontophoresis of SNP compared with the response in the control group. However, although not significant, both groups displayed a difference between the flux values obtained at 50% and 100% pressure delivery, unlike the delivery of ACh, where the flux values obtained at 100% pressure application were similar to those found with 50% pressure delivery.

5.8 LIMITATIONS OF THE STUDY

5.8.1 Same day assessment for all elements of this study

One of the major decisions concerning gathering of data for this study was the choice between gathering all data on the same day, and spreading the data gathering over two or more visits. By gathering all data in the same visit it ensured that for each element of the study, the pre-study background was absolutely identical with food and caffeine intake etc., however in order to allow recovery between each test it did make the visit quite lengthy. It also ensured that the actual testing could be completed for each subject without the need to return. Initial volunteers were questioned, and it was the general consensus that a single lengthy visit was preferable to returning on another occasion. However with this decision it has to be noted that although time was given for recovery between tests, and the results do not indicate any residual effects before the next element of the assessment, there may have been some impact in assessing all elements on the same day such as mental stimulation of the subjects due to possible boredom.

5.8.2 Subject recruitment and implications for the study

Recruitment of subjects with Type 2 diabetes was carried out by a written invitation sent out to a random sample of subjects located on the diabetes centre at Ninewells Hospital SCI-DC database system. The return rate was quick and good and of those who had agreed in writing, all of them following a telephone discussion agreed to participate with the exception of one individual who had a pacemaker fitted and who was unsure about the iontophoresis process. However, the group were generally free from complications and neuropathy, and it could be noted that those who do take an interest in their health issues are more likely to volunteer to help with studies than those who do

have some difficulties with either the management of their diabetes, or with complications. It may have been useful to target a group with known neuropathy as a comparison and perhaps this would be a useful follow up study in the future.

5.8.3 Number of subjects included in the study

The number of subjects included in this study was limited because of possible time allocation to a total of 60. As a result the two groups contained a mix of sexes which did reflect the mix normally found with Type 2 diabetes with a slightly higher number of male subjects than female. However, with a group number of 30 in total, it did preclude any analysis of the data with regard to gender. Eun (1995) noted that men have been reported as having slightly higher cutaneous blood flow than women, and it would have been interesting to see if gender had any influence on the findings in this study.

5.8.4 Skin thickness variations

It has been noted that skin thickness does vary between individuals, but Ferrell *et al.* (2002) found no significant correlation between the calculated resistance and skin fold thickness as measured with calipers and reported that subcutaneous fat did not affect resistance. More importantly for this study, they observed that there was no significant correlation between skin fold thickness and the ACh perfusion/time integral. There is a debate around how much the presence of plantar callus influences plantar pressure. Some authors argue that it leads to an increase in pressure (Boulton 1987; Young *et al.* 1992; Masson 1992; Abouaesha *et al.* 2001), while others have found that it does not increase plantar pressure at all (Potter & Potter 2000). As a precaution, any overlying callus found in this study was removed using a sterile scalpel prior to the study being

carried out. However, Hashmi *et al.* (2006) found the epidermis itself to be thicker in Type 2 diabetes when compared to controls without diabetes, and to display a reduced plasticity when using high frequency ultrasonography. As this was a comparative study with Type 2 diabetes and healthy controls, it may have been useful to measure the thickness of the plantar epidermis.

5.8.5 Choice of true pressure values rather than a set pressure value

It would have proven much quicker and easier to select a set pressure value to use for all subjects in this study, rather than measuring their own maximum pressure for application with the process of iontophoresis and vasodilation. However, a meaningful single pressure value would have been very difficult to select and for some subjects would have been set either too high or too low in comparison with their own maximum pressure.

5.8.6 Subject related movement artefacts

Although the limb was held securely in a boot to stabilise and ensure that the foot did not move with the addition of pressure, it was still possible for small movements to occur during assessment which might have caused some movement artefacts. During PORH on release of the cuff it was also quite possible for the participant to move their foot slightly altering the actual area of interest pre and post occlusion.

5.8.7 Pre testing intake of food and beverage

While clear, written instructions were provided for each participant concerning their intake of food and caffeine related drinks prior to the assessment, there is no guarantee that every participant remembered to follow the instructions carefully. A way of preventing this would have been to ask the participants to refrain from eating from midnight onwards i.e. fast overnight and on arrival early morning to provide a suitable breakfast as carried out by Beer *et al.* (2008).

5.8.8 Mental stimulation during the assessment session

As well as being a temperature controlled environment, the area was a quiet and peaceful room with no direct entry for anyone other than the participant and the researcher. Eun (1995) noted that mental stimulation could influence cutaneous blood flow. However, although every physical element was controlled in the environment, when the participant was in a supine position for a lengthy period of time there is no guarantee that they were not mentally doing shopping lists or other activities which may have influenced cutaneous blood flow.

5.8.9 Possible human error on the part of the researcher

It is very possible for human error to have occurred in several elements. The equipment was carefully positioned for continuity of positioning; however it is quite possible that it was not absolutely exact in every case. Utilisation of a single researcher to position the equipment who was very aware and well versed in the positioning required did help to reduce this possible error. Calculation and addition of the pressure values was also a

possible source of human error from the initial reading of the values obtained with the Emed-x/E system to transference of the values to the pressure delivery system for each individual. There is little room for human error in reading the flux values obtained ether by the LSCI system or the LDF system as both provide the readings clearly. However, the time taken to reach maximum peak pressure with the LSCI system is a judgement, and again limitation of possible error is by the single researcher carrying out all readings and standardising the readings with pre-tester training.

The vasodilator substances and solutions were weighed and mixed by the researcher using an electronic balance, following training, and sterile syringes were used to ensure the correct amount of deionised water was added. A magnetic stirrer was also utilised for mixing the 2% solution of methylcellulose to ensure no bubbles were present in the mixture. Care was taken to ensure continuity of the solutions used which were freshly made for each session. Although every care was taken, it was still a possible source of human error.

Chapter Six

Conclusions

6 CONCLUSIONS

The aim of this study has been to investigate endothelial function in the superficial vessels situated in the plantar aspect of the forefoot, when placed under normal walking pressure. The initial focus was around the development of equipment as well as to ascertain the optimum protocols to allow meaningful investigations utilising the equipment to proceed.

A pressure delivery system was successfully developed which could repeatedly deliver a known pressure to the area under study, while also acting as a housing unit for the LDF probe. This dual activity of the system meant that simultaneous delivery of pressure could be carried out along with assessment of endothelial function, in combination with measurement of the blood flux on the plantar aspect of the foot. With the LDF probe not only measuring blood flux, but also being the delivery point for pressure, it was possible to measure both in the same locality under study simultaneously. This study was centred on the plantar aspect of the foot, and as such it was paramount that time and effort was dedicated to ensuring that the equipment could be optimised for utilisation in the area. The most suitable vehicle for the endothelium-dependent and independent vasodilators (ACh and SNP), given the position of the foot and the size of the preferred iontophoresis chamber, was fully investigated and considered, with a 2% solution of the vasodilator and methylcellulose in deionised water chosen. The optimum protocol for the delivery of the drugs via the process of iontophoresis was also fully explored, with the better test-retest reliability in combination with the shortest duration being the features of the favoured protocol for the study (Tables 3.4 and 3.5).

The utilisation of LSCI is a recent development in measuring endothelial function, but is thought to offer advantages such as measuring dynamic changes in blood flow over wider areas, coupled with good resolution, so ideal for investigating areas such as the forefoot. In this study it was successfully tested for suitability to investigate PORH in the forefoot, and was also compared with LDF. A strong, significant positive association was found between the established LDF for resting flux under 3^{RD} MPJ and LSCI (Table 4.14. and Figures 4.31 to 4.36). Thus, LSCI was found to be suitable for assessing endothelial function in superficial vessels on the plantar aspect of the forefoot.

The equipment and protocols were then utilised in vivo for two groups with matching age and sex distribution, 30 subjects with Type 2 diabetes and 30 control subjects. Of the parameters tested with PORH, peak flux following release of occlusion was the only one displaying a significant difference between the groups. The subjects with Type 2 diabetes displayed significantly higher peak flux value than the control subjects (Table 4.4, and Figure 4.9). Published literature would suggest that this finding could be related to a reduction in prostaglandin production by the group with Type 2 diabetes, in that diabetes is thought to have an impact on the vasoconstriction of the arterioles rather than releasing mediators to dilate the vessels.

Plantar pressure exerted on the areas of the forefoot across the groups in this study was found not to be significantly different (Table 4.17). As the group with Type 2 diabetes did not display any clinical signs of neuropathy this was not a surprising finding, and additionally no correlation was found between the natural walking pressure values across the 6 forefoot areas under study and baseline blood flux (Table 4.19). This would indicate that pressure did not influence baseline blood flux values in this study.

A noticeable impact of Type 2 diabetes in this study was that the group displayed higher flux values than the control group throughout all stages of the study. There is some

evidence in the literature that this could be related to involvement of sympathetic fibres sustaining damage prior to clinical signs and symptoms of neuropathy, with increased arteriovenous shunting of blood flow combined with increased nutritive capillary flow at rest. It could also relate to a reduction in pre/post capillary resistance with tissue hypoxia related to increased demand or altered oxygen transportation.

When the endothelium-derived vasodilator ACh was applied to the superficial vessels supplying 3rd MPJ using iontophoresis, a reduction in blood flux was found in both groups generally at 50% of normal walking pressure. No significant difference was found for either group between 50% and 100% pressure delivery, so the main impact on blood flux occurred in both groups at 50% application of normal walking pressure (Tables 4.21. and 4.22). A difference was found in the ability of the groups to deal with 50% pressure with baseline flux. The control group displayed similar flux values with 0 and 50% pressure addition (56.88 ± 68.48 AU and 55.11 ± 77.83 AU), which would indicate that they could maintain blood flux when placed under 50% normal walking pressure, whereas the group with Type 2 diabetes were unable to do so (79.72 ± 81.87 AU and 63.01 ± 58.49 AU).

When SNP, the NO donor vasodilator was applied to the superficial vessels, pressure was found to have a greater impact on blood flux on the subjects with Type 2 diabetes in comparison to the control group. Without the addition of pressure, with 50% pressure and 100% pressure addition, the control subjects responded better, and achieved higher blood flux values (Figure 4.65, 4.67 and 4.69). With this reduced capacity the group with Type 2 diabetes did not respond to pressure application as well under the vasodilator SNP as the control group could. The literature would suggest that this reduction in response by the group with Type 2 diabetes to SNP could be interpreted as structural change within the superficial vessels leading to a reduction in the capacity to

dilate, or a reduction in the activity of NO when e.g. high oxidative stress reduces the release of NO.

6.1 CONTRIBUTION TO KNOWLEDGE

The contribution to knowledge of this study has been to successfully develop and test equipment which has allowed for the first time simulated plantar pressure application to an area of the forefoot known to receive high pressure levels during walking, with simultaneous assessment of endothelial function. The development of a device which could deliver known pressure repeatedly to the chosen area was a vital element of this study. The novel utilisation of the DP2 probe as a component of that pressure delivery system ensured that the pressure delivered and blood flux measurements were both produced in the exact same location. Iontophoresis is a method of drug delivery which is normally carried out on the upper limbs or on occasion the dorsum of the foot where the skin is much finer. This study successfully utilised the process on the plantar aspect of the foot, and in a vertical position, which meant that 2% methylcellulose as a thickening agent was required.

PORH is a well utilised method of assessing endothelial dysfunction in the lower limb in conjunction with LDF. However, the recent development of LSCI offered the possibility of measuring rapid changes in blood flux over a larger area, coupled with good resolution, particularly useful for an area like the forefoot. On examination of the literature, LSCI had not been used on the plantar aspect of the forefoot before, and published research indicated that further evidence of the comparability with LDF was also required.

6.2 UTILITY AND APPLICATION OF THIS KNOWLEDGE

This knowledge adds to the understanding of two of the important elements, namely plantar pressure alteration and endothelial dysfunction known to be involved in the development of forefoot plantar ulceration associated with diabetes. As elevated plantar pressures and endothelial dysfunction are commonly cited as having a major role in the development of plantar ulceration and tissue viability in the foot with Type 2 diabetes, it is hoped that the development of this equipment may help with improving knowledge around the interaction of the two elements.

6.3 SUGGESTIONS FOR FURTHER RESEARCH IN THE AREA

On completion of this study there are some areas which have become apparent where further investigation would be beneficial. It would be useful to investigate further a group of subjects with diabetes who are known to present with neuropathy, thus elements like the effect of sensory nerve blocking could be investigated further, as well as the greater impact of increased plantar pressure on the forefoot.

Further work should be undertaken regarding the higher blood flux found in areas of increased pressure with Type 2 diabetes, with perhaps a comparison study of areas which do not receive high pressure on the plantar aspect of the foot.

Given the complexity of PORH and the debate concerning the elements of the test, it would be useful to investigate further the effect on the lower limb as well as carrying out further comparative work with LSCI and LDF.

Further work should be carried out regarding measurements of skin thickness and how this affects blood flux, as well as fully investigating the role that callus formation and removal may have on flux values.

A larger group containing more subjects of each gender would allow further work on the impact of gender on blood flow.

An interesting area of further study could be to investigate the impact of mental stimulation on peripheral blood flow during the assessment process.

Finally, as there is so much variation found in the protocols used for iontophoresis delivery, further work in the development of protocols would be useful.

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Appendix One

Friends, students,colleagues

LEND ME YOUR FEET!

Participants required for Research

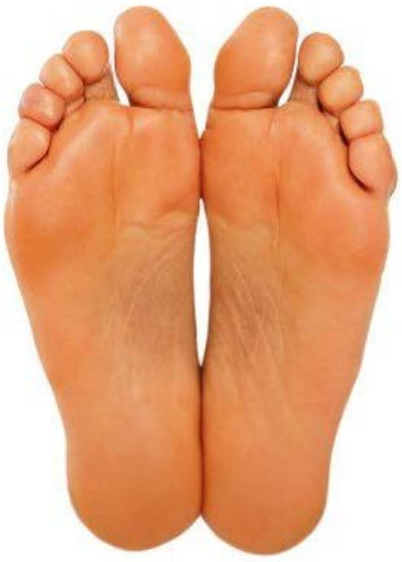
I am currently investigating the effect of foot pressure on microvascular function in the diabetic foot and looking for healthy volunteers to constitute a control group for the study.

As a participant in this study, you will be invited to attend the podiatry clinic in QMU where blood flowing through the superficial skin blood vessels will be measured on the sole of the foot. You will be asked to sit comfortably and a cuff will be inflated around your calf for 4 mins. The reactive hyperaemia will be measured using a laser speckle machine.

Your participation would involve the attendance of one session, lasting approximately 40 mins..
All information will be treated with the strictest confidence.

If you are

- ✓ ***Male/female aged 18 - 75***
- ✓ ***In general good health***
- ✓ ***Have no foot problems***
- ✓ ***Would like to volunteer for this study and would like further information please contact:***



Queen Margaret University
EDINBURGH

telephone Lynne Flynn on **07876762072**
email lflynn@qmu.ac.uk

QMU and Tayside Committee on Medical Research Ethics has examined this proposal and has raised no objections from the point of view of medical ethics

Appendix Two

Queen Margaret University
EDINBURGH

Healthy Volunteer Information Sheet

Study Title – The effect of pressure on microvascular function in the diabetic foot.

You are being invited to take part in a research study which we believe will contribute to the explanation of why people with diabetes suffer with foot ulcerations. This study needs healthy volunteers so that we can compare the responses of their local blood flow to pressure in the feet, to the response in people with diabetes. Before deciding to take part, it is important that you understand why the research is being carried out and what the study will involve. Please make sure you take the time to read the following information carefully and talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Foot ulceration is a problem which can be found with diabetes. Two factors which are thought to contribute to foot ulcers are changes which occur to the small blood vessels found just under the skin surface, and the pressure placed on the sole of the foot when walking. The aim of this study is to investigate these 2 factors together to try to find out if there are differences in the way the small blood vessels respond under pressure with diabetes when compared to a group of people without diabetes. The component of the study you are being asked to help with is in assessing the reactive hyperaemia which occurs in the skin blood vessels following occlusion of the local vessels.

What will happen to me if I take part?

You will be invited to attend the podiatry clinic at Queen Margaret University for a single visit only.

You will be seated in a chair with your feet up. A small box will be placed about 30cms above your foot to measure the blood flowing through your skin. After 2 minutes a pressure cuff will be inflated around your leg, just like the cuff used when you have your blood pressure measured. This will be inflated for 4-5 minutes and then removed. The small box will stay in place above your foot for a further 15 minutes to measure your blood flow without contact with your skin, or causing any discomfort. During this process you will be asked to sit still with your feet up. The process will take approx. 40 mins. to complete.

Are there any side effects?
There are no known side effects or problems with the equipment used in this study. On occasion a mild tingling sensation can be felt at the start of the procedure, and if this should continue then the testing would be stopped.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will you taking part in the study be kept confidential?
Yes, all the information about your participation in this study will be kept confidential. The details are included in Part 2.
A contact point for further information about this study is
Mrs Lynne Flynn, Subject Area of Podiatry, Queen Margaret University or Institute of Motion Analysis and Research, Ninewells Hospital, Dundee DD1 9SY.

This completes Part 1 of the information sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2
What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the study. If this happens the researcher will tell you about it and discuss whether you want to continue with the study. If you decide not to carry on then the procedure will be stopped and any data gathered from you will be destroyed. If you decide to continue in the study, you will be asked to sign an updated consent form.

What will happen if I don't want to carry on with the study?
You can withdraw from the study at any time and any data already gathered will be destroyed.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak with the researcher Lynne Flynn who will do her best to answer your questions (contact number 0131 474 0000 email lflynn@qmu.ac.uk). If you remain unhappy need any further information or wish to complain formally, you can contact Dr Rami Abboud, Director of the Institute of Motion Analysis and Research Ninewells Hospital Dundee DD1 9SY tel no. 01382 496276.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is
due to someone's negligence then you may have grounds for a legal action for compensation against University of Dundee but you may have to pay your legal costs.

Will my taking part in this study be kept confidential?
If you join this study the data collected about you will be coded and anonymous and will be securely stored. It will be used only for this study and then destroyed. During the course of the study some parts of the data will be looked at by the supervisory team for the study to ensure that the study is being carried out correctly. All individuals with access to the data will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site. The procedures for handling, processing, storage and destruction of the data will be compliant with the Data Protection Act 1998.

What will happen to the results of the research study?
The results of the study will be published in the form of a thesis for submission for PhD, and may also be published in one of the journals dedicated to diabetes. You will not be identified in any report or publication.

Who has reviewed the study?
The Tayside Committee on Medical Research Ethics, and Queen Margaret University Ethics committee have examined this proposal and have raised no objections from the point of view of medical ethics. It is a requirement that the research records are made available to monitors from NHS Tayside whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.
Thank you for considering taking part or taking time to read this information sheet. If you decide to take part in this study you will be given a copy of the information sheet and a signed consent form to keep.

Appendix Three

Queen Margaret University
EDINBURGH

Consent Form

Study Title – The effect of pressure on micro vascular function in the diabetic foot.

I have read and understood the information sheet and this consent form. I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in this study.

I understand that I have the right to withdraw from this study at any stage without giving any reason.

I agree to participate in this study.

Name of participant: _____

Signature of participant: _____

Signature of researcher: _____

Date: _____

Contact details of the researcher

Name of researcher: Lynne Flynn

Address: Subject Area Podiatry,
School of Health Sciences
Queen Margaret University, Edinburgh
Queen Margaret University Drive
Musselburgh
East Lothian EH21 6UU

Email / Telephone: lflynn@qmu.ac.uk / 0131 474 0000

Appendix Four

UNIVERSITY OF DUNDEE

Department of Orthopaedic and Trauma Surgery

COLLEGE OF MEDICINE, DENTISTRY & NURSING

LF/SM

6 July 2011

Dear

As you will be aware patients with diabetes can sometimes develop problems with their feet, and the problems can occur at differing rates and severity and can be related to circulation. We are contacting you following your assistance with projects in the past, to ask if you would consider volunteering to help us with our current project which will be looking at subtle changes in the blood flow through the skin when different pressures are applied to the foot. A separate sheet containing full details regarding this project is enclosed.

This is a non invasive study which has no known side effects or problems, but we hope will provide some valuable information about how the small blood vessels function in diabetes. It will involve a single visit to our vascular laboratories in Ninewells Hospital and **travel costs will be refunded for the visit**. All you have to do at this stage is to fill in the return slip and send it in the Freepost addresses envelope provided.

Should you decide to volunteer, your cooperation and support for this study would be very much appreciated and would be a great contribution to ongoing research related to the lower limb and foot problems which can be associated with diabetes.

Yours sincerely

Prof. R J Abboud
Head of Department, Orthopaedics&Trauma Surgery
Director, Institute of Motion Analysis and Research (IMAR)
Chairman BMSc (Hons) Degree
Co-Director, Centre of Academic Clinical Practice
Tel: ++44-1382-425746
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Mrs L M Flynn
PhD student
Department of Orthopaedics and Trauma Surgery
University of Dundee
Tel: ++44-1382-496276
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E mail: l.m.flynn@dundee.ac.uk

Version 1 6th July 2011

UNIVERSITY OF DUNDEE

Department of Orthopaedic and Trauma Surgery

COLLEGE OF MEDICINE, DENTISTRY & NURSING

RETURN SLIP

I am happy to participate in the research project entitled

The effect of pressure on microvascular function in the diabetic foot

Full Name	

Telephone Number	
Mobile number	
Email address	

Postal address	

Please return to:

Mrs Sheila MacDonald
Institute of Motion Analysis and Research (IMAR)
University Department of Orthopaedics and Trauma Surgery
Tayside Orthopaedic and Rehabilitation Technology (TORT) Centre
Ninewells Hospital and Medical School
Dundee DD1 9SY
Email: s.a.z.macdonald@dundee.ac.uk

Version 1 6th July 2011

Research Participant Information Sheet
Study Title- The effect of pressure on microvascular function in the diabetic foot.

You are being invited to take part in a research study. Before deciding to take part, it is important that you understand why the research is being carried out and what the study will involve. Please make sure you take the time to read the following information carefully and talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?
Foot ulceration is a problem which can be found with diabetes. Two factors which are thought to contribute to foot ulcers are changes which occur to the small blood vessels found just under the skin surface, and the pressure placed on the sole of the foot when walking. The aim of this study is to investigate these 2 factors together to try to find out if there are differences in the way the small blood vessels respond under pressure with diabetes when compared to a group of people without diabetes. The activity of the blood vessels will be assessed by placing a substance on the skin surface which when absorbed causes the small local vessels to enlarge temporarily and the amount of blood flowing through the vessels to increase. This increase in local blood flow can be measured on the skin surface by placing a probe against the skin. This study is being carried out by a researcher who is studying towards a PhD.
You have been chosen to take part in this study as you are on the diabetes centre database at Ninewells Hospital and Medical School and therefore have the type of condition which is required for this study.

Do I have to take part in this study?
No, it is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?
You will be invited to attend the vascular unit at Ninewells Hospital for a single visit only. Note that you should eat breakfast or lunch as usual before travelling to the hospital, but avoid eating or drinking for 1-2 hours before the tests are carried out. It is expected that this period of time will be taken up in the time it takes to travel to the hospital and in preparing you for the tests You will be asked to walk barefoot at

your normal pace over a walkway which will measure the pressure you have on the sole of your foot. If you have any hard skin on the sole of your foot in the area under study this will be removed by the researcher who is a registered podiatrist. You will then be seated in a chair with your feet up. A small box will be placed about 30cms above your foot to measure the blood flowing through your skin. After 2 minutes a pressure cuff will be inflated around your leg, just like the cuff used when you have your blood pressure measured. This will be inflated for 4-5 minutes and then removed. The small box will stay in place above your foot for a further 15 minutes to measure your blood flow without contact with your skin, or causing any discomfort. Equipment will then be placed onto the sole of your foot just behind your toes which will allow the jelly like substance to be absorbed into your skin. A small pressure device will be placed against your skin and will place the skin under the same pressure measured when you were walking. A skin surface probe will be attached to your skin and will measure the amount of blood flowing through the surface blood vessels when they are under pressure.
The pressure will be reduced to half and then to a quarter of your normal pressure and measurements will be repeated. This process will be repeated using a second jelly like substance also designed to expand your local small blood vessels. During this process you will be asked to sit still with your feet up. The process will take approx. 2 hours to complete. Time will be given between measurements for you to stretch or move around for your comfort.

Expenses and payment
As this visit will be for the study only and not for your normal treatment, expenses for attending will be reimbursed.

Are there any side effects?
There are no known side effects or problems with the equipment used in this study. On occasion a mild tingling sensation can be felt at the start of the procedure, and if this should continue then the testing would be stopped.

What are the possible benefits of taking part?
We cannot promise that this study will help you directly, but the information we get might help to prevent foot ulcers in the future for people with diabetes by providing us with a better understanding about why they happen.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will you taking part in the study be kept confidential?
Yes, all the information about your participation in this study will be kept confidential. The details are included in Part 2.
A contact point for further information about this study is
Mrs Lynne Flynn, Institute of Motion Analysis and Research, Ninewells Hospital, Dundee DD1 9SY phone 07876762072.

This completes Part 1 of the information sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2

What if relevant new information becomes available?

Sometimes during the course of a research project, new information becomes available about the study. If this happens the researcher will tell you about it and discuss whether you want to continue with the study. If you decide not to carry on then the procedure will be stopped and any data gathered from you will be destroyed. If you decide to continue in the study, you will be asked to sign an updated consent form. If the study is stopped for any other reason you will be told why, and this will have no effect on any future care for your condition.

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time and any data already gathered will be destroyed.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researcher Lynne Flynn who will do her best to answer your questions (contact number 07876762072). If you remain unhappy and wish to complain formally, you can contact Dr Rami Abboud, Director of the Institute of Motion Analysis and Research Ninewells Hospital Dundee DD1 9SY tel no. 01382 496276.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against University of Dundee but you may have to pay your legal costs.

Will my taking part in this study be kept confidential?

If you join this study the data collected about you will be coded and anonymous and will be securely stored. It will be used only for this study and then destroyed. During the course of the study some parts of the data will be looked at by the supervisory team for the study to ensure that the study is being carried out correctly. All individuals with access to the data will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site. The procedures for handling, processing, storage and destruction of the data will be compliant with the Data Protection Act 1998.

Informing the family doctor

With your consent your own GP will be notified that you are participating in this study.

What will happen to the results of the research study?
The results of the study will be published in the form of a thesis for submission for PhD, and may also be published in one of the journals dedicated to diabetes. You will not be identified in any report or publication.

Who has reviewed the study?
The Tayside Committee on Medical Research Ethics, which is responsible for scrutinising proposals for medical research on humans, has examined this proposal and has raised no objections from the point of view of medical ethics. It is a requirement that the research records are made available to monitors from NHS Tayside whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.
Thank you for considering taking part or taking time to read this information sheet. If you decide to take part in this study you will be given a copy of the information sheet and a signed consent form to keep.
Information sheet version 3
Date 20.03.09

Appendix Five

Friends, students, countrymen

LEND ME YOUR FEET!

Participants required for Research

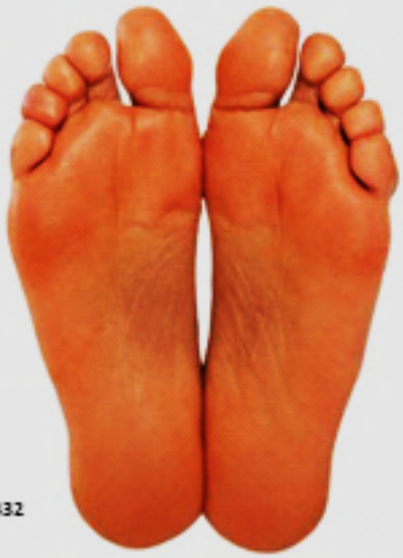
We are looking for healthy volunteers to constitute a control group in a study investigating the effect of foot pressure on microvascular function in the diabetic foot.

As a participant in this study, you will be invited to attend the vascular unit at Ninewells Hospital and Medical School, Dundee where blood flowing through the superficial skin blood vessels will be measured when the sole of the foot is placed under your normal walking pressure. You will be asked to sit comfortably and a probe will be placed against the skin surface on the sole of your foot to measure your blood flow. A solution will be placed in contact with the skin to dilate the blood vessels along with a device to simulate your own normal foot pressure when walking.

Your participation would involve the attendance of one session, lasting approximately 2 hours.
All information will be treated with the strictest confidence.

If you are

- ✓ *Male/female aged 18 - 75*
- ✓ *In general good health*
- ✓ *Have no foot problems*
- ✓ *Would like to volunteer for this study and would like further information please contact:*



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telephone Lynne Flynn on 07876762072
email L.M.Flynn@dundee.ac.uk

telephone Professor RJ Abboud on 01382 496332
email jimar@dundee.ac.uk

The Tayside Committee on Medical Research Ethics has examined this proposal and has raised no objections from the point of view of medical ethics

Healthy Volunteer Information Sheet

Study Title – The effect of pressure on microvascular function in the diabetic foot.

You are being invited to take part in a research study which we believe will contribute to the explanation of why people with diabetes suffer with foot ulcerations. This study needs healthy volunteers so that we can compare the responses of their local blood flow to pressure in the feet, to the response in people with diabetes. Before deciding to take part, it is important that you understand why the research is being carried out and what the study will involve. Please make sure you take the time to read the following information carefully and talk to others about the study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

Please ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Foot ulceration is a problem which can be found with diabetes. Two factors which are thought to contribute to foot ulcers are changes which occur to the small blood vessels found just under the skin surface, and the pressure placed on the sole of the foot when walking. The aim of this study is to investigate these 2 factors together to try to find out if there are differences in the way the small blood vessels respond under pressure with diabetes when compared to a group of people without diabetes. The activity of the blood vessels will be assessed by placing a substance on the skin surface which when absorbed causes the small local vessels to enlarge temporarily and the amount of blood flowing through the vessels to increase. This increase in local blood flow can be measured on the skin surface by placing a probe against the skin. This study is being carried out by a researcher who is studying towards a PhD.

What will happen to me if I take part?

You will be invited to attend the vascular unit at Ninewells Hospital for a single visit only. Note that you should avoid eating or drinking for 1-2 hours before the tests are carried out. You will be asked to walk barefoot at your normal pace over a walkway which will measure the pressure you have on the sole of your foot. If you have any hard skin on the sole of your foot in the area under study this will be removed by the researcher who is a registered podiatrist. You will then be seated in a chair with your feet up. A small box will be placed about 30cms above your foot to measure the blood flowing through your skin. After 2 minutes a pressure cuff will be inflated around your leg, just like the cuff used when you have your blood pressure measured. This will be inflated for 4-5 minutes and then removed. The small box will stay in place above your foot for a further 15 minutes to measure your blood flow without contact with your skin, or causing any discomfort. Equipment will then be placed onto the sole of your foot just behind your toes

which will allow the jelly like substance to be absorbed into your skin. A small pressure device will be placed against your skin and will place the skin under the same pressure measured when you were walking. A skin surface probe will be attached to your skin and will measure the amount of blood flowing through the surface blood vessels when they are under pressure. The pressure will be reduced to half and then to a quarter of your normal pressure and measurements will be repeated. This process will be repeated using a second jelly like substance also designed to expand your local small blood vessels. During this process you will be asked to sit still with your feet up. The process will take approx. 2 hours to complete. Time will be given between measurements for you to stretch or move around for your comfort.

Are there any side effects?
There are no known side effects or problems with the equipment used in this study. On occasion a mild tingling sensation can be felt at the start of the procedure, and if this should continue then the testing would be stopped.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will you taking part in the study be kept confidential?
Yes, all the information about your participation in this study will be kept confidential. The details are included in Part 2.
A contact point for further information about this study is
Mrs Lynne Flynn, Institute of Motion Analysis and Research, Ninewells Hospital, Dundee DD1 9SY phone 07876762072.

This completes Part 1 of the information sheet.
If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decision.

Part 2
What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the study. If this happens the researcher will tell you about it and discuss whether you want to continue with the study. If you decide not to carry on then the procedure will be stopped and any data gathered from you will be destroyed. If you decide to continue in the study, you will be asked to sign an updated consent form. If the study is stopped for any other reason you will be told why, and this will have no effect on any future care for your condition.

What will happen if I don't want to carry on with the study?
You can withdraw from the study at any time and any data already gathered will be destroyed.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researcher Lynne Flynn who will do her best to answer your questions (contact number 07876762072). If you remain unhappy and wish to complain formally, you can contact Dr Rami Abboud, Director of the Institute of Motion Analysis and Research Ninewells Hospital Dundee DD1 9SY tel no. 01382 496276.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against University of Dundee but you may have to pay your legal costs.

Will my taking part in this study be kept confidential?

If you join this study the data collected about you will be coded and anonymous and will be securely stored. It will be used only for this study and then destroyed. During the course of the study some parts of the data will be looked at by the supervisory team for the study to ensure that the study is being carried out correctly. All individuals with access to the data will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site. The procedures for handling, processing, storage and destruction of the data will be compliant with the Data Protection Act 1998.

What will happen to the results of the research study?

The results of the study will be published in the form of a thesis for submission for PhD, and may also be published in one of the journals dedicated to diabetes. You will not be identified in any report or publication.

Who has reviewed the study?

The Tayside Committee on Medical Research Ethics, which is responsible for scrutinising proposals for medical research on humans, has examined this proposal and has raised no objections from the point of view of medical ethics. It is a requirement that the research records are made available to monitors from NHS Tayside whose role is to check that research is properly conducted and the interests of those taking part are adequately protected.

Thank you for considering taking part or taking time to read this information sheet. If you decide to take part in this study you will be given a copy of the information sheet and a signed consent form to keep.

Appendix Six

UNIVERSITY OF DUNDEE

Institute of Motion Analysis & Research

ORTHOPAEDIC & TRAUMA SURGERY

INSTITUTE OF MOTION ANALYSIS & RESEARCH

Consent Form

Study Title –
The effect of pressure on micro vascular function in the diabetic foot.

I have read and understood the information sheet and this consent form. I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in this study.

I understand that I have the right to withdraw from this study at any stage without giving any reason.

I agree to participate in this study.

Name of participant: _____

Signature of participant: _____

Signature of researcher: _____

Date: _____

Contact details of the researcher

Name of researcher: Lynne Flynn

Address: University Dept of Orthopaedics and Trauma
TORT Centre
Ninewells Medical and Teaching Hospital
Dundee DD1 9SX

Email / Telephone: lflynn@qmu.ac.uk / 0131 474 0000

Institute of Motion Analysis & Research (IMAR), Department of Orthopaedic and Trauma Surgery,
College of Medicine, Dentistry and Nursing, Tayside Orthopaedic and Rehabilitation Technology (TORT) Centre
Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, United Kingdom
tel ++44 (0)1382 496332 *email* imar@dundee.ac.uk *email* orthol@dundee.ac.uk *fax* ++44 (0)1382 496200
www.dundee.ac.uk/orthopaedics/imar/

Appendix Seven

LYNNE FLYNN MPHIL[1], RAMI J, ABBOUD PHD HON FRCS(ENG)[2], FAISEL KHAN PHD BSC(HONS)[3]

1. PhD student, Department of Orthopaedics &Trauma Surgery, Ninewells Hospital & Medical School, Dundee DD1 9SY UK.

2. Prof., Head of Department, Orthopaedics & Trauma Surgery, Director, Institute of Motion Analysis and Research (IMAR), Ninewells Hospital & Medical School, TORT Centre, Dundee DD1 9SY UK.

3. PhD, BSc(Hons), Reader, Vascular & Inflammatory Diseases Research Unit, Ninewells Hospital & Medical School, Dundee DD1 9SY UK.

Vascular Assessment and Microcirculation in Lower Limb Wounds

Abstract

A good blood supply is paramount to initiate healing of lower limb wounds regardless of whether these are in an acute or a chronic phase, and it is vital that the method chosen for vascular assessment is accurate, user friendly and if possible non invasive. Several methods of vascular assessment are currently available for both clinical as well as research use. Included in this review are laser Doppler flowmetry and perfusion imaging with the utilisation of iontophoresis and reactive hyperaemia, laser speckle contrast imaging, photoplethysmography, thermal infra red imaging, transcutaneous oxygen tension as well as the ankle brachial pressure index and nailfold capillaroscopy. Although the techniques function in a variety of ways, the measurements offer an understanding of the role of microvasculature and endothelial function in the development of lower limb ulcers, as well as in wound assessment and the determination of the possibility of healing as an outcome of management strategies. This review outlines the techniques for assessment and given the important role microvasculature plays in the repair and healing of wounds, examines the available evidence for the role of the various measurements in this process.

Keywords: Lower limb wounds, Microcirculation, Laser Doppler flowmetry, Laser Doppler perfusion imaging

Introduction

Regeneration and repair of tissue in healing wounds involves multiple complex and interrelated mechanisms, with the microcirculation playing a significant role. Initially, microvascular perfusion in the wound is reduced to help limit blood loss, but this is then superseded by an increase in microvascular perfusion as inflammation ensues, which brings in immunological cells, growth factors, and fibroblasts to the wound and provides it with nutrients. Accordingly, it can be appreciated that a lack of perfusion to the lower limb can lead to ulceration and gangrene, often with an outcome of amputation. Potential causes of poor perfusion include peripheral arterial disease, venous insufficiency and commonly, diabetes mellitus.[1,2] Given the important role of the microcirculation in wound healing, there is interest in being able to assess this effectively in order to aid better prediction of wound healing, to monitor progression of repair, and also to understand underlying mechanisms. For example, dysfunction of the vascular endothelium has widely been investigated in the pathogenesis of peripheral arterial disease.[3,4] The vascular endothelium is responsible for several functions including controlling the vascular tone and inhibiting platelet aggregation, as well as modulation of the vascular wall permeability, however, in the main, endothelial function refers to the ability of the endothelium to release compounds which induce relaxation of smooth muscle within the vessel wall such as nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factors.[3] Several non-invasive methods are available to assess microvascular perfusion *(Table 1),* either indirectly or directly and these can aid in the diagnosis, assessment and management strategies for lower limb pathologies and associated complications such as ulceration and gangrene. The aim of this review is to evaluate some of the common methods currently in use.

Laser Doppler Flowmetry (LDF)

BACKGROUND

While many techniques can provide an indirect indication of microvascular perfusion, laser Doppler flowmetry is one of the few that can assess the microcirculation directly. Changes in microvascular perfusion of the skin can be measured relatively easily utilising the non invasive technique of laser Doppler flowmetry.[4-7] The technique utilises the motion generated from a light source and captured by a detector, this is known as the Doppler effect. With LDF the moving erythrocytes, or in fact tissues, cause a scattering of the laser light, and the velocity of this back-scattered light is detected providing the Doppler frequency information.[5,8,9] The density and speed of the red blood cells when measured with a regular or constant haematocrit within the sample tissue, is known to be proportional to red blood cell flow, hence the utilisation of the term 'flux' as a unit of flow measurement.

INSTRUMENT AND MEASUREMENT TECHNIQUES

Traditional LD flowmeters utilise a helium-neon laser light source of wavelengths varying between 630nm and 670nm with a fibre optic cable in contact with the skin surface. They provide an estimated perfusion depth of 1-1.5mm into the dermis and detect dermal blood flow without influence from the flow

TECHNIQUE	FUNCTION	ADVANTAGES/DISADVANTAGES
Laser Doppler Flowmetry	Uses laser light. Velocity of this back scattered light is detected providing Doppler frequency information.	**Advantages** • Effective and reliable method for measurement of blood perfusion in the microvasculature. • Provides continuous, noninvasive and real-time measurement capabilities. • Versatile and easy to use. **Disadvantages** • Motion artefact noise, lack of quantitative units for perfusion, lack of knowledge of depth of measurement and the biological zero signal.
Laser Speckle Contrast Imaging	Interference when laser light has been reflected from different parts of an illuminated surface.	**Advantages** • Rapid skin blood flow measurements over wide areas coupled with good resolution. • Full-field imaging of skin perfusion in near real time. **Disadvantages** • Relationship between speckle contrast and velocity is highly non-linear, thus a lack of quantitative units.
Photoplethysmography	Optical reflection mode system measuring scattering and absorption characteristics of tissue associated with changes in blood flow, with near infrared spectroscopy.	**Advantages** • Non invasive, Easy to use, cost effective and portable. **Disadvantages** • Blood volume, movement of the vessel wall and orientation of erythrocytes may have an influence on the amount of light received. • Cannot easily be calibrated.
Thermography	Thermal infrared imaging for the measurement of cutaneous circulation.	**Advantages** • Can be used over large areas. • Allows for real time recording. **Disadvantages** • If temperatures are very close in range, infrared imaging camera may misread the information and individual objects can become indistinguishable. • Current technology in thermography only allows for imaging to be applied to surface temperatures.
Transcutaneous Oxygen Tension	Transcutaneous oxygen and carbon dioxide sensors directly measure tissue gas tensions with a short sensor response times.	**Advantages** • Non invasive and continuous. **Disadvantages** Measurements do not equate directly to arterial blood flow due to skin perfusion.
Ankle Brachial Pressure Index	Systolic pressure is measured at the ankle using either the posterior tibial artery or dorsalis pedis and the value in mmHg obtained here is divided by the brachial systolic pressure, utilising a pressure cuff and a hand held Doppler probe. A normal index value at rest is equal or higher than 1.	**Advantages** • Easy to perform. • Non invasive. • Equipment is cheap and readily available. **Disadvantages** Not useful for small vessel disease or with calcification of vessels.
Nailfold Capillaroscopy	Under 10x to 20x magnification has been used to assess digital microcirculation, and reproducibility measurements of capillary width and number of loops/millimeter have been measured.	**Advantages** • Simple to carry out and non invasive, safe and cost effective. • Allows direct observation of nail fold capillaries.

Table 1. Available techniques for the assessment of microvascular perfusion with advantages and disadvantages.

to the underlying skeletal muscle.[5,9-12] An issue had been noted in that the optical fibres produce low frequency noise artefacts; further developments have ensued to overcome this problem.[9]

Semi conductor laser diodes operating with near infrared light of 780nm to 820nm as a light source have been developed to replace the helium-neon lasers.[5,9,11] The main advantages over traditional flowmeters being deeper penetration and better reproducibility.[5,9]

In contrast to single point measurements, laser Doppler perfusion imaging affords non-contact assessments (therefore desirable for wound assessments) with the laser beam →

position controlled by a system of mirrors. Currently, there are two main commercial laser Doppler imaging systems, produced by Lisca Development AB, Sweden and Moor Instruments Ltd UK. A digital image is produced, being developed as the combination of many single-point recordings of a two dimensional matrix of the blood flow over an extended skin surface, and over a larger area, thus producing a detailed perfusion map.

Both LDF and LDPI have been found to be user-independent by utilising established protocols and maintaining external factors which can influence the readings, such as controlling environmental temperature, reducing movement as well as reflection artifacts where possible, removal of topical agents, as well as refraining from smoking and caffeine ingestion for a period of at least 2 hours prior to assessments.[5,10]

UTILISATION OF LASER DOPPLER FLOWMETRY

LDF has been utilised for many applications including dermatology, wound healing, diabetology and peripheral vascular aspects.[9,10] LDI and capillary microscopy have been used to assess subpapillary and nutritive microcirculation in 17 patients with arterial occlusive disease and chronic venous insufficiency ulcers in 4 regions, a non-granulating tissue area, a granulating tissue area, an area of skin adjacent to the ulcers (within a distance of 1-8mm), and a more distant area of 9-25mm.[13] Laser Doppler flux was significantly higher in granulating areas than in areas without granulation tissue and intact skin. In scar tissue flux was found to be higher than in both intact skin and in ulcers without granulation tissue being present. Thus; ulcers without granulation tissue had poor perfusion and did not display healing, whereas when granulation tissue was present, high perfusion was found. They also noted that the blood supply in the intact skin adjacent to the ulcers was affected by an altered microcirculation found in the ulcers. Thus, it was noted that LDI flux measurement could provide information to aid the optimum evaluation of microcirculation in ulcers of mixed aetiology. Krupa (2003)[14] investigated the relationship between plantar pressure and skin perfusion on the foot of type 11 diabetic, peripheral neuropathic patients with a history of plantar ulceration. Although the heel area was found to display high pressures, in comparison to the forefoot areas in the study it displayed a higher skin perfusion, thus findings indicated that understanding of the skin vasculature response to plantar pressures could add valuable insight into re-ulceration issues.

Pressure ulcers were investigated as a complication of tissue ischaemia resulting from diabetes. When comparison was made between subjects with diabetes and controls, skin blood flow decreased markedly in subjects presenting with diabetes without neuropathy, as measured with single point LDF in response to initial locally applied increasing pressure. This inability of the microcirculation to respond to local pressure was noted as a possible factor in ulcer development in this group.[2] Figoni *et al.* (2006)[15] assessed the utility of LDI versus transcutaneous gases ($TcPCO_2$ and $TcPO_2$) in cases with severe ischaemia, for the assessment of limb perfusion in order to determine the degree and progression of the pathological process leading to possible amputation. They compared 31 patients who required amputation with 29 age–matched non ischaemic controls and found that LDI could detect a proximal to distal gradient of perfusion of the lower limb which was not discernible with $TcPO_2$. LDI was also found to discriminate between ischaemic and non ischaemic skin which was not possible with $TcPO_2$ thus overall LDI proved to perform better than $TcPO_2$. Skin perfusion in the hallux utilising LDI with 14 patients with peripheral arterial occlusive disease was compared with 11 healthy volunteers.[16] Significantly lower digital artery pressure and skin perfusion pressure was found in the patient group. They noted that LDI provided an easy and non-invasive method of simultaneously assessing digital artery and skin perfusion, enabling the severity of peripheral arterial occlusion to be assessed thus predicting possible healing of foot ulcerations.

Laser Doppler Flowmetry and Iontophoresis

Iontophoresis is a useful technique where small doses of vasoactive substances are required to stimulate skin microvessels locally without systemic effects to gain a better understanding of mechanisms of vascular dysfunction. Utilising iontophoresis with LDF provides a non-invasive method using a small electric charge to aid migration of charged substances across the skin surface.[5,12,17-19] The polarity of the active electrode used has to be the same charge as the substance itself.[12,20] During the process of iontophoresis, the electric dose, which is calculated from duration and current strength, is thought to correlate with the amount of substance delivered across the skin[21].The most commonly utilised substances for this purpose are those for investigating endothelial function, namely acetylcholine (ACh) an endothelium-dependent agonist, and sodium nitroprusside (SNP) an endothelium–independent vasodilator, although other substances have also been used such as bradykinin and substance P.[5,12,17,19,21,22] However, when using iontophoresis, several methodological issues need to be considered, which have been reviewed elsewhere.[12]

UTILISATION OF IONTOPHORESIS

Iontophoresis in conjunction with LDI has been utilised to investigate many pathological conditions, in particular diabetes mellitus and peripheral arterial disease, but only those studies relating to factors potentially affecting wound microcirculation will be described below Sensory neuropathy is recognised as a contributory factor to development of wounds. Kelly *et al.* (2001)[23] studied endothelial responses in the diabetic foot with sensory neuropathy. Analysis of the ACh and SNP response revealed no significant differences between groups of subjects with diabetes and neuropathy, without neuropathy and controls without diabetes. However this study utilised a protocol with a higher current for a shorter duration of iontophoresis than found in most other protocols and with a small number of subjects in that n=6 in each of the groups with diabetes with and without neuropathy, as well as the healthy controls studied.

Khan *et al.* (2000)[24] studied children, adolescents and young adults with type 1diabetes and measured skin microvascular function using LDF with iontophoresis of ACh and SNP in the feet, areas prone to development of ulcers and wounds. Findings indicated that early reductions in the microvascular function in the form of endothelium-dependent and independent vascular responses, as well as a reduction in maximal vasodilator capacity, was present prior to any manifestation of clinical presentation.

Impaired pressure induced vasodilation (PIV) in young adults with type 1 diabetes was investigated by Koitka *et al.* (2004)[22] who noted that vascular and neurological mechanisms were both likely to be involved in foot ulceration. They found an impaired vasodilation to ACh but not to SNP between the group with diabetes and the control group with n=12 in each. Although Sigaudo-Roussel *et al.* (2004)[25] carried out their study on streptozo-

tocin-induced diabetic mice and healthy control animals; they also found that endothelial impairment was enough to severely alter PIV. PIV suppression was noted as possibly leading to complications such as foot ulceration in their study utilising n=10 in both groups under study.

A pilot study to investigate the effects of local pressure on microvascular function in the diabetic foot, with 16 patients and 8 healthy controls was carried out.[18] The subjects with diabetes displayed higher plantar pressures than the control group, but no significant difference was found in basal skin perfusion or in ACh response. Within the patient group, however, baseline flow was increased (p=0.041) but the acetylcholine response reduced (p=0.03) at the high-pressure compared with the low-pressure site; this was most apparent in those who were particularly at risk of ulceration due to high plantar pressures. The authors concluded that further work is required to determine whether, and under what conditions, this additional hyperaemia is protective or maladaptive.

LDF AND REACTIVE HYPERAEMIA

An additional tool for assessing the integrity of the microvasculature is to measure the reactive hyperaemic response, which is defined as a temporary increase in skin and muscle blood flow following an arterial occlusion.[26,27] Post occlusive reactive hyperaemia (PORH) has been specified as a useful, non-invasive and sensitive indicator for microvascular dysfunction in that the maximum peak blood flow and the time to reach this point following release of the occlusion are related to nitric oxide (NO) and as such are indicators of endothelial function.[26] The ability to continuously monitor perfusion has made LDF an important mode for study for post-occlusive reactive hyperaemia (PORH).[27-29] Useful parameters for reactive hyperaemia include time to peak flow and the value of peak flow. These parameters have been found to provide early detection of vascular alterations ie prior to the development of clinical symptoms in diabetes mellitus, and also to show the effects of smoking and peripheral vascular disease.[27,28,30] PORH was compared in subjects with Fontaine classification of stage 11 PAOD ie intermittent claudication occurring, but with no pain on resting, with healthy controls.[30] In PAOD patient's skin perfusion was not impaired during baseline ie prior to occlusion, which was thought to be a result of compensatory mechanisms related to endothelial, myogenic and sympathetic activities. During hyperaemia however, patients displayed reduced vasoreactivity suggesting perhaps an exhaustion of compensatory mechanisms.

Although LDF is useful for monitoring the PORH response, until recently this has only really been possible with single point instruments (which have their inherent limitations as mentioned in a previous section) since these instruments are able to continuously monitor perfusion and capture the rapid changes in perfusion post occlusion. A recent development, however, has been the introduction of laser speckle contrast imaging which has the capacity to perfusion from larger areas of tissue in near real time.

Laser Speckle Contrast Imaging (LSCI)

BACKGROUND

In 1960s a 'grainy' pattern was discovered when laser light was viewed on a matt surface such as glass, paper or dull metal, and this speckle pattern lead to the term being adopted.[31] It has been described as an interference when light has been reflected from different parts of an illuminated surface.[31,32]

With movement of an object, changes are noted in the speckle pattern. When a solid object undergoes small movements the speckles appear to move with the object, however with large movements the pattern undergoes complete change and frequency of the fluctuations is dependent on the velocity of the motion. This phenomenon is known as 'time-varying' speckle and Stern in 1975 was the first to note the potential for speckle changes when assessing blood flow. Royl *et al.* (2006)[32] noted that 'during increased blood flow the intensity variations of the speckle pattern are more rapid'. The group found that in the exposure time the contrast of the pattern was reduced and quantification of the blurring effect was in fact possible with analysis of adjacent areas within the illuminated area. The speckle contrast K was then calculable thus; the mean velocity of moving elements could be calculated using this speckle contrast. It was noted that this offered a faster way of signal processing which allowed full-field imaging of skin perfusion in near real time.[29,31,33]

UTILISATION OF LSCI IN BLOOD FLOW MEASUREMENT

The reproducibility of laser speckle contrast imaging (LSCI) was compared with LDF as a method of assessing skin microvascular reactivity when utilising PORH.[29] It was found to offer both rapid skin blood flow measurements over wide areas coupled with good resolution. LSCI also displayed very good inter-day reproducibility when assessing forearm PORH and local thermal hyperaemia. Although the findings were positive, the study had some limitations such as limited number of subjects and the authors concluded that more data would be required to clearly evaluate any linearity between LSCI and skin blood flow.

A comparison of inter-day reproducibility utilising POHR with single point LDF integrated probe LDF with LSCI was carried out.[33] In agreement with Roustit *et al.* (2010)[29] they found LSCI faired better than LDF due probably to the larger skin area under measurement, however they did find that LDF was more sensitive to changes in skin blood flow at lower levels found on the finger pad area. It was also noted that the LSCI signal was more sensitive to changes in red blood cell velocity than concentration, and in agreement with Nakagami *et al.* (2010)[34] they found that the speckle contrast was not linear to either velocity or perfusion, and in fact the relationship between speckle contrast and velocity was highly non-linear. It was however concluded that this did not preclude the use of LSCI for quantitative analysis of perfusion, but that careful calibration and or interpretation would be required. However, the known percentage change in velocity did produce the same change in speckle contrast, thus LSCI was found to be more useful in measuring changes in perfusion rather than absolute measurements.[33] The advantages over LDF are that it offers high spacial and temporal resolution and is relatively user friendly and cheap to use.[35]

An investigation into the use of LSCI for the assessment of circulation in pressure ulcers was undertaken.[34] Although this work was utilising animal studies and small numbers were used, nevertheless it was concluded that LSCI could have great potential for assessing the severity of tissue damage in deep tissue injury, although much more work would be required in the area.

Photoplethysmography

BACKGROUND

Photoplethysmography (PPG) is an optical technique which →

measures blood volume changes in the microvascular bed in a non invasive, portable and cost effective manner. Pioneered by Alrick Hertzman in 1937, the first paper on PPG described the use of a reflection mode system used to measure blood volume changes in the fingers. PPT requires only a few basic components, a light source for the skin and a photo detector to measure variations in light intensity.[36] The light is initially absorbed, then scattered and reflected in the skin and the blood and some of the reflected light is detected by the photo detector.[37] Depth of light penetration is dependent upon the wavelength (red or near infrared) and the distance between the light source and the detector. The signal can in fact be divided into two separate parts, an AC and a DC signal. The AC signal relates to blood flow in the vascular bed and DC reflects variations in total blood volume.

INSTRUMENT AND MEASUREMENT TECHNIQUES

PPG currently uses low cost semi conductor technology with LED and matched photo detector devices working at red and near infrared wavelength. There are two main operating configurations, transmission where the skin sample is between the light source and detector and reflection where the LED and detector are side by side. Transmission provides more restrictions on the location of the tissue under study than the reflection mode.[36] It should also be noted that blood volume, movement of the vessel wall and the orientation of the erythrocytes may have an influence on the amount of light received.[37]

UTILISATION OF PPG

PPG was utilised as a clinically useful quantitative tool for healing of diabetic wounds by measuring scattering and absorption characteristics of tissue with near infrared spectroscopy.[38] Their study was carried out on 20 female hairless rats with 10 streptozotcin induced diabetic rats displaying 38 full thickness wounds. Cross and parallel polaraisation pictures were taken to evaluate the area and contraction of the wounds. Wound healing rate was half that in the diabetic group in comparison to the controls with the average absorption coefficient being twice as high, and the scattering coefficient 30% higher in the diabetic wounds. During healing both scattering and absorption coefficients increased faster with diabetes. The technique was found to differentiate between healing diabetic and non-diabetic wounds, however the study was carried out in animals, and a small sample size was utilised even though 38 wounds were assessed.

PPG was suggested as useful for many clinical settings including cardiovascular assessment of both arterial and venous disorders.[36] Arterial assessment such as vessel compliance and ageing could be assessed effectively using PPG as well as endothelial function, which was noted as an early event in atherosclerosis that correlated well with major risk factors associated with cardiovascular disease. The amplitude of the PPG pulse was noted as often correlating with LDF in the assessment of microvascular blood flow and the assessment of the viability of tissues. The ability to assess the tissue perfusion was suggested to be of particular importance for the clinical assessment of tissue viability and the healing potential when tissue breakdown had occurred.

PPG and LDF were utilised in a single probe when assessing blood flow at different tissue depths, in pressure ulcers on 17 individuals of both sexes, over 60 years of age.[37] They noted that blood flow was crucial in ensuring the tissue requirements for nutrition and oxygenation, as well as for the removal of waste products from the area, were fulfilled. Their dual probe system was found to successfully detect the relevant blood flow responses at different depths and when combining the two methods of assessing blood flow, LDF complemented the PPG.

Thermography

Temperature measurement has commonly been utilised for the assessment of cutaneous circulation, thus the use of thermal infrared imaging has been indicated for assessing microcirculation, and in fact skin temperature measurement has become common practice for the assessment of the vasculature function in critical illness.[39,40] The FLIR PM695 system was used to assess chronic venous stasis ulcers before and during treatment with water-filtered infrared-A ie a special form of heat radiation with a high tissue penetration.[41] This afforded the group the opportunity to take image sequences at 1 second intervals during the treatment process, providing a valuable visual impression of the heat distribution on and in the skin around the ulcer. The results showed healing either completely or near completion in 7 out of 10 patients (5 males and 5 females with chronic venous stasis ulcers, and a reduction in size in another 2 patients.

Thermography was compared with LDI in the assessment of primary Raynauds phenomenon (PRP) and secondary to systemic connective tissue disorders like systemic sclerosis (SSc) in a group of 7 subjects with PRP, 33 with SSc and 17 control subjects.[42] Patients with SSc are known to present with structural microvascular disease which can lead to ulceration and gangrene often resulting in amputation, therefore assessment and thus early indication of irreversible tissue damage is vital. They found a poor correlation between flux and temperature gradients along fingers. This was thought to be due to the fact that LDI is a more direct measurement of blood flow, thus more sensitive to changes in flow. It was noted that despite this poor correlation, neither technique was negated, rather that the different parameters of measurement with each system was required to be emphasised.

Transcutaneous Oxygen Tension $TcPO_2$

$TcPO_2$ is carried out with probes positioned on the leg and foot and a chest probe acting as a reference site, the probe uses an electrochemical technique. It has a gold cathode surrounded by an anode and a heater ring. Normal levels are approx. 60mmHg with values of 20mmHg or less indicative of severely reduced blood flow, and values may be used to predict vascular disease, the need for revascularisation, as well as providing an indication for successful healing following amputation.[43] Zimny *et al.* (2004)[44] utilised $TcPO_2$ to assess the skin oxygen supply in 21 patients with type 2 diabetes who were at risk of ulceration, but with no previous history of ulceration and compared this group with 20 subjects with diabetes with no foot lesions or neuropathy and 21 healthy controls. They found $TcPO_2$ to be significantly reduced in the at risk group in comparison to the other two groups and noted that $TcPO_2$ was a useful tool in the identification of the foot at risk in diabetes.$TcPO_2$ and $TcPCO_2$ (transcutaneous partial pressure of carbon dioxide) were compared with LDI in 31 subjects displaying severe leg ischaemia and 29 healthy control subjects as a method to compare pre-amputation assessments. The findings indicated that $TcPO_2$ could be considered an index of skin perfusion only when blood flow was reduced, and there was a lack of correlation between $TcPO_2$ and LDI in the normal controls.[15]

When $TcPO_2$ was compared with ankle brachial pressure index (ABPI) for the evaluation of percutaneous transluminal angioplasty in 60 subjects, it was found to display a positive correlation with ABPI both before and 4 weeks after angioplasty. However, 24 hours after the intervention, there was a lack of correlation, thus they concluded that ABI was still the most favorable method to indicate success of this revascularization procedure, and the use of $TcPO_2$ was not recommended as a predictor to success on account of it being representative of the microcirculation of a volume of tissue rather than the macrocirculation supplying a digit or a limb.[45]

$TcPO_2$ was evaluated with toe pressure in the management of chronic critical leg ischaemia. Neither method displayed any advantages over the clinical judgment of vascular surgeons, and thus was found not to lead to a better clinical outcome.[46]

Ankle Brachial Pressure Index (ABPI)

This method is often used in the initial stage of non-invasive vascular assessment[43] and is described as a simple, accurate and reproducible method.[47] The systolic pressure is measured at the ankle using either the posterior tibial artery or the dorsalis pedis and the value in mmHg obtained here is divided by the brachial systolic pressure, utilising a pressure cuff and a hand held Doppler probe.[43] A normal index value at rest is equal or higher than 1. This value decreases in relation to the severity of vascular disease with subjects with ulcers and gangrene displaying rest pain and tissue loss with an ABPI of less than 0.5.[43] If the ABI is found to be abnormal, segmental pressures can be used to define the level of the disease, with the cuff placed at upper and lower thigh, upper calf and ankle levels. Toe pressures utilising cuffs around the toes can be useful. A toe to brachial index of 0.75 is considered normal with 0.25 being found in association with sever arterial disease.[43,47]

Nailfold Capillaroscopy (NFC)

Capillaroscopy of the nailfold with less than 10x to 20x magnification has been used to assess digital microcirculation, and reproducibility measurements of capillary width and number of loops/millimeter have been measured. Nailfold capillaroscopy (NFC) was compared with fingertip lacticemy and LDI when assessing digital microcirculation in 44 patients presenting with systemic sclerosis and 40 controls.[48] There was a lack of correlation found with functional and morphological microvascular abnormalities measured by fingertip lacticemy and NFC. However, they concluded that it was appropriate to consider LDI and NFC as complementary tools for evaluating different aspects of microcirculation, with early detection of changes being paramount as microvascular abnormalities could result in reduction in the vessel lumen, decreased blood flow and hypoxia resulting in digital ulceration and ultimately in severe cases gangrene of the extremities.

Conclusion

In this review we have discussed the current and frequently used methods of vascular assessment that can be utilised for the evaluation of lower limb wounds. In the main the techniques can be useful for both clinical as well as for research purposes. LDF and LDPI both offer relatively user friendly, quick, non invasive and with LDPI, non contact direct methods of measuring blood flow. Multiple studies have utilised LDF and LDPI in the analysis of ulceration in the limbs, and have combined iontophoresis as well as reactive hyperaemia to investigate the mechanisms of vascular dysfunction, particularly for wounds resulting from diabetes mellitus and peripheral arterial disease. The more recent development of LSCI has added the possibility of full-field imaging of skin perfusion in near real time, and although studies are not yet abundant, do display this as a development with useful possibilities for the future. PPG is an optical technique which uses low cost, semi conductor technology and matched photo detector devices to measure blood volume changes in the microvascular bed. This makes it very cost effective as well as being portable and non invasive. Thermal infra red imaging has become common practice as a method of assessing vascular function in critical illness; it can be used over larger areas and in common with LSCI allows real time recording. $TcPO_2$ has proven particularly useful for the identification of the foot at risk in diabetes; it is another non invasive method which provides a continuous measurement. ABPI continues to be simple, effective, reproducible, user friendly and cost effective and is very much utilised as a quick clinical tool for the assessment of the arterial supply to the lower limb, as does NFC for the assessment of digital perfusion as it affords direct observation of the nailfold capillaries. Non of the methods discussed offer a panacea for vascular assessment related to the formation of ulceration in lower limbs as each measure different components (compartments) of the vasculature, either tissue O_2, macro or microcirculation, however each have strengths as well as drawbacks for the practitioner to evaluate prior to considering their utilisation. ■

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