# An Investigation into the Association Between Skin Microcirculation and Small Fibre Function in the Foot: Potential Implications in Assessing the Cutaneous Neurovascular Response in Diabetic Foot Disease

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A thesis submitted in partial fulfilment of the requirements of Staffordshire University for the award of the degree of Doctor of Philosophy by supervised research.

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I am convinced and confident of this very thing, that He who has begun a good work in us will [continue to] perfect and complete it until the day of Christ Jesus [the time of His return]."

- Holy Bible Philippians 1:6

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Sincerely,

Gayathri (Victoria) Balasubramanian

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## LIST OF ABBREVIATIONS

ABI – Ankle Brachial Index

AGEs - Advanced Glycation End Products

AH - Area of Hyperaemia

AH/AO - Area of Hyperaemia/Area of Occlusion; Hyperaemia repayment ratio

AO - Area of Occlusion

AV - Arteriovenous

BZ - Biological Zero

DFU - Diabetic Foot Ulcers

EDA – Electrodermal Activity

EDHFs - Endothelium-Derived Hyperpolarizing Factors

GDP - Gross Domestic Product. Laser

GSR – Galvanic Skin Response

Intraclass Correlation Coefficient - ICC

LASCA - Speckle Contrast Image Analysis

LDF - Laser Doppler flowmetry

LDI - Laser Doppler Imager

LDPM - Laser Doppler Perfusion Monitoring

LDPM - Laser Doppler Perfusion Monitoring

LSCI - Laser Contrast Speckle Imager

LSCI - Laser Speckle Contrast Imaging

PAD - peripheral arterial disease

PAOD - Peripheral Arterial Obstructive Disease

PF - Peak Flow

PU - Perfusion Units

PIV - Pressure-Induced Vasodilation

PORH - Post-Occlusive Reactive Hyperaemia

QSART - Quantitative Sudomotor Axon Reflex Testing

QST – Quantitative Sensory Testing

RF - Rest Flow

RSA – Respiratory Sinus Arrythmia

RSS - Risk Stratification Systems

SC – Skin Conductance

T – Temperature

TBI - Toe Brachial Index

TcPo2 - Transcutaneous Oxygen Pressure

TH1 - Time to Half Before Hyperaemia

TH2 - Time to Half After Hyperaemia

TL - Time to Latency

TM - Time to Max

TR - Time to Recovery

# ABSTRACT

# Abstract

Diabetes is a global public health problem as it is associated with various complications. One of the major complications of diabetes is diabetic foot syndrome, which leads to catastrophic events such as ulceration and amputation. The triggers of ulcerations are multifactorial, including cutaneous microcirculatory changes in the foot of people with diabetes. The cutaneous microcirculation of the foot is strongly influenced by the small fibres that mediate the sensation of heat and pain, in addition to sympathetic activities such as thermoregulation and vasodilation. However, there is a lack of knowledge on the subject of microcirculation, small fibre nerves, their relationship, evaluation and possible role in ulceration in the context of diabetic foot. This research aimed to investigate the relationship between cutaneous microvascular and small fibre nerve functions in the foot. The review of the existing literature, which was undertaken as a part of this thesis, revealed that there is a relationship between microcirculation and the functions of the small nerve fibres. The first study of this thesis highlighted that the skin microcirculation in the foot can be systematically and reliably assessed with the Post-Occlusive Reactive Hyperaemia (PORH) test with an occlusion time of 30 seconds, which makes the test potentially viable in a clinical setting for diabetic foot assessment. The minimal time occlusion can be safe for people with underlying complications and be easily measured alongside ABI or TBI. This study also confirmed that small fibre nerves play an important role in regulating skin temperature, which affects cutaneous perfusion. It was concluded that there is a strong relationship between cutaneous microcirculation and foot skin temperature. In addition, it was found that the skin temperature is an independent predictor of microcirculation, meaning it can be a surrogate method of assessing microcirculation. In summary, this research has contributed to a thorough understanding of the relationship between microcirculatory and both sensory and autonomic functions of the small fibre nerves and their interdependence. Risk assessment of diabetic foot requires comprehensive assessment as one parameter alone cannot help to understand the foot microclimate and identify a foot at risk. The results of the current thesis contribute to the understanding of soft tissue biomechanics and to help develop strategies for a comprehensive assessment of the diabetic foot using time-efficient methods such as PORH and foot temperature measurement. The findings have clinical implications as simple, non-invasive techniques can be instrumental in determining a foot at risk of ulceration, as temperature changes have been associated with foot complications. Such simple assessment techniques can be used in both high and low resource settings for mass screening or even self-screening of the foot. This, in turn, can aid in the prevention and early detection of ulcers, thereby reducing amputations.

# CHAPTER I

## INTRODUCTION

A part of this chapter was presented as:

An oral presentation on “Neurovascular and biomechanical aspects of diabetic foot: current research gaps”, 6th Postgraduate Research Conference, Staffordshire University, UK, 2018

An Oral presentation on “An Overview of PhD research project: Assessing the Risks of Diabetic Foot Ulcers Using Neurovascular and Biomechanical Parameters”, Research Seminar Series and meeting of Visiting Professors/Fellows, Staffordshire University, UK, 2019

An Oral presentation on “An assessment of skin microcirculatory responses in the diabetic foot, a critical analysis of the current literature”, 7th Postgraduate Research Conference, Staffordshire University, UK, 2019

A 3 minutes thesis presentation on “Assessing the Risks of Diabetic Foot Ulcers Using Vascular, Neurological and Biomechanical Parameters”, 7th Postgraduate Research Conference, Staffordshire University, UK, 2019 and Semi-finalist at a National level in the UK

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# Chapter 1 Introduction

## 1.1 Introduction

This chapter gives an overview of the doctoral thesis on the topic of "An Investigation into the Association Between Skin Microcirculation and Small Fibre Function in the Foot: Potential Implications in Assessing the Cutaneous Neurovascular Response in Diabetic Foot Disease". This is a topic of pivotal importance in the prevention and early diagnosis of diabetic foot ulcers (DFU) as it is said "Time is tissue" or "Time is limb". There are many groups in the scientific and commercial communities trying to improve the lives of people with diabetes, especially those with foot problems. Despite the wealth of literature available in the field of diabetes, there is a paucity of research specifically aimed at understanding complex event=s that can lead to foot ulcers, such as skin microcirculatory dysfunction. Firstly, microcirculation problems have been studied in relation to wound healing and are well documented. However, its role as a precursor in ulcer events and the importance of its assessment for the prevention and early detection of DFU has received limited attention in the literature. The underlying mechanisms driving tissue degradation in DFU have only recently begun to unfold. A better insight into these mechanisms would provide useful information about a foot at risk of ulceration.

Secondly, there has been a large array of improvements achieved in the last few years in terms of developing non-invasive techniques to assess skin microcirculation. In recent years there has been an increased interest in exploring methods for comprehensive assessment of the foot but that which is less-time consuming, practical and reliable. This research has focused on both key elements. In this Chapter 1, a comprehensive overview of diabetes, diabetic foot-related problems, anatomy of neurovascular structures of the skin and underlying pathology is provided. The chapter unravels the key research gaps in existing literature. Furthermore, the rationale for the current research, scope and boundary has also been included. Therefore, Chapter 1 lays a foundation for the current doctoral research work.

## 1.2 Diabetes – the burden and the disease

### 1.2.1 Diabetes

Diabetes continues to be a global public health concern. More than the disease itself, it is the associated chain of complications that are the dominant features of diabetes. Diabetes and its complications not only impose a huge health burden but also significantly impacts the economies of the patient, families, health service systems and nations through direct medical costs and indirect costs. It is estimated that the global direct health expenditure on diabetes in 2019 is USD 760 billion and is expected to grow to a projected USD 825 and 845 billion by 2030 and 2045, respectively (R.

Williams et al., 2020). This indicates that diabetes imposes a large economic burden on the global healthcare system and the wider global economy.

There is an annual upsurge in the number of patients being diagnosed with diabetes. The global diabetes prevalence in 2019 is estimated to be 9.3% (463 million people), rising to 10.2% (578 million) and 10.9% (700 million) by 2030 and 2045, respectively (Saeedi *et al.*, 2019). Research also shows that the prevalence is higher in urban (10.8%) in comparison to rural (7.2%) areas, and in high-income (10.4%) than in low-income countries (4.0%) (Saeedi *et al.*, 2019). Diabetes is a chronic, metabolic disease characterised by raised blood glucose (blood sugar) levels in the blood. There are many types of diabetes, of which type 1, type 2 and gestational diabetes are the most common. The other types of diabetes include latent autoimmune diabetes of adulthood (LADA), diabetes insipidus, Maturity Onset Diabetes of the Young (MODY) and others. Although there are various types of diabetes known, the most discussed are the two types namely, type 1 and type 2 diabetes. Type 1 diabetes is most commonly observed in childhood and is caused due to the autoimmune destruction of β-cells of the pancreas resulting in insufficient or no production of insulin (Gillespie, 2006). Thus, these patients require insulin replacements through injections or nasal administration. Type 2 diabetes is a metabolic disorder observed in a later age (most commonly in people over 40 years), which is caused by a combination of two main factors: defective insulin secretion by pancreatic β-cells and the inability of insulin-sensitive tissues to respond appropriately to insulin (Peate and Nair, 2016; Galicia-Garcia *et al.*, 2020). Glucose homeostasis requires tight regulation of processes associated with insulin release and activity. Any defects in any of the mechanisms involved in these processes can lead to a metabolic imbalance responsible for the development of the type 2 diabetes, which is characterised by insulin resistance or reduction in the ability of β-cells of the pancreas to produce insulin in response to the demand (Peate and Nair, 2016; Galicia-Garcia *et al.*, 2020). This leads to the increase in blood glucose levels that in turn damage the β-cells of the pancreas resulting in further decrease in the production of insulin. Thus, type 2 diabetes requires vigilant care and management to maintain glucose homeostasis. Some of the strategies used in the management of type 2 diabetes involves lifestyle modifications, use of oral hypoglycaemic agents and administering insulin replacement therapies (Peate and Nair, 2016). Despite constant care and attention, due to various factors a plethora of complications may arise. One of the major mechanisms that cause these complications is glycation.

### 1.2.2 Glycation

In diabetes patients, because of insulin deficit or resistance there is a prolonged hyperglycaemic state, which is the increased glucose levels in the blood. Various tissues and cells respond to the constant hyperglycaemic state in several ways that trigger a complex cascade of events which leads to cellular dysfunction (Singh et al., 2014). During this period, glucose forms covalent bonds with the plasma

proteins and lipids in the blood through a process referred as glycation or non-enzymatic glycosylation. Glycation can happen both within the body (endogenous) and outside the body (exogenous). Glycation is commonly associated with some of the changes that occur with ageing and metabolic disorders (Bhagavan, 2002; Singh et al., 2014). Diabetes accelerates the ageing process through endogenous glycation.

Various structural and functional proteins including plasma proteins and collagen are glycated (Singh et al., 2014). As a result of glycation of plasma proteins such as albumin, fibrinogen and globulins there are various deleterious effects such as alteration in drug binding in the plasma, platelet activation, generation of free radicals, formation of advanced glycation end products (AGEs), impaired fibrinolysis and impairment in immune system regulation (Singh et al., 2014; Rubin, 2017). Furthermore, despite the sequence of events leading to bone fragility is yet to be unveiled, it is known that the glycation of collagen causes its structural impairment, reduced bone mass and decreased integrity of the microarchitecture in the bones resulting in skeletal fragility. (Singh et al., 2014; Rubin, 2017).

It is important to understand the basics of glycosylation of proteins to appreciate the changes that occur in diabetes. During the formation of glycated proteins, a series of biochemical reactions takes place within the body that produces harmful end products. A nucleophilic addition reaction initiates a hyperglycaemia induced non-enzymatic glycation between a free amino group of a protein and a carbonyl group from a reducing sugar to form a freely reversible Schiff base. This reaction occurs over a period of hours and once a labile Schiff base is formed it rearranges to form a more stable Amadori product (Bhagavan, 2002; Ansari and Dash, 2013; Yamamoto and Sugimoto, 2016). A glucose derived Amodori product (ketoamine or fructosamine) reacts with itself or primary amines, such as the ε-amino-lysine and undergoes further reactions to form AGEs (Ansari and Dash, 2013). It is found that the degree of glycation achieved in a protein is determined by the concentration of sugar in the environment of the protein and in case of diabetes it will be the sugar levels in the blood (Bhagavan, 2002; Singh et al., 2014). As glycation-induced changes are linked to the duration of diabetes, studies assume that structural changes in blood vessels and nerve fibres and the associated functional changes within the body can commence during the pre-diabetes stage itself (Boulton, Cavanagh and Rayman, 2006; Bansal, 2015). The various stages of progression related to diabetes are summarised in Figure 1.1.

An association between prediabetes and complications of diabetes such as early nephropathy, small fibre neuropathy, early retinopathy and risk of macrovascular disease exists and cardiac symptoms such as reduced heart rate variability in patients with prediabetes (Bansal, 2015). As the elevation of blood sugar is a continuum, prediabetes cannot be neglected (Bansal, 2015). Prediabetes is often

defined as an intermediate state of hyperglycaemia with glycaemic parameters above normal but below the diabetes threshold (Levin *et al.*, 2008; Bansal, 2015). While prediabetes is commonly an asymptomatic condition, it always precedes the onset of diabetes and elevates the risk for developing diabetes. Most studies suggest that the yearly conversion rate of prediabetes to diabetes is between 5%-15% (Al-dajah et al., 2019; Fazli et al., 2020; C. Lorenzo et al., 2010). Understanding these changes that occur at a prediabetic state in addition to diabetes might aid early interventions such as lifestyle interventions and pharmacotherapy (high risk candidates) to prevent or delay the development of diabetes prevention. Nevertheless, if the prediabetes state is not reversed, it progresses to diabetes.

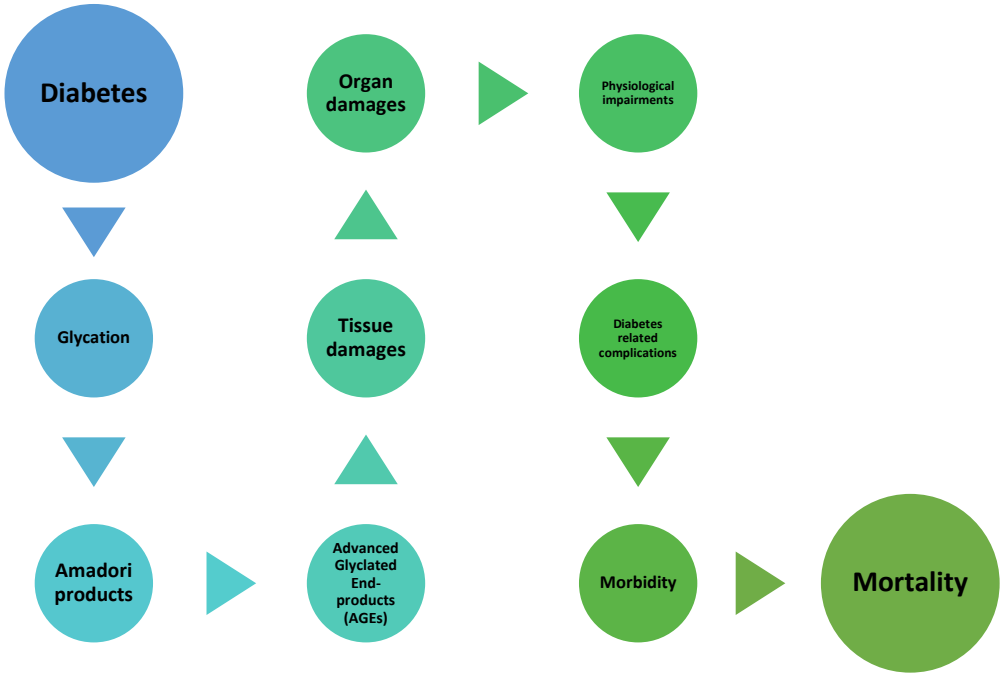



*Figure 1.1 Stages of diabetes-related complications*

### 1.2.3 Diabetes-related complications

As discussed earlier, the Advanced glycation is one of the major pathways involved in the development and progression of different diabetic complications (Singh et al., 2014). Studies show that pathogenesis of complications like retinopathy, nephropathy, neuropathy, cardiomyopathy, and vascular diseases are attributed to protein glycation and formation of AGEs (Goh and Cooper, 2008; Singh et al., 2014; Bhat *et al.*, 2017). Glycation alters the cell functions through denaturation of the protein and lipids. This results in functional changes of the protein or lipid and organopathy due to accumulation of AGEs in tissue, activation of receptor-mediated signal pathway in cells, generation of oxidative stress and carbonyl stress (Miyata, Ishikawa and van Ypersele de Strihou, 2003; Singh et al., 2014; Lankin and Tikhaze, 2016). AGEs activate specific receptors, like the receptor for AGEs (RAGE)

and indirectly binds to these sites, which is present on the surface of all cells (Dods, no date; Bhagavan, 2002; Singh et al., 2014; Stirban, Gawlowski and Roden, 2014). This binding causes the release of chemical substances that alter the function of the molecules in contact, stimulating inflammation, atherosclerotic changes, oxidative stress (which is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species in cells and tissues and the ability of a human biological system to detoxify these reactive products), and apoptosis (cell death) (Dods, no date; Stirban, Gawlowski and Roden, 2014; Pizzino *et al.*, 2017; Bitto *et al.*, 2018). AGEs are found to accumulate intracellularly; thereby, interrupting the function of intracellular proteins (Singh et al., 2014). Such AGE-related changes play a significant role in the development of complications in patients with diabetes (Singh et al., 2014). The most common sites of diabetes complications such as the kidney, retina, and atherosclerotic plaques have accumulated AGEs (Singh et al., 2014). The glycation related changes observed in various structures or organs of the body are summarised in table 1:1.

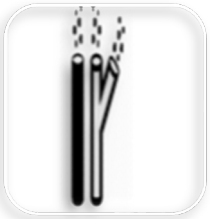
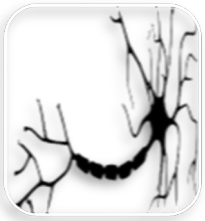
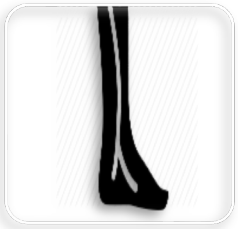
As mentioned above and in the table below AGEs themselves can contribute to inflammation and oxidative stress, which are implicated in vascular dysfunction. In the microcirculation of the foot, this can contribute to a pro-inflammatory environment, further exacerbating tissue damage. Overall, the presence of AGEs is associated with a range of detrimental effects on multiple physiological systems in individuals with diabetes.

(Bhagavan, 2002; Singh et al., 2014; Stirban, Gawlowski and Roden, 2014; Bansal, 2015; Rubin, 2017)

*Table 1.1 Potentials Changes Induced by AGEs in Various Structures of the Body*

| Structure | AGEs related changes |
|---|---|
| *Eyes*<br>  | • Increase in AGEs in the vitreous contributes to the development of diabetic retinopathy<br>• AGE-RAGE interaction play a role in the sustained inflammation, neurodegeneration, and retinal microvascular dysfunction<br>• AGEs progressively accumulate in the lens and retina affecting the vision<br>• AGE in the retinal blood vessel walls contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage. Increased microvascular permeability is one of the key changes observed in retinopathy |

| | |
|---|---|
| | • Crosslinking of proteins by AGE in the vessel wall results in increased vascular stiffness<br>• Apoptosis of pericytes is triggered by AGEs. Pericytes are functionally significant and loss of pericytes results in the vessels becoming haemorrhagic and hyperdilated leading to oedema (inflammation) and diabetic retinopathy<br>• AGEs play a pivotal role in the loss of lens transparency, which is the development of cataract |
| *Heart*<br>  | • AGE-immune complexes may play a role in atherogenesis, which is the formation of plaques or atheromas on the vascular walls. These changes lead to the development of atherosclerosis<br>• Diastolic dysfunction is related to HbA1c levels, and the most likely reason for this is the accumulation of AGEs in the myocardium<br>• AGE play an important role in diabetic myocardial fibrosis<br>• AGEs affect the physiological properties of proteins in the extracellular matrix by inducing the formation of cross-links<br>• AGEs also cause intracellular changes in vascular and myocardial tissue via interaction with AGE receptors<br>• The diabetic myocyte is exposed to several metabolic disturbances that contribute to contractile dysfunction |
| *Kidneys*<br>  | • AGEs interact with RAGE activating a series of intracellular signalling pathways, thereby, the AGE-RAGE aplaysplay a role in the development of diabetic nephropathy.<br>• AGEs trigger ian mbalance between the synthesis and degradation of extracellular matrix components, leading to the pathologic accumulation of collagens, fibronectins, and laminins<br>• The glycation of collagen followed by the formation of inter and intramolecular cross-links leads to structural changes resulting in increased stiffness, reduced thermal stability, and resistance to proteolytic digestion within the renal organs |

| | |
|---|---|
| *Vascular vessels*<br>  | Glycation damages the collagen and elastin throughout the body. AGEs-related intermolecular collagen cross-linking leads to:<br>• diminished arterial and myocardial compliance<br>• increased vascular stiffness and rigidity increase<br>• increase in diastolic dysfunction and systolic hypertension<br>• Changes in microvessels and macrovessels that may further contribute to major complications |
| *Nerves*<br>  | • Increased tissue and cellular glucose levels stimulate glycolytic and polyol pathways in the peripheral nerve<br>• The modification of proteins with AGEs causes structural and functional alterations in the peripheral nerve<br>• AGEs induce pathogenesis of sensory neuron damage<br>• The fibre loss or demyelination (the stripping of the myelin sheath) of nerve fibres in human diabetic peripheral nerves may be attributed to the accumulation of AGE<br>• The interruption of axonal transport contributes to the development of atrophy and degeneration of nerve fibres<br>• Microvessels in the peripheral nerve are affected by AGEs and reduces nerve blood flow and induces hypoxia in the peripheral nerve<br>• Glycation of collagen and laminin alters the electric charge of the basement membrane to increase the permeability of blood vessels, and cause thickening of the basement membrane |
| *Foot*<br>  | • The AGE-RAGE axis has a key role in the pathogenesis of diabefoot associated with diabetic neuropathy<br>• AGEs decrease nitric oxide, a mediator for vasodilatation, thereby, causing vascular issues related to diabetes foot<br>• The AGEs induced microvessel damage affect the microcirculation of the feet |
| *Bones* | • The cortical thickness and volumetric bone mineral density are decreased<br>• Bone strength is decreased<br>• The microvasculature of the bones are affected |

| | |
|---|---|
|  | • Bone Material Strength index (measured through microindentation procedure) is known to be reduced<br>• The quality is reduced because of the changes to the microarchitecture<br>• Hyperglycaemia or poor glycaemic control leading to accumulation of AGEs and microvascular changes leads to an impairment of bone formation. This, in turn, may result in reduced bone turnover because of coupling with bone resorption prolonging the life span of type 1 collagen, which then might become damaged by further accumulation of AGEs. Such impairment in material properties increases bone fragility and risk of fracture risk |

### 1.2.4 Diabetic Foot Syndrome and Diabetic Foot Ulcers

#### *1.2.4.1 Epidemiology and the burden of disease*

One of the complications that is associated with significant impairment of quality of life, decreased mobility, decreased independence, increased morbidity, and mortality and with impact on health care resources is diabetic foot syndrome. The diabetic foot syndrome or disease includes several pathologies, mainly diabetic peripheral neuropathy and peripheral arterial disease (PAD) which result in foot ulceration (Amin, Doupis and Paleou, 2016). Studies show that the global prevalence of diabetic foot varies from 3% in Oceania to 13% in North America, with a global average of 6.4% (Zhang *et al.*, 2017; Chun *et al.*, 2019). A diabetic foot ulcer (DFU) is a localised injury to the skin and/or underlying tissue, below the ankle, in a person with diabetes (Figure 1.2). Diabetic foot ulceration coupled with other comorbidities contribute to higher mortality. For instance, evidence shows a very high prevalence of ulceration amongst the group of people with diabetes on dialysis because of end-stage renal disease and the mortality in this patient group is found to be higher than for most forms of cancer ((Boulton and Whitehouse, 2000)). The annual incidence of DFU or necrosis in people with diabetes is known to be about 2% to 5% and the lifetime risk of developing an ulcer in that population ranges from 15% to 20% (Chun et al., 2019; Mayfield *et al.*, 2003; Jeffcoate & Harding, 2003; Levin et al., 2008; N. C. Schaper et al., 2003). The common sites for ulceration that are widely recognised are dorsal or plantar aspects of the toes, plantar metatarsal heads, and heel. In some patients the ulcers heal with re-epithelialisation, which is the restoration of epithelium in the denuded wound area (Levin *et al.*, 2008). The process wound healing involves the phases of haemostasis and coagulation, inflammation, proliferation, and maturation as depicted in figure 1.3. Once the skin on the foot is ulcerated, it is susceptible to becoming infected, leading to an urgent medical problem and in the

absence of healing, there can be infections and adverse outcomes such as amputation (Bakker *et al.*, 2016).

In the presence of diabetic foot problems, the impact is not only at the foot level. The mental health status of these people with diabetic foot problems worsens even more than in those suffering from other comorbid conditions. Studies show that people with diabetic foot problems are more likely to have depression and anxiety than people with diabetes without foot complications (Chapman, Shuttleworth and Huber, 2014; Prinz *et al.*, 2017). Particularly, diabetic foot ulcers disrupt patients' daily lives, including changes in sleep patterns, limited mobility, and impairment of certain aspects of life such as sexuality, feelings of loneliness, powerlessness, anxiety, and depression (Flett, Alpass and Harcourt, 1994; Persoon *et al.*, 2004; Herber, Schnepp and Rieger, 2007; Ahmad *et al.*, 2018). Furthermore, physically restrictive regimes like the use of lower extremity offloading measures could result in increased psychological pressure and emotional instability (Schram, Baan and Pouwer, 2009). Apart from this, foot problems can also cause financial issues because they can incur direct costs like treatment, medical supplies, food and nutrition, hospitalisation, surgery, nursing services, podiatric care, wound care, therapeutic footwear, orthotics and rehabilitation equipment and indirect costs such as absence from work, transportation and reduced productivity. This needs significant attention and interventions to prevent or treat DFUs at the earliest.

Delayed detection of skin lesions and signs of trauma or injury can result in a wound that, in turn, can become susceptible to infection. Delayed detection of skin lesions and signs of trauma or injury can result in a wound that, in turn, can become susceptible to infection. Often, infection, reduced supply, or complete disruption of vascular supply can lead to gangrene. Once the foot has become susceptible to gangrene, it leads to amputation (major or minor). Existing literature shows that delayed referrals can be an issue causing a lot of adverse complications in people with diabetes (Meloni and Apelqvist, 2018; Pankhurst and Edmonds, 2018). Ulcer severity also increases with time and delay in seeing a podiatrist or a foot specialist (Meloni and Apelqvist, 2018). It has been recognised that even a delay of hours (as a progression from an initial scratch to gangrene can take as little as 48 hours) can cost people with diabetes a limb as ulcers are highly susceptible to infection which can spread rapidly and cause overwhelming tissue destruction or gangrene requiring major amputation (Philips *et al.*, 2022). There may different reasons that cause these delays such as failure to recognise the adverse event or unappreciation of its severity by the patients or healthcare professionals, lack of awareness and education among people with diabetes about the examination of the feet or with regard to the recognition of warning signs like injury, infection, colour temperature and lesions, unawareness of the need for referral, inability to access to a specialist or multidisciplinary care, shortage of resources including time, scarcity of tools/equipment and a lack of education or training of both people with

diabetes and healthcare professionals and either the absence or failure of implementation of foot care guidelines (Meloni and Apelqvist, 2018; Pankhurst and Edmonds, 2018; Philips *et al.*, 2022). Many medical professionals fight for time. Especially when demand increases, physicians and nurses struggle to devote sufficient clinical time to meet the needs of diabetics in the community. Even in such cases, the waiting time for an appointment and a consultation can vary greatly, which can lead to delays. The literature shows that there were difficulties in accessing specialist vascular teams and noted a longer wait for people with diabetes and peripheral artery disease to see a specialist following the reorganization of vascular care in the UK (Pankhurst and Edmonds, 2018). The use of telephone consultation as an alternative to face-to-face consultation has become widespread in many general practices, partly because it is perceived to be more time efficient and can alleviate primary care access problems for housebound patients or those who work (Hammersley *et al.*, 2019). Although telephone consulting is viewed by both healthcare professionals and patients as improving normal access to care, there are safety concerns, largely due to the loss of formal investigation, although these concerns can be mitigated by the familiarity between patients and doctors and the condition (McKinstry *et al.*, 2009) Whether telephone consultations save time is widely debated (McKinstry, 2002; McKinstry *et al.*, 2002, 2009; Richards *et al.*, 2002). Also, telephone consultations may not be appropriate for complex issues, so such health issues may require in-person consultations, which are time-consuming. However, in this current times, post-pandemic era, the use of e-Health tools for healthcare professionals-based management and self-management has become increasingly common. For instance, there are mHealth tools such as mobile apps and personal digital assistants such as that have diabetes skills app integrated within them to help with the self-management of diabetes (Balasubramanian, Beaney, et al., 2021; Bonoto et al., 2017; Chambers et al., 2020; Dincer & Bahçecik, 2020; Jeffrey et al., 2019). Using these tools could help improve HbA1c control and improve perceptions of self-care by contributing to better information and health education (Bonoto et al., 2017). Developing and implementing efficient apps and tools in eHealth and mHealth for foot care is an ongoing research area (Bonoto *et al.*, 2017; Dincer and Bahçecik, 2020; Kilic and Karadağ, 2020). But existing literature expresses a clear demand for them (D. Wallace et al., 2019). Be it in person or remote consultation, the need for a simplistic, time-effective and non-invasive tool for comprehensive foot assessment is apparent. The use of such tools can not only help assessment by healthcare professionals but also for people with diabetes for self-assessment.

It is estimated that only two-thirds of DFUs will eventually heal, but approximately 28% may result in some form of lower extremity amputation (Bakker *et al.*, 2016). Approximately, 40–70% of all non-traumatic amputations of the lower limbs occur in patients with diabetes (Alexiadou and Doupis, 2012). Moreover, it is estimated that around 85% of non-traumatic amputations are preceded by

diabetic foot ulcer (Alexiadou and Doupis, 2012). Effective evidence-based prevention programme with strategies for early detection and control can reduce the amputation rate by 50% (Apelqvist and Larsson, no date). Hence, understanding the risks associated with foot ulcer development and its course is crucial. This can potentially throw light on some of the predictive factors to develop strategies for early interventions. Eventually, relevant interventions can be implemented to improve the quality of life in diabetic patients and prevent adverse complications such as amputation.

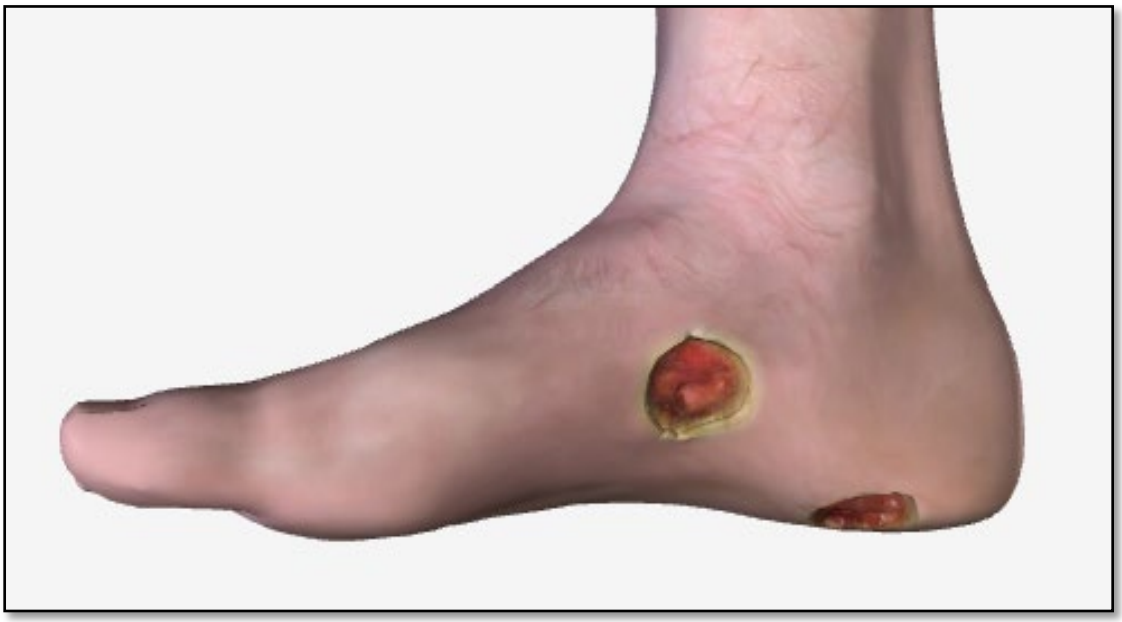


*Figure 1.2 Diabetic Foot Ulcer*

The image was taken from Biodigital platform

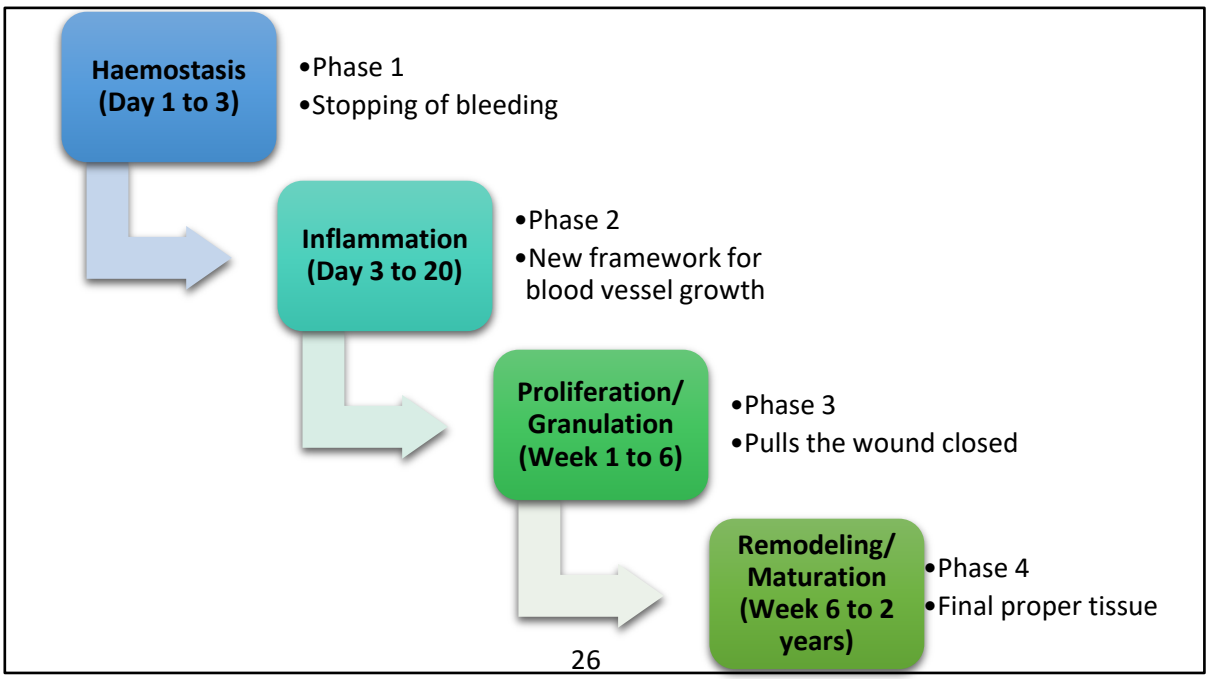





*Figure 1.3 Phases of wound healing*

#### *1.2.4.2 Pathophysiology of DFU*

The pathway to foot ulcer is complex and multifactorial as depicted in Figure 1.4. So, rarely a foot ulcerates due to a single underlying cause (Armstrong and Lavery, 2016). The well-known triad behind the occurrence of a DFU incident are peripheral neuropathy, peripheral vascular disease and trauma. The trauma could be due to various extrinsic and intrinsic risk factors that trigger the diabetic foot to ulcerate (Armstrong and Lavery, 2016). Extrinsic factors include trauma, ill-fitting shoes, walking barefoot and the like. Intrinsic factors include pathologies within the body like neuropathy or vascular disease. A combination of two or more of these factors results in ulceration as shown in Figure 1.4 below (Armstrong and Lavery, 2016). However, one of the major factors for diabetic foot ulceration is neuropathy (Armstrong and Lavery, 2016). In order to better understand the potential chain of events that lead to DFU, it is important to discuss the neurovascular aspects of the foot.

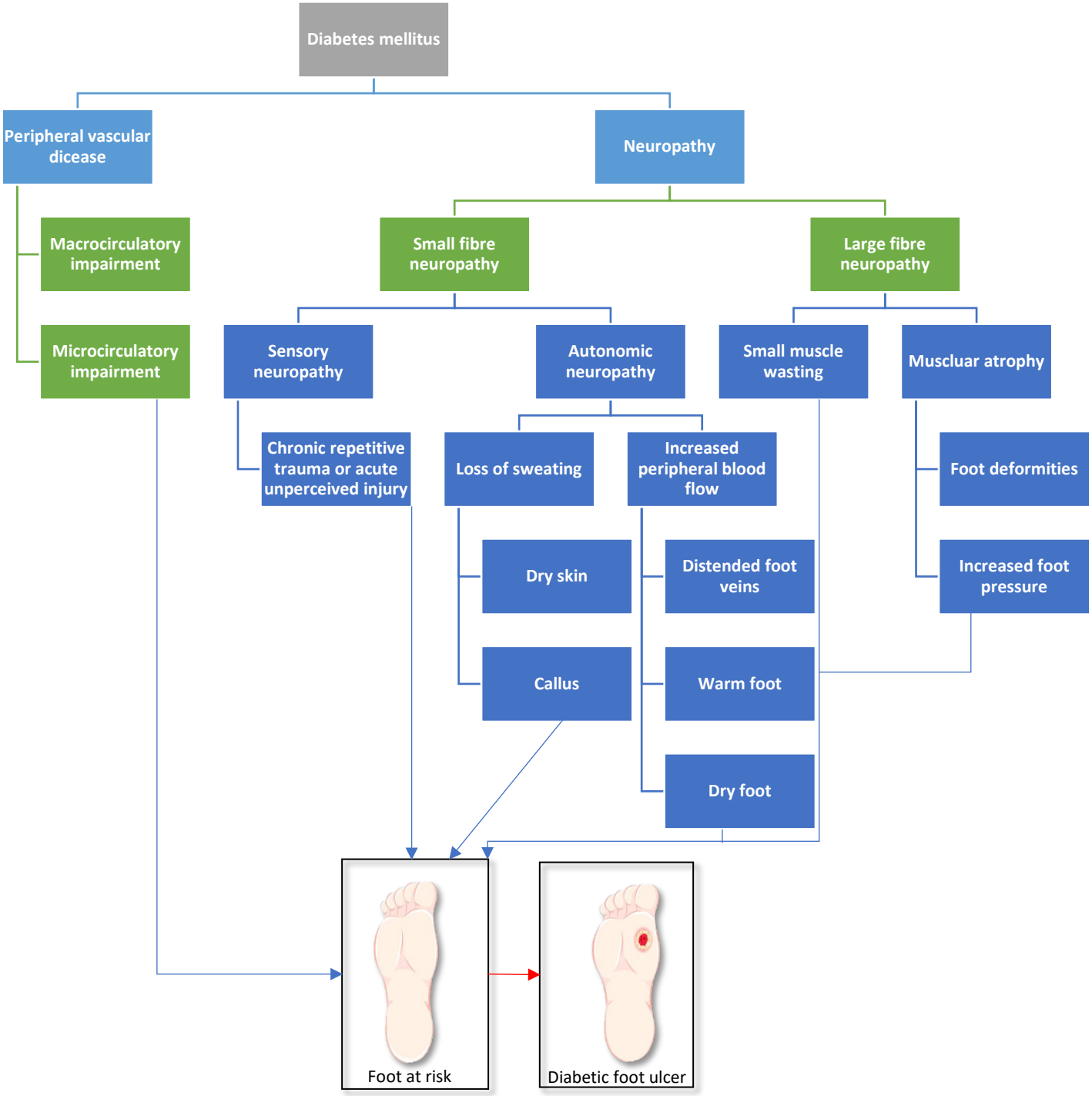



*Figure 1.4 Pathway to foot ulcer in people with diabetes*

Image adapted from **(Boulton and Whitehouse, 2000)**

### *1.2.4.3 Diabetic Neuropathy*

Diabetic neuropathy is defined as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes" (Levin *et al.*, 2008). Diabetic neuropathy can be both clinical and subclinical. Confirmed clinical neuropathy is defined as the presence of clinical neuropathy signs and confirmation through abnormal quantitative neurological function tests (Levin et al., 2008). Subclinical neuropathy is the presence of abnormal quantitative neurological functional tests but the absence of clinical signs during examination (Levin *et al.*, 2008). There is a large volume of published studies describing the diverse classifications of diabetic neuropathy. Anatomically, neuropathy can be classified as small or large fibre neuropathy, based on whether the large or small fibres of the nerves are affected. These nerve fibres are classified based on their thickness. The large fibre nerve cells have diameters of more than about 5 micrometres, whereas the small fibres are thinner. Depending on the involvement of the type of nerve fibres, the manifestation of the symptoms, signs and Electrodiagnostic features varies (Misra, Kalita and Nair, 2008). The symptoms of both the types of neuropathies (small and large), the functions and structural differences observed between the small and large nerve fibres are summarised in Figures 1.5, 1.6 and 1.7 below. Understanding these symptoms can aid in diagnosing peripheral neuropathy (Misra, Kalita and Nair, 2008).

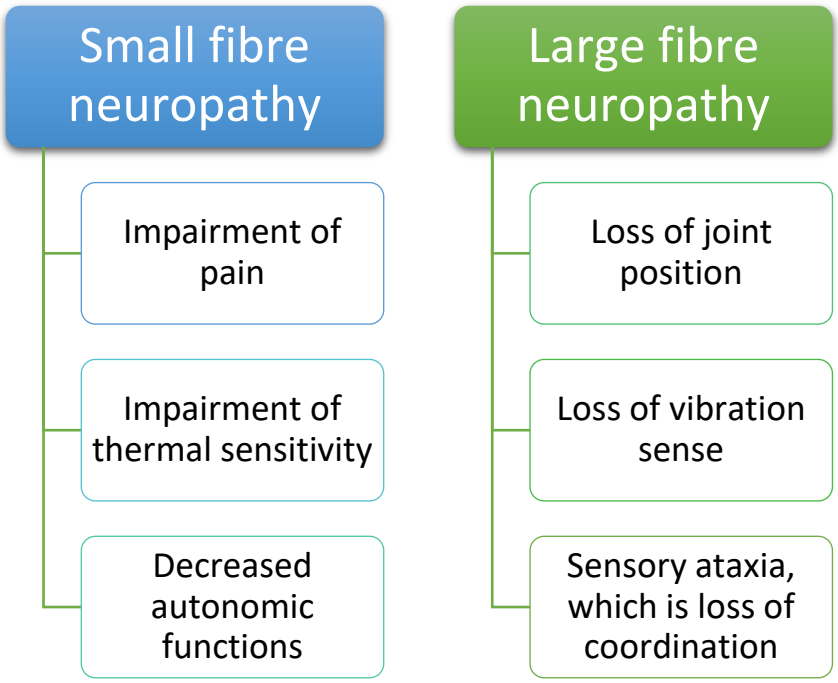



*Figure 1.5 Small and large fibre neuropathy*

Image was created using the data from (Misra, Kalita and Nair, 2008)

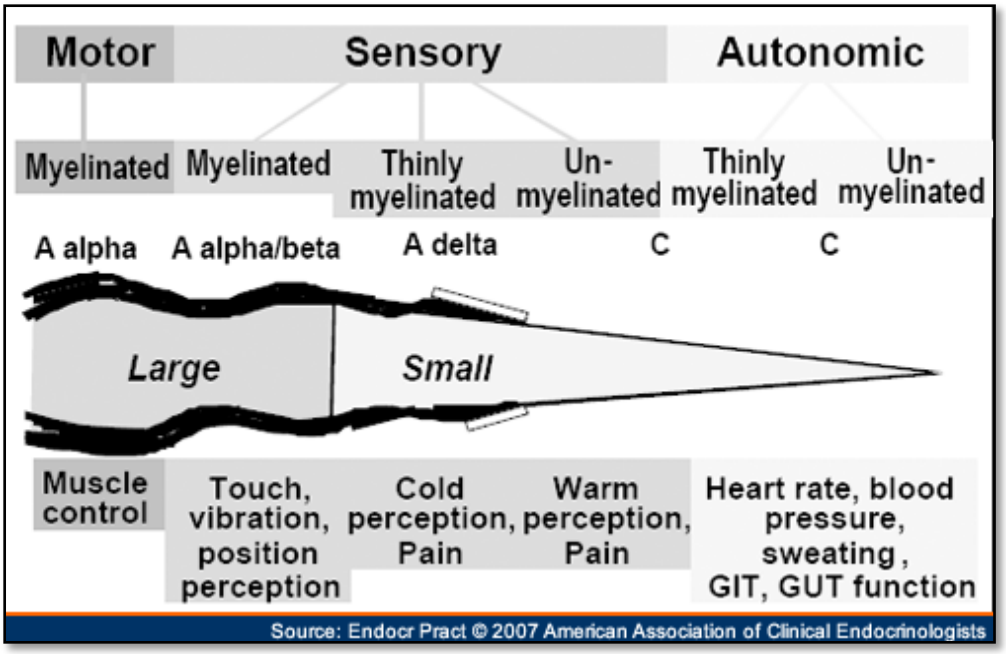



*Figure 1.6 Large and Small Nerve Fibres*

Image Source: Endocrine Practice© 2007 American Association of Clinical Endocrinologists

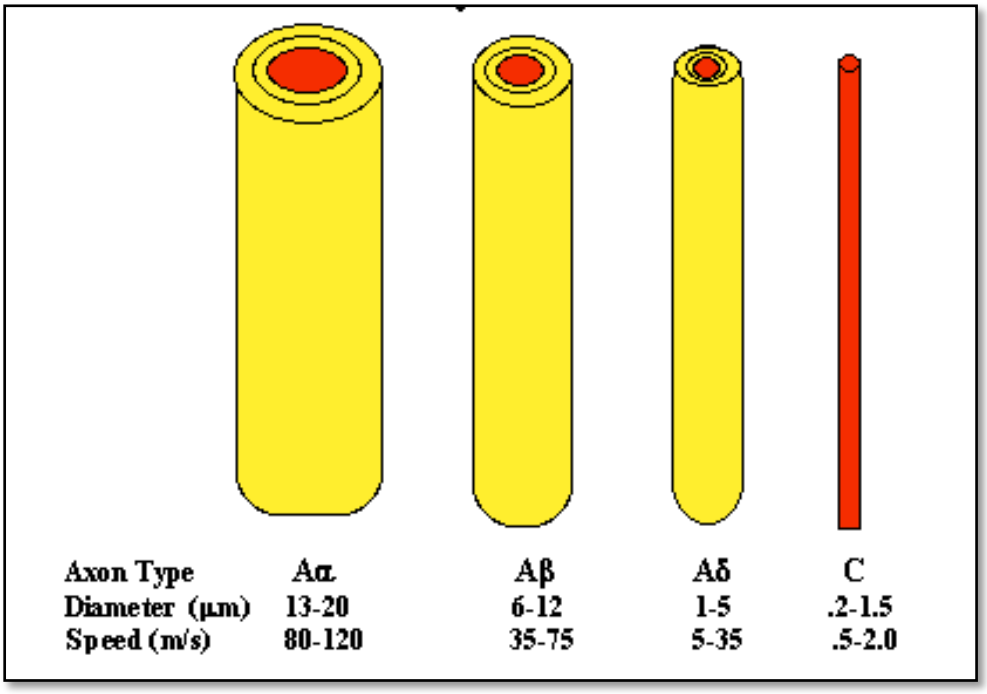



*Figure 1.7 Difference in nerve fibres by size*

Image copyright Eric H. Chudler 1996-2019 (Washington University, no date)

#### 1.2.4.3.1 Peripheral Neuropathy

Peripheral Neuropathy is the most common type of neuropathy in people with diabetes. It usually manifests as distal symmetrical neuropathy, where the sensory loss begins with the toes moving upwards to the feet and legs (Veves, Giurini and LoGerfo, 2006). Patients with distal symmetrical neuropathy can have numb or dead feeling (Veves, Giurini and LoGerfo, 2006). In contrast, some patients can also experience pain or tingling sensations (Veves, Giurini and LoGerfo, 2006). Peripheral neuropathy can be diagnosed by examination of the foot, using various scoring systems to assess neuropathy and related disability, and monofilament test (Armstrong and Lavery, 2016). The pathogenesis for distal symmetrical neuropathy is associated with metabolic factors and vascular factors (Schramm, Dinh and Veves, 2006; Veves, Giurini and LoGerfo, 2006). The common hypothesis for diabetic peripheral damage is that the chronic hyperglycaemic state triggers non-enzymatic glycation, increased free radical formation, Protein Kinase C (a regulator for vascular smooth muscle function) hyperactivity, and the related changes. These changes affect the blood vessels and the nerves. The nerves are supplied by many microvessels. The endothelial dysfunction in the microvasculature results in decreased blood flow to the nerves causing damage and abnormalities in nerve growth (Veves, Giurini and LoGerfo, 2006; Obrosova, 2009). Microcirculation and related dysfunction in people with diabetes will be discussed in detail later in this thesis. In summary, the duration of diabetes, glycaemic control and vascular factors are observed to be associated with neuropathy (Veves, Giurini and LoGerfo, 2006).

The peripheral neuropathy is a common complication of diabetes and DFU is found to be associated with somatic and autonomic neuropathy (Armstrong and Lavery, 2016). As discussed previously, changes due to neuropathy causes various problems. Patients with sensory loss can be vulnerable to mechanical, thermal or chemical injuries that can result in a foot ulcer incident (Veves, Giurini and LoGerfo, 2006). For instance, when patients experience sensory loss in their feet, it enhances the risk to develop foot ulcer by seven times in comparison to non-neuropathic diabetic patients (Armstrong and Lavery, 2016). As sensory loss advances there can be loss of proprioception and ankle or knee reflexes (Veves, Giurini and LoGerfo, 2006). Proprioceptive loss is the lack of ability to sense stimuli arising within the body regarding position, motion, and equilibrium (Armstrong and Lavery, 2016). Therefore, proprioceptive loss makes the patient more vulnerable to trauma and injuries. Peripheral autonomic dysfunction at a sympathetic level results in a dry skin (Armstrong and Lavery, 2016). In the presence of autonomic neuropathy, the skin becomes dry owing to the reduction in sweating. The skin microcirculation plays a vital role in sweating as well. One of the key roles of skin microcirculation is thermoregulation, which is controlled by neural mechanisms, hormones and chemicals (Guyton and Hall, 2015). For instance, during an event of heat, sweating is facilitated through microcirculation to

regulate the body temperature and cool the surface of the skin (Charkoudian, 2010; Guyton and Hall, 2015). But, due to the co-existing microcirculatory issues in patients with diabetes, the skin maybe dry and the neurovascular structures may not respond adequately to the increased physiological demand such as thermal stress. Consequently, dry skin paves the way for infections (Veves, Giurini and LoGerfo, 2006). In the absence of a peripheral vascular disease, the foot may still be warm with distended dorsal foot veins but pain-free due to neuropathic complications (Armstrong and Lavery, 2016). Although many patients with diabetes are neuropathic, it remains undiagnosed for a long time because of the pain-free state. Sometimes the diagnosis may be delayed until patients present themselves when further complications arise.

As narrated above, neuropathy and vascular changes in diabetes are interlinked. Neuropathy seen in conjunction with ischaemia makes the foot vulnerable to pressure from footwear (Armstrong and Lavery, 2016). A compromised peripheral sympathetic system increases the blood flow to the limbs thereby, elevating the precapillary load (Archer, Roberts and Watkins, 1984; Rayman, Hassan and Tooke, 1986). This results in capillary basement membrane thickening as elicited in the haemodynamic theory of microcirculation (Boulton et al., 2020). Sympathetic denervation can also result in arteriovenous shunting of the blood, also known as “capillary steal syndrome”, where the blood bypasses the capillaries depriving the tissues from nutrients (Boulton et al., 2020). Thus, a comprehensive understanding of the vascular aspects is essential to resolve the complex issues of DFU.

#### *1.2.4.4 Peripheral Vascular Disease*

##### 1.2.4.4.1 Macrocirculation

Vascular complications are widely observed in diabetic patients. PAD has deleterious effects on the foot health. Two major leg artery issues arise in diabetic patients (Levin *et al.*, 2008). Firstly, the atherosclerotic occlusion, a commonly observed problem, which affects the popliteal more in comparison to the femoral and iliac arteries (Levin *et al.*, 2008). This occlusive disease leads to enlargement of the arteries below the knees (Levin *et al.*, 2008). Also, the ankle arteries pressure is found to be low and such lower arterial predisposes to infections (Levin *et al.*, 2008). Ankle Brachial Index (ABI) is a non-invasive vascular screening test to identify large vessel, PAD by comparing systolic blood pressures in the ankle to the higher of the brachial systolic blood pressures. ABI is measured using a continuous wave Doppler, a sphygmomanometer and pressure cuffs to measure brachial and ankle systolic. The ABI is derived by dividing the highest ankle systolic pressure by highest brachial systolic pressure and 0.91 to 1.3 is considered to be within a healthy range (Potier *et al.*, 2011). Secondly, arterial wall calcification is associated with diabetes that pries with the leg blood pressure (Levin *et al.*, 2008). The arterial calcification modifies the vascular flow in the legs and feet thereby

altering blood pressure (Levin *et al.*, 2008). The wall expansion, which is essential for the prevention of atherosclerotic plaque formation is also compromised (Levin *et al.*, 2008). Additionally, increased intraluminal pressure makes the patient susceptible for having atherosclerosis (Levin *et al.*, 2008). Sometimes there is development of thrombosis and occlusion in distal arteries, which leads to infection and gangrene of the toes (Levin *et al.*, 2008). Arterial Stiffening also interferes with the blood flow in the veins. Diabetic neuropathy that leads to muscle loss further reduces the blood return to the heart (Levin *et al.*, 2008). Furthermore, the autonomic neuropathy damages the sympathetic nerves that control the blood flow to the feet. There is a reduced vasoconstrictive response whilst standing in diabetic patients (Levin *et al.*, 2008). The unusual higher flow of blood in the legs during standing position, coupled with the rise in the intraluminal pressure due to gravity stretches the arterial wall compromising its expansion during a systole (Levin *et al.*, 2008). The continued higher flow of blood with reduced wall movement favours the development of atherosclerotic plaque (Levin *et al.*, 2008). The autonomic neuropathy that permits persistently high blood flow in the foot in standing posture is probably the responsible for lowering nutritional flow relative to shunt microvessel in the foot in long-standing diabetes (Levin *et al.*, 2008).

#### 1.2.4.4.2 Microcirculation

The microvasculature is the network of finer arteries, arterioles, capillaries, and venules that supply and drain blood from every tissue and organ in the body (Figure 1.8). These microvessels that are devoid of a muscular layer and their diameter ranges from 5 to 200 µm (Yamaguchi, Ishikawa and Imai, 2018). The blood circulation within the microvessels or microvasculature, is the microcirculation. Microcirculation plays a vital role in nutrition exchange to the tissues, removal of waste and last but not the least, thermoregulation (Guyton and Hall, 2015; Boulton et al., 2020). Cutaneous blood flow can increase or decrease substantially in response to thermal stress. Vasodilation and increased skin blood flow are essential to heat dissipation during exposure to heat and exercise (Charkoudian, 2003). In contrast, vasoconstriction and decreased skin blood flow to prevent heat loss to protect against hypothermia during exposure to cold is necessary (Charkoudian, 2003). These skin blood flow mechanisms both local and reflex are controlled by nerves, endothelial derivatives and metabolic factors (Charkoudian, 2003). Such responses are a vital aspect of normal thermoregulation. These observations demonstrate that the skin blood flow is affected by skin temperature and neurovascular interactions (Fromy *et al.*, 2002). Hence, it could conceivably be hypothesised that vascular changes due to abnormal neuronal control are reflected in the cutaneous thermal changes. Prior studies that have noted that the skin temperature on the feet increases in the presence of complications (Bharara, Cobb and Claremont, 2006; Gatt et al., 2018). As previously discussed, glycation, which is defined as the cohesion of a carbohydrate to another biomolecule, such as a protein, lipid, or DNA either

enzymatically or non-enzymatically, causes both structural and functional damage to the neurovascular system. It damages the collagen and elastin throughout the body. AGEs related intermolecular collagen cross-linking leads to increased vascular stiffness and rigidity (Bhagavan, 2002; Singh et al., 2014). AGEs induce pathogenesis of sensory neuron damage, the fibre loss or demyelination of nerve fibres, atrophy and degeneration of nerve fibres and reduced blood flow to the nerves through microvasculature inducing hypoxia (depriving oxygen and causing suffocation) in the peripheral nerves (Bhagavan, 2002; Singh et al., 2014). Overall, one of the issues that emerges from the critical review of literature is that foot microcirculation and nerve functions are impaired in people with diabetes. Therefore, the neurovascular aspect at the plantar surface plays a vital role to prevent injuries or trauma and their pertinent function ensures an optimal environment in terms of thermoregulation at the soles (Flynn, Tooke and Flynn, Michael D, 1990; Guyton and Hall, 2015; Charkoudian, 2003).

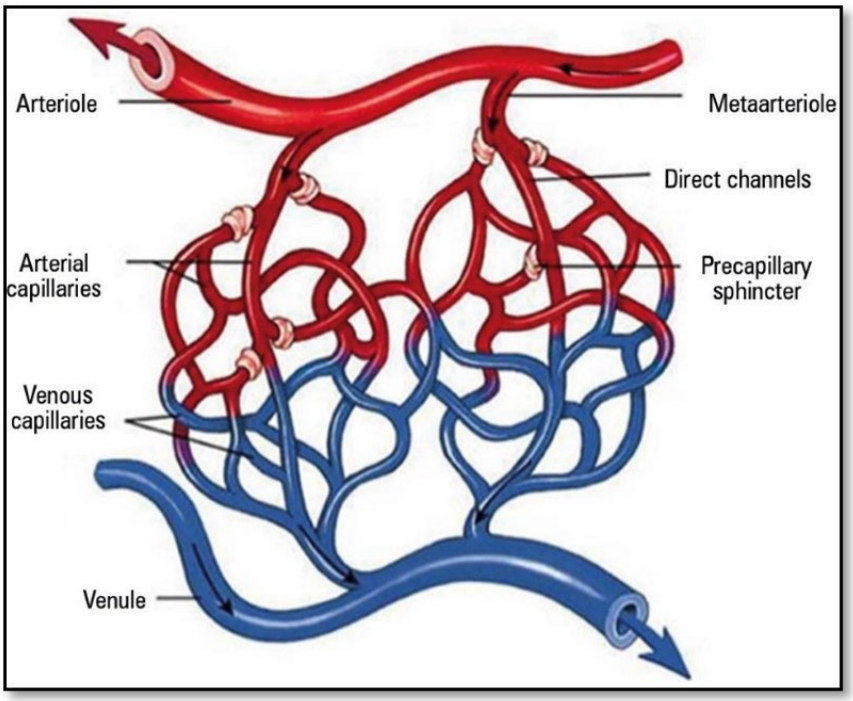



*Figure 1.8 Microvasculature*

Image taken from (Hoff, Gregersen and Hatlebakk, 2009)

There are many theories put forth by several studies on microcirculation. While the concept of 'small vessel diseases is widely debated, the structural damage and functional abnormalities of microcirculation is irrefutable (Flynn and Tooke, 1992; Korzon-Burakowska and Edmonds, 2006a; Veves, Giurini and LoGerfo, 2006). History of evidence on microcirculation and related disturbances exist. Previous research into microcirculation has focused on both structural and functional

dysfunction at microvascular level in people with diabetes (Flynn and Tooke, 1992; Boulton et al., 2020; Veves, Giurini and LoGerfo, 2006; Körei et al., 2016). Structural damage to the endothelium was reported in early 1959 (Goldenberg *et al.*, 1959). Historically, in 1983 Parving introduced the "haemodynamic theory" to explain microangiopathy in diabetes (Flynn and Tooke, 1992; Veves, Giurini and LoGerfo, 2006). He proposed that the increased microvascular blood flow triggers endothelial injury response, followed by microvascular sclerosis (Flynn and Tooke, 1992; Veves, Giurini and LoGerfo, 2006). This in turn may lead to functional abnormalities such as impaired maximum hyperaemic response, reduced tissue response to injury or trauma, autoregulation of blood flow and changes to vascular tone (Flynn and Tooke, 1992; Boulton et al., 2020; Veves, Giurini and LoGerfo, 2006). Another theory on microcirculation is "capillary steal syndrome", which proposes that the sympathetic denervation causes arteriovenous shunting (Boulton et al., 2020). As a consequence of the blood flow is shunted away from the capillaries depriving the tissue from nutrition despite the increase in peripheral blood flow (Flynn and Tooke, 1992; Boulton et al., 2020; Korzon-Burakowska and Edmonds, 2006a; Chao and Cheing, 2009). Debatable evidence exists to identify the contribution of either of these theories and their operation resulting in microvascular impairments (Chao and Cheing, 2009). However, it is implied that under stress such as thermal, mechanical or injury related, when demand is increased, the microcirculation needs are not met. In view of all that has been mentioned so far, one may suppose that microcirculatory dysfunction plays a role in diabetic foot complications.

#### *1.2.4.5 Neurovascular interactions in the foot*

Microcirculation is known to contribute to the pathogenesis of neuropathy and an association between the degree of microvascular dysfunction and severity of neuropathy has been identified (Veves, Giurini and LoGerfo, 2006; Vas, Green and Rayman, 2012). Thus far, presence of microvascular complications is directly linked to small fibre dysfunction (Vas, Green and Rayman, 2012; Körei et al., 2016). Neuropathy with small fibre involvement results in altered pain perception and impaired thermal sensation to both cold and heat stimuli. As discussed previously, this in turn affects microcirculatory responses of the feet. In line with this statement, evidence shows reduced nerve-axon reflex upon local heating in the foot of people with diabetes (Vas, Green and Rayman, 2012). Small fibre dysfunction is known to precede large fibre complications in people with diabetes (Körei et al., 2016). As sensory loss advances, the large fibres are involved with loss of proprioception and ankle or knee reflexes (Veves, Giurini and LoGerfo, 2006). It can thus be suggested that people with loss of protective sensation due to neuropathy can be vulnerable to injuries leading to a foot ulcer incident (Boulton and Whitehouse, 2000; Veves, Giurini and LoGerfo, 2006). Peripheral autonomic nerve dysfunction at a sympathetic level results in a dry skin (Khalfallah et al., 2012; Körei et al., 2016;

Armstrong and Lavery, 2016). Furthermore, autonomic dysfunction co-existing with microcirculatory dysfunction resulting in decreased activity of sweat glands gives dry feet (Armstrong and Lavery, 2016). Consequently, dry skin along with cracks and fissures paves the way for infections (Veves, Giurini and LoGerfo, 2006). In summary, structural microcirculatory disease can affect the blood supply to nerves promoting neuropathy and on the other hand small fibres play a major role in the microcirculatory responses. Therefore, it can be concluded that there is an unambiguous relationship between microcirculation and small fibre dysfunction relating to diabetic foot complications.

The foot is continuously under mechanical stress due to weight-bearing activities of daily living such as walking, exercise, and standing. It is exposed to various trauma, physical injury due to sudden or violent action, exposure to dangerous toxins or repetitive mechanical stress. Some of the extrinsic factors for trauma are thermal (Example: hot surfaces), mechanical (Example: repetitive insult from ill-fitted shoes), and chemical (Example: corn treatments) (Boulton, 2000; Boulton et al., 2020; Armstrong and Lavery, 2016). On the other hand, some of the intrinsic factors that contribute to the risk of trauma are foot deformity and glycation-related changes in case of diabetes. The small fibres, Aδ-fibres and C-fibres innervate the skin and are responsible for cutaneous (skin) sensations, mediating thermal sensations, and autonomic functions such as cutaneous blood flow and sweating (Krämer *et al.*, 2004; Misra, Kalita and Nair, 2008; Themistocleous *et al.*, 2014), whereas the large fibres aids in muscle control, touch and vibration sensation, and proprioception (Vinik *et al.*, 2003). The loss of these protective sensations in the feet increases the risk of injuries (Boulton et al., 2020; Armstrong and Lavery, 2016). The role of microcirculation in foot complications is evident. However, there has been no detailed investigation of its relationship to ulcer. The microcirculatory mechanisms that underpin ulceration incident are not fully understood. From previous research, it is well-established that the triad, macrovascular disease, neuropathy, and mechanical stress are involved in the pathogenesis of diabetic foot ulceration. Although microvascular disease or microangiopathy cannot be a standalone cause for a DFU incident, the interaction of the triad with microcirculation and its involvement cannot be denied. Several studies have attempted to investigate the role microcirculation in DFU, however, research to date has not yet determined a causal relationship. In the absence of macrovascular issues and occlusive arterial diseases, a neuropathic foot with palpable pulses may imply microcirculation as a causative factor in the development of ulceration (Flynn and Tooke, 1992; Korzon-Burakowska and Edmonds, 2006a; Veves, Giurini and LoGerfo, 2006). Whilst more research is mandated to understand structural damage of microcirculation and its direct association to DFU incidents, evidence shows that functional damage may impair normal vasodilatory mechanisms in response to injury and threaten cutaneous perfusion (the term cutaneous perfusion means the passage of blood or similar fluid through the blood vessels in the skin; also the term

cutaneous microcirculation and perfusion is used interchangeably), which may promote tissue breakdown (Flynn and Tooke, 1992). According to the "haemodynamic theory", increased microvascular blood flow triggers endothelial injury response (Flynn and Tooke, 1992; Veves, Giurini and LoGerfo, 2006). This results in functional abnormalities such as impaired maximum hyperaemic (defined as an excess of blood in the vessels of an organ or tissue usually caused by a physiological demand instigated through a stimulus) response, reduced tissue response to injury or trauma, autoregulation of blood flow and changes to vascular tone (defined as the tissue integrity or degree of constriction experienced by a blood vessel relative to its maximally dilated state) (Flynn and Tooke, 1992; Boulton et al., 2020; Veves, Giurini and LoGerfo, 2006). With respect to DFUs, it is proposed that the impaired microcirculatory response may induce microcirculatory failure, resulting in tissue necrosis and ulceration (Flynn and Tooke, 1992; Korzon-Burakowska and Edmonds, 2006a). The role of microcirculation in the development of ulceration, gangrene, necrosis and wound healing is previously denoted (Flynn and Tooke, 1992; Boulton et al., 2020; Levin *et al.*, 2008; Lanting et al., 2017). It is therefore likely that connections exist between microcirculatory dysfunction and DFUs. Thus, understanding functional abnormalities is of importance when studying diabetic foot syndrome.

Post-occlusive reactive hyperaemia (PORH) can be defined as a transient increase in microvascular blood flow following a brief period of arterial occlusion. It is one such hyperaemic response known to be influenced by endothelial, myogenic, neurogenic, and metabolic factors. Therefore, PORH allows a combined assessment of endothelial dependent and independent function (Lanting et al., 2017). Additionally, the decreased PORH response reflects decreased tissue response to injury. Studies have shown reduced vasodilatory capacity on heating and implied the association between structural microvascular disease and C fibre dysfunction (Krishnan and Rayman, 2004; Vas, Green and Rayman, 2012). The study suggested that structural microcirculation is more likely to impact tissue nutrition and reduced blood supply to the nerves may result in nerve ischaemia leading to neuropathy (Vas, Green and Rayman, 2012). The same heat provocation test has also been used for early detection of small fibre neuropathy in people with impaired glucose tolerance without apparent microvascular disease (Green et al., 2010). The most obvious finding to emerge from these results is that this test reflects the microcirculatory response to increased demand in times of thermal stress and associated nerve dysfunction. In the presence of impaired microcirculatory function, there is a possibility that repetitive stress and increased tissue demand may lead to reperfusion related injuries leading to DFU (Flynn and Tooke, 1992). A further study with more focus on exploring microcirculatory responses in relation to DFU is therefore needed. Similar to pressure ulcer development, reduced physiological responses may induce local ischaemia and reperfusion injury in the foot (Flynn and Tooke, 1992; Coleman et al., 2014). A similar role of reduced microcirculatory responses in the foot ulcer

development is widely discussed in literature (Flynn and Tooke, 1992; Boulton et al., 2020; Korzon-Burakowska and Edmonds, 2006a). Thus, the knowledge of the interaction between microcirculatory responses and small fibre nerve functions can potentially be translated to DFU prediction or diagnosis. Additionally, a PORH study that has demonstrated that every one-second increase in time to Peak is associated with a 1.5% increased likelihood of active or previous diabetic foot complication and with a 2% increased likelihood of previous foot complication (Lanting et al., 2017). Studies suggest that thermal assessment of the foot, which is linked to microcirculation may be useful to assess the risk of DFU and needs further investigation (Bharara, Cobb and Claremont, 2006; Balbinot et al., 2012). These findings support the need for further investigation into the relationship between measures of microvascular function and their role in DFU development. Such a study will offer some important insights into predicting DFU incidents and prevention of adverse complications.

#### *1.2.4.6 The skin of the foot and nocioceptors*

The skin is the largest and the most accessible organ to study microcirculatory and nerve functions. The skin microcirculatory bed is rich in capillaries and supplied by various nerves whose functional assessment facilitates understanding of pathophysiological mechanisms that lead to microvascular and small fibre dysfunction (Figure 1.9). The skin has an intrinsic ability to auto-regulate its blood flow that depends on some of the external or internal factors. Such functions are facilitated by a complex regulatory system that includes local regulation of cutaneous microcirculation involving sensory and autonomic fibres (Stirban, 2014). The skin can be either glabrous without hair such as in palmar or plantar surfaces or it can be non-glabrous with hair as found in the hand, feet or other regions of the body. Glabrous skin has highly innervated arteriovenous shunts and plays a major role in thermoregulation (Stirban, 2014). In contrast, non-glabrous hairy skin has fewer arteriovenous shunts and is primarily involved in defence and nutrition (Stirban, 2014).

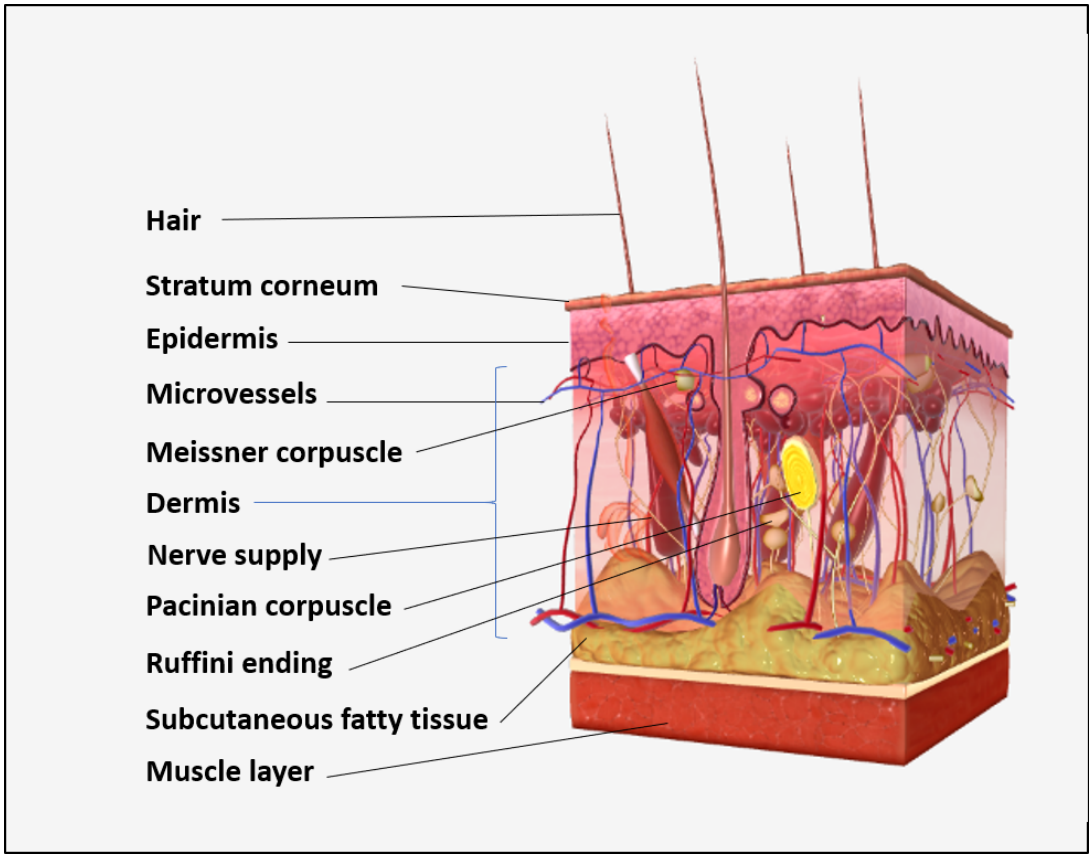



*Figure 1.9 Skin tissue*

The image was adapted from BioDigital Platform but annotated according to the purposes of this study

Nociceptors are localised sensory receptor neurons which are sensitive to a noxious (harmful) stimulus or a prolonged stimulus that eventually becomes noxious (Dubin and Patapoutian, 2010). The nociceptors that can be found in the skin, joints and viscera exchange messages respond to a wide range of noxious stimuli (Dubin and Patapoutian, 2010; Gangadharan and Kuner, 2013). Nociceptors detect signals from tissues susceptible to injuries or from damaged tissue (Dubin and Patapoutian, 2010). Most nociceptors are either C fibres with small diameter unmyelinated axons which support conduction velocities of 0.4–1.4 m/s) or A fibres whose axons are myelinated and support conduction velocities of approximately 5–30 m/s (Aδ range) (Djouhri and Lawson, 2004; Dubin and Patapoutian, 2010). The diameter of axons of sensory neurons and whether they are myelinated determines the speed of transmission of signals.

Following an incident of injury and inflammation, the nociceptors are sensitised by various pro-nociceptive mediators, such as prostaglandins, glutamate, kinins, cytokines, extracellular ATP, protons and other tropic factors (Gold and Gebhart, 2010; Ricciotti and Fitzgerald, 2011; Amaya *et al.*, 2013). Based on the site of stimuli application and the type of the stimuli such as chemical, thermal and mechanical, there are subcategories of nociceptors that respond (Dubin and Patapoutian, 2010;

Pinho-Ribeiro, Verri and Chiu, 2017). Additionally, the polymodal nociceptors of the skin respond to high-intensity stimuli such as mechanical, thermal and to chemical substances (Rosenberg and Pascual, 2014; Dafny, 2020). The skin nociceptors are categorized by their function in response to the noxious stimuli as illustrated in Figure 1.10 (Dubin and Patapoutian, 2010; Rosenberg and Pascual, 2014; Dafny, 2020). Stimulation and activation of the terminal branches of the sympathetic and nociceptor fibres result in axon reflex mediated neurogenic inflammatory reaction, sweating and vasodilation (Gibbons, Wang and Freeman, 2010). The pain mediated by the skin nociceptors can be protective in nature and the skin microcirculation responds to these stimuli. Furthermore, several humoral, neural and external factors are involved in the regulation. In particular, cutaneous microcirculatory disturbances in diabetic neuropathy are of interest to understand diabetic foot syndrome and adverse complications such as ulceration and delayed wound healing.

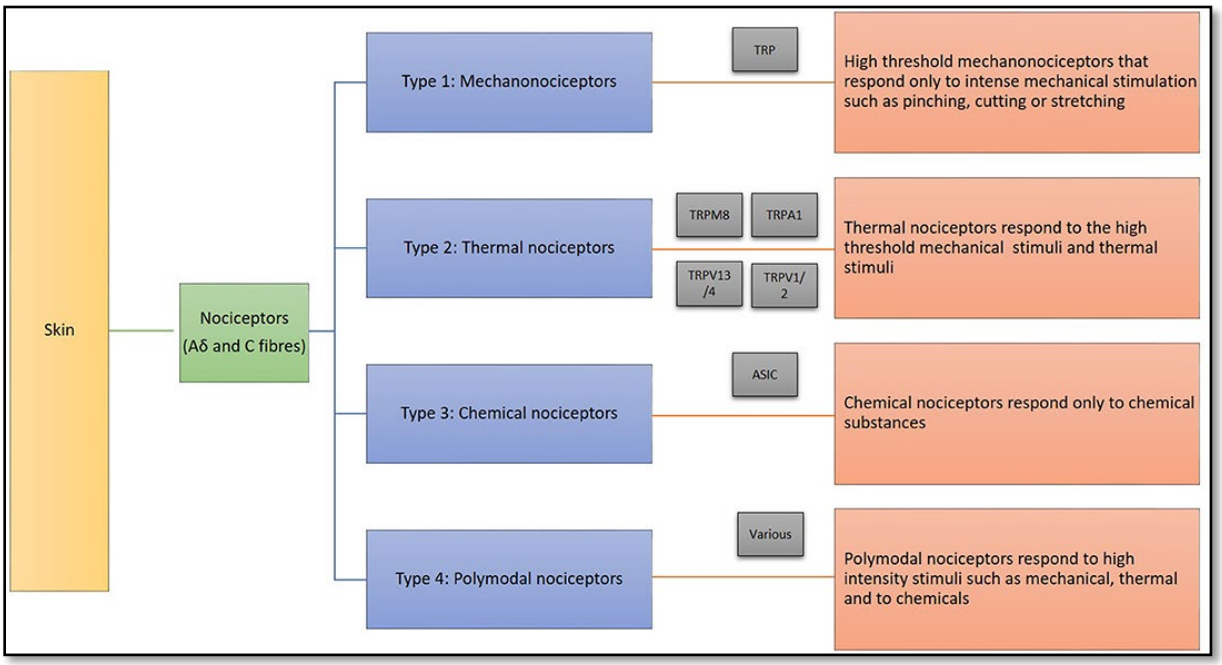



*Figure 1.10 The skin receptors and their functions*

### *1.2.4.7 Injury, inflammation, and soft tissues*

To gain a better understanding of microcirculatory function and recognise relevant methods to evaluate it, especially in the foot, it is vital to look at the bigger picture of the body's defence, injury, inflammation, and repair mechanisms. In the body defence mechanism, both lymphatic and blood vessels play an important role in an inflammatory response ('Compendium of Inflammatory Diseases', 2016; Granger & Rodrigues, 2016). It is known that changes in inflammatory mediators such as kinins, histamines and prostaglandins are known to correlate with the risk of developing a diabetic foot ulcer and inflammation is one of the earliest signs of ulcer (Lanys et al., 2021). Inflammation is a tissue response to extrinsic and intrinsic stimuli which is microcirculation-dependent (Granger and

Rodrigues, 2016). During an inflammatory response, the cardinal signs of inflammation that can be observed are heat (calor), pain (dolor), redness (rubor), and swelling (tumor), which may eventually lead to the loss of tissue function (function laesa). The microcirculation reacts strongly to inflammatory reactions and plays a crucial role in this. All components of the microvasculature such as the arterioles, capillaries, and venules respond and work towards the delivery of inflammatory cells to the injured or infected tissue/site (Granger & Senchenkova, 2010). The infected or injured region is isolated from the healthy tissue and the systemic circulation by the microvasculature to facilitate tissue repair and regeneration (Bentov & Reed, 2014; Granger & Senchenkova, 2010; P. C. Johnson et al., 1976). Microcirculation-related inflammatory responses include impaired vasomotor function, decreased capillary perfusion, adhesion of leukocytes and platelets, activation of the coagulation cascade, increased thrombosis, increased vascular permeability, and increased proliferation rate of blood and lymphatic vessels (Granger & Senchenkova, 2010). Apart from this, other microcirculatory changes lead to shunts and hypoxia (reduced tissue oxygen capacity) caused by endothelial cell injury. This endothelial injury is induced by a severe form of infection such as sepsis, red blood cell stasis due to vascular resistance, increased distances in oxygen diffusion in case of oedema owing to capillary leak syndrome (Guven et al., 2020). Defence mechanisms (stimulation–response) play a crucial role in the foot. For instance: Despite the causes of ulcer occurrence, the role of microcirculation in the healing process remains the same and the subpapillary perfusion promotes the formation of granulation tissue in patients with venous ulcers (Ambrózy *et al.*, 2013). Microvasculature aids in tissue perfusion, fluid homeostasis, cutaneous oxygen delivery and recruiting collateral vessels to facilitate healing process (Bentov & Reed, 2014).

Although the responses of the inflammatory system are regarded as defence mechanisms (stimulation–response) it may also be considered as a homeostatic system that operates continually to maintain organ and organism function (Tracy, 2006). Based on the dual nature of inflammation, stimulation–response and homeostatic, C-reactive protein or interleukin-6 are used as biomarkers to assess the level of activity of the inflammatory process (Tracy, 2006). These biomarkers are useful indicators because they can represent normal homeostatic function, a response to a pathological condition, or both, to varying degrees depending on the individual, time, and condition (Tracy, 2006). While biomarkers are likely to represent homeostasis in younger or healthier people, with increasing age and the presence of underlying pathology such as chronic inflammatory changes due to diabetes or induced atherosclerotic changes in cardiovascular disease, these biomarkers may indicate a stimulation–response type of inflammation (Pahwa et al., 2020; Payne, 2006; Tracy, 2006). According to the literature, there is consensus that inflammation biomarkers are independent predictors of the future occurrence of chronic disease outcomes and events (Tracy, 2006). Similarly, physiological

markers such as skin temperature, galvanic skin response and perfusion measurements that indicate homeostatic and stimulation-response in relation to microcirculation may be pertinent to predict the future occurrences of chronic disease outcomes or events such as ulcers.

#### *1.2.4.8 Neurovascular sensations in the skin*

In polyneuropathy, the small nerve dysfunction presents with symptoms such as pain, burning, numbness, and autonomic dysfunction characterised by lack of sweating show a stocking-glove distribution (Tavee and Zhou, 2009). The literature shows that the order of sensation loss is as follows: The loss of pain sensation, cold, warmth, touch and deep pressure upon application of local anaesthetics as the smallest fibres respond first (Prevoznik, 1986; Bigley, 1990; Vlckova-Moravcova *et al.*, 2008; Tavee and Zhou, 2009). However, the sequence of loss of sensation in case of pathological conditions like diabetes may be different. This depends on the distribution and the number of receptors and of their sensory nerve fibres which are impacted by both aging and diabetes (Dyck *et al.*, 2013). Although the evidence strongly suggests that small fibre neuropathy precedes large fibre neuropathy, there is too little evidence to say which sensory loss mediated by the small fibres is the first one to be lost. Research shows an association between microvascular impairment and small fibre neuropathy; microvascular dysfunction contributes to small fibre neuropathy and vice versa as microvessels supply the small fibres and small fibres innervate blood vessels (Barwick et al., 2016). Based on the severity of the neuropathy and the extent of the small fibre functions lost, the related microcirculatory response may be compromised. If the neurovascular elements are related to the impairment of small fibre nerve functions, the corresponding microcirculatory responses may be compromised. Thus, the impairment of the microcirculation may follow a similar trend corresponding to small fibre nerve dysfunction.

The sequential order of sensory loss upon application of local anaesthetics is usually pain, temperature, touch and deep pressure based on the sequential sensory block and for the purpose of this review the neurovascular tests were discussed in this sequence (Lightner, Lin and Yoo, 2010). Furthermore, only test that were non-invasive, less time-consuming and allows for an objective quick assessment of both neuro and vascular function were appraised. This review aimed at exploring the role of the microcirculation and the neurovascular interactions by appraising the microcirculatory responses mediated by the small fibre nerves and its significance. This review intended to understand some of the neurovascular interactions, especially in relation to small fibres and microcirculation in the diabetic foot. The scope of this review was to identify the microcirculatory responses through functional assessment of small fibre nerves and to discuss cutaneous neurovascular interactions in the foot. Only studies that discussed small fibre nerve function in relation to microcirculatory responses or vice versa using combined neurovascular testing through brief methods were reviewed. However,

the review boundaries are that conventional tests such as Quantitative Sensory Testing (QST), Quantitative Sudomotor Axon Reflex Testing (QSART), electrochemical skin conductance (using SUDOSCAN®), iontophoresis, and skin biopsies used to test small fibre nerve functions and microcirculation were not reviewed in this first part of the review. As mentioned earlier, microvascular functional changes are detectable even in the prediabetes state and progress over time with diabetes (Stirban, 2014). Moreover, in the presence of peripheral diabetic neuropathy, a higher degree of dysfunction is observed (Stirban, 2014). The understanding microvascular disease progression and tailored investigation may aid in the early diagnosis of microcirculatory and accompanied small fibre dysfunctions. This knowledge can be translated to effectively predict diabetic foot complications. Consequently, the practical implications from this review can be valuable for screening, early diagnosis, treatment, and enhancing prognosis by devising management and adapting prevention strategies that can change the paradigm of diabetic foot care in future. The results from the review published in a peer-reviewed journal (Appendix 1).

#### *1.2.4.9 Investigating neurovascular responses*

The investigation of the microcirculation in patients with diabetes is an increasing field of interest. Currently, there are various novel integrated research techniques used specifically to test microvascular function. Besides, these investigations identify the role of small fibre nerves in relation to the microcirculatory responses. This paves the way to understand the neurovascular interaction and its role in diabetic foot complications.

Impaired neurogenic blood flow regulation contributes to capillary hypertension, endothelial dysfunction leading to oedema and skin damage, as indicated by decreased Transcutaneous Oxygen Pressure (TcPo2) in patients with type 2 diabetes with a foot at risk of ulceration (Zimny *et al.*, 2001). Other non-invasive methods such as the measurement of pulse volume, skin perfusion, skin perfusion pressure (SPP) allow to assess healing (wound healing is likely when pressure is above 30 mmHg) and to determine amputation levels (Sarin *et al.*, 1991; Yamada *et al.*, 2008; Shapiro and Nouvong, 2011a). These assessments can be made using plethysmography and Laser Doppler Flowmetry (LDF) systems. Newer imaging technology such as Laser Speckle Contrast Imaging (LSCI) or Laser Speckle Contrast Analysis (LASCA) allows visualisation of the blood in the microvasculature in and around the ulcer area, which may indicate the ability to heal (Shapiro and Nouvong, 2011a). However, LSCI images are limited to a skin depth of 1 mm (Shapiro and Nouvong, 2011a). While recent research focuses on assessing microcirculation to predict ulcer outcomes, further studies are needed to gain a deeper understanding of the microcirculatory changes in the ulcers with respect to inflammatory responses, the stages of healing and duration for better prediction of wound healing or even to assess a foot at risk. For

instance: A better understanding of the microcirculatory perfusion changes during calor, dolour or rubor could help to identify a foot at risk through non-invasive methods such as the use of LDF or LSCI. In the past, the most important signs of inflammation were predominantly recognised by mere observation. However, nowadays pain-free non-invasive and even contactless (imaging) techniques have facilitated objective assessment of inflammatory signs, tissue injury responses, repairs and healing. LDF technique is one such non-invasive technique, which allows assessment of microvascular blood flow when reflection and scattering of the laser light occurs due to the movement of the red blood cells (Balasubramanian et al., 2020; Nakamoto et al., 2012). Although the depth the laser penetrates is relatively low (~1 mm), it is a practical and useful device for assessing cutaneous microcirculation. This device is gaining popularity in the field of research in diabetes, cerebrovascular conditions, Raynaud's phenomenon, dental problems and others. Various provocation tests such as heating, cold, pressure, postural changes and iontophoresis are used to assess the impairment of microcirculation. These provocation tests dependent on the stimulation of the small nerve fibres and are mediated by them to invoke the respective microcirculatory responses, which can be observed using LDF or LSCI. Since small fibre nerve functions and thermal changes affect microcirculation, methods such as quantitative sensory testing, assessment of skin electrodermal activity, and thermography are used in conjunction with microvascular testing from time to time to explore the underlying mechanism of action.

- Thermal stimuli trigger a microcirculatory response. This can be either using heat or cold stimulus.
    - Heat-induced pain and the threshold is one of the parameters measured which help to assess C fibre functions and microcirculatory response (Themistocleous *et al.*, 2014). A local heating stimulus induces nociceptive stimuli-mediated vasodilation and a neurogenic flare by an axon reflex response involving the C fibres. Whilst the intensity of the hyperaemic response depended on the microvascular ability to vasodilate, the size of the flare was dependent on the small fibre function (Krishnan and Rayman, 2004; Green et al., 2010; Vas and Rayman, 2013).
    - Cold Perception mediated by the small fibres can be studied using infrared thermographic cameras, infrared handheld thermometers, Laser Doppler Flowmetry systems and more recently the in-shoe temperature-based sensors designed to fit in prescribed footwear or offloading devices (Bus, 2016). Plantar thermography is used as a complementary diagnostic method for various foot-related complications (Balbinot *et al.*, 2012). By using a cold stress test, the microcirculation with respect to thermal changes, especially when cold, can be monitored. Through the cold stress

test, the afferent nerves that mediate pain and thermal perception in the skin and sympathetic efferent vasoconstrictor aspect and reduced perfusion are evaluated. The response following the cold stress test might be reflective of a sympathetic vasoconstrictor and the protective vasodilatory activities (Van den Brande, De Coninck and Lievens, no date; Pollock *et al.*, 1993). However, there are no studies that identify and isolate the neurogenic and vascular components in the image such as LASCA (Balbinot et al., 2012; Kamshilin et al., 2018; J. J. Van Netten et al., 2013). Such studies may help to further the knowledge of the neurovascular relationship.

- There are pressure sensations mediated by small fibre nerves. The polymodal mechanothermal receptors in the foot (previously discussed) respond to mechanical stimuli such as application of local pressure in addition to thermal stimuli. The microcirculatory responses that correspond to the pressure changes are reflected as a change in skin perfusion. The autoregulation of blood flow upon application of extrinsic pressure-based stimuli is known as reactive hyperaemia and the most commonly used provocation tests are Post-Occlusive Reactive Hyperaemia (PORH) and Pressure-Induced Vasodilation (PIV).
    - PORH is a measure of the reactive hyperaemia to arterial occlusion with pneumatic cuffs. During a PORH test, at occlusion, the blood flow goes to a biological zero followed by a PORH response when the pressure is released. PORH is a transient increase in blood flow because of the induced vasodilation in the organ or tissue following that brief period of the arterial occlusion. During hyperaemia, the tissue becomes re-oxygenated, and reperfusion occurs. PORH is considered to be both endothelial dependant and independent (myogenic, metabolic and neuronal in nature) (Barwick, Lanting and Chuter, 2015; Barwick et al., 2016; Marche et al., 2017). The maximum peak flow or magnitude of the PORH response could be attributed to the slower conduction speed of the sensory nerves (Larkin and Williams, 1993; Yamamoto-Suganuma and Aso, 2009). The sensory nerve function seems to influence the peak perfusion and decrease in time to peak (Barwick et al., 2016; Marche et al., 2017). Thus, in a PORH output, the magnitude and duration of hyperaemia can be considered as the neurogenic component and the increase in blood flow which is the maximum perfusion or hyperaemia as the vascular component.
    - PIV is one of the cutaneous microcirculatory reactive mechanisms to low pressure (Fouchard *et al.*, 2019). PIV works through a vascular and neuronal mechanism (Koïtka, Legrand-Fernandez, *et al.*, 2004; Fouchard *et al.*, 2019). The local application of pressure over a particular threshold at a specific location over time in a progressive

manner such as 5.0 mmHg/min for 20 minutes may act as a stimulus and the sensations are mediated by the afferent nociceptive C fibres (Klabunde, 2012; Koïtka, Abraham, et al., 2004). PIV is considered to be more than a transient phenomenon rather an important physiological response allowing the skin to respond adequately to a mechanical stimulus (Abraham et al., 2001). Cutaneous receptors in the skin respond to local mechanical stresses such as local pressure strain (Fromy *et al.*, 2002). These receptors are found to be of mechanothermal nature as the PIV response required certain optimal cutaneous thermal condition (lower skin temperature in known to interfere with PIV response) (Fromy *et al.*, 2002; Klabunde, 2012; Koïtka, Abraham, et al., 2004). Whilst it is apparent that PIV is mediated by C fibres, studies need to isolate the role of the small nerve fibres and the endothelial component possibly using imaging methods.

#### *1.2.4.10 Various Microcirculatory Responses and Their Association in Diabetes Foot-Related Complications*

The foot is constantly subjected to mechanical stress from weight-bearing activities of daily living, such as walking, exercise and standing. It is subject to various traumas, physical injuries due to sudden or violent impact, exposure to dangerous toxins or repetitive mechanical stress. Some of the extrinsic factors for trauma are thermal (Example: hot surfaces), mechanical (Example: repetitive damage from ill-fitted shoes), and chemical (Example: corn treatments) (Boulton, 2000; Boulton et al., 2020; Vanderah, 2007; Hawke and Burns, 2009; Armstrong and Lavery, 2016). On the other hand, some of the intrinsic factors contributing to the risk of trauma are foot deformities and glycation-related changes in the case of diabetes.

Both neuro- and vascular aspects are essential for healthy foot function. In the foot, the nerves are designed to respond to the thermal, mechanical, and chemical stimuli and evoke a reflex withdrawal from the noxious (harmful) stimulus (Hawke and Burns, 2009). For instance, yanking your foot off a sharp object or stepping away from a hot surface. This protective mechanism may be absent due to neuropathy in people with diabetes (Boulton, Cavanagh and Rayman, 2006; Hawke and Burns, 2009). For such mechanisms, the microcirculation is equally important. The ability of the microvasculature to produce a tissue injury response to stimuli such as local heat or pressure is a protective response (Abraham *et al.*, 2001; Korzon-Burakowska and Edmonds, 2006a). For instance: For example: increase in perfusion and autoregulation of temperature in local tissues after exposure to heat are neurovascular mechanisms of the foot that appear to play an important role in preventing tissue injury.

Research into microcirculation has a long history. Certain protective microcirculatory responses to stimuli, which are controlled by neural mechanisms, metabolic aspects, hormones and chemicals appear to be protective in nature such as a hyperaemic response (Guyton and Hall, 2015). A microcirculatory hyperaemic response can be induced upon application of a stimulus. This transient hyperaemic response to various stimuli, witnessed by an increase in blood perfusion is one of the measures used to assess microcirculatory function and is termed reactive hyperaemia. Reactive hyperaemia is an indicator of the intrinsic ability of an organ or tissue to locally autoregulate its blood supply, which is found to be impaired in people with diabetes (Flynn and Tooke, 1992; Korzon-Burakowska and Edmonds, 2006a; Merrill, 2008; Klabunde, 2012). For the purpose of this review, the observed microcirculatory responses have been stratified based on the selected stimuli as follows and their response is discussed based on the presence of a pathological condition such as diabetes or other injuries/inflammation:

1. Neurovascular responses to iontophoresis, vasoactive substances and topical anaesthetics
2. Endothelium-dependant and independent vasodilation have been studied using the laser Flowmetry through the method of iontophoresis (Agarwal *et al.*, 2010; Hellmann *et al.*, 2015; Loader *et al.*, 2017). The indirect effect of the vasoactive substance on skin microcirculation results from the stimulation of C fibres, typically through a nerve-axon-related hyperaemic response (Schramm, Dinh and Veves, 2006; Stirban, Gawlowski and Roden, 2014). Thus, the axon flare-reflex, which is a C fibre mediated microcirculatory response can be measured. For instance: Capsaicin is a powerful vasodilator known to significantly increase skin perfusion and induces pain mediated by the small fibres. Capsaicin-evoked axon flare responses are currently visualised by LASCA or photoplethysmography (Gibbons, Wang and Freeman, 2010; Kamshilin, Mamontov and Almazov, 2018; Unal-Cevik, 2018). The characteristics of the flare which depended on the amount of activated small nerve fibres and the function mediated by the C fibres (Unal-Cevik, 2018). Two components can be isolated from the flare response. The size of the flare and the maximum perfusion represented the neurogenic and vascular components, respectively. The use of EMLA cream was found to decrease the responses and pain symptoms, however, it did not completely block the small fibre functions possibly because of only partial inhibitions of the lower layers of skin innervations (Unal-Cevik, 2018).
3. Laser-Doppler measurements of the skin microcirculation following postural change help to understand vascular disturbances in the form of reduced capillary blood flow, observed as an enhanced reduction in skin blood flux, and impaired fluid filtration after sitting up (Schramm, Dinh and Veves, 2006). This is a measure that helps to observe the microcirculatory response to an autonomic function of the c fibres. The evidence generated from such studies shows

that sympathetic innervation plays a major role in the regulation of skin microcirculation by opening and closing arteriovenous anastomoses and pre-capillary arterioles during postural changes (Schramm, Dinh and Veves, 2006; Veves, Giurini and LoGerfo, 2006; Stirban, 2014). Impairment of endothelium-dependent microvascular regulation is known to correlate closely with the presence of sudomotor dysfunction (Stirban, 2014). This microcirculatory impairment is of importance as autonomic neuropathy caused by sympathetic denervation can play a pathogenic role in the development of a diabetic foot; as skin dryness that eventually cracks paves way for infections and ulceration (Schramm, Dinh and Veves, 2006). Therefore, neurovascular investigations are useful in understanding and evaluating the association between somatic/autonomic neuropathy and microcirculatory changes.

4. Vasodilation in response to occlusive ischemia or Post-Occlusive Reactive Hyperaemia (PORH)
5. Microcirculatory response to locally applied pressure:
    a. Pressure-induced vasodilation (PIV)
    b. Reduced skin blood flow
6. Interplay between microcirculation and temperature-vasodilation in response to local heating

The literature review in the previous chapter 2 indicated that in people with diabetes, the ability of cutaneous microcirculation to respond normally to non-painful stimulation, such as the application of pneumatic pressure, local pressure and local heating were impaired (Abraham *et al.*, 2001; Fromy *et al.*, 2002; Klabunde, 2012; Koïtka, Abraham, et al., 2004; Krishnan and Rayman, 2004; Lanting et al., 2017). This may be significant in understanding tissue response to injuries. Persistent pressure, injury, or infection place more physiological demands on capillary circulation (Flynn and Tooke, 1992; Abraham *et al.*, 2001). Due to microcirculatory dysfunction, the hyperaemic response may be compromised and tissue demands are not met (Flynn and Tooke, 1992). Vascular insufficiency leads to tissue breakdown and contributes to adverse complications and increases the risk of ulceration (Flynn and Tooke, 1992). Figure 1.11 shows this chain of events.

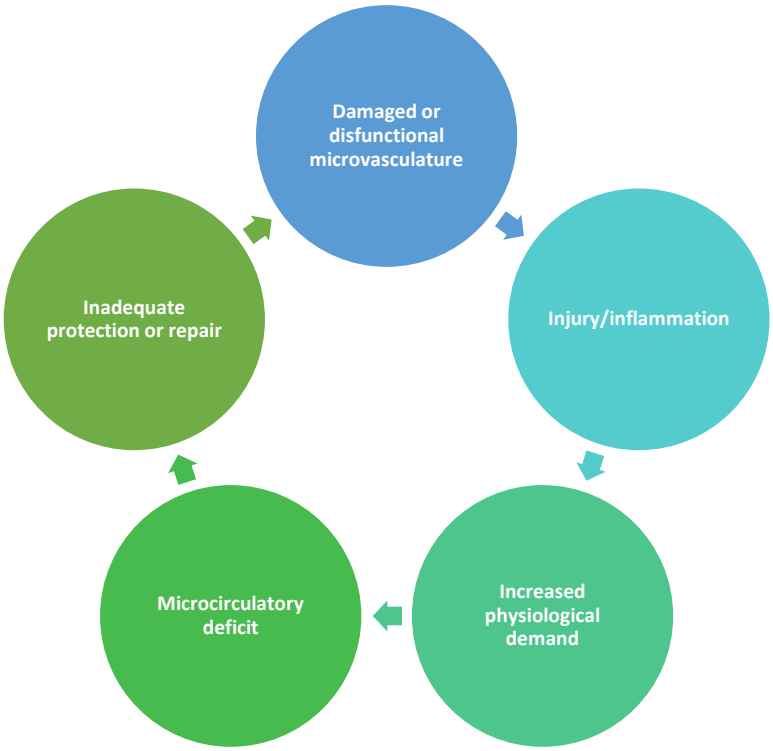



*Figure 1.11 Chain of events with microcirculatory responses*

#### 1.2.4.10.1 Vasodilation in Response to Occlusion or Post Occlusive Reactive Hyperaemia (PORH)

PORH, which was discussed in Chapter 2, is a reactive hyperaemic response to occlusion for a brief period of arterial occlusion using pneumatic cuff. During occlusion, the blood flow goes to a biological zero that is defined as the "no flow" Laser Doppler signal and following the release of the occlusion, blood flow rapidly increases. Tissues are reoxygenated and reperfusion occurs during the hyperaemic phase. Concurrently, the vasodilator metabolites are removed from the tissue restoring the vascular tone of the resistant vessels, thereby returning blood flow to normal (Klabunde, 2012). The reperfusion rate, peak hyperaemia, and maximal vasodilation depend on the occlusion duration, the stimulus for vasodilation, and the intrinsic capacity of the tissue or organ (Guyton and Hall, 2015; Larkin and Williams, 1993; Klabunde, 2012).

PORH is predominantly an endothelial-dependent process, however, it also aids in the combined assessment of both endothelial-dependent and endothelial-independent function (Wierzbowska et al., 2014; Lanting et al., 2017). Hyperaemia occurs because of the shear stress, the tangential frictional force acting at the endothelial cell surface caused by arterial occlusion (Wierzbowska et al., 2014). A mechanical stimulation occurs when the shear stress vector is directed perpendicular to the long axis of the arterial vessel.(Wierzbowska et al., 2014) The endothelium responds to this mechanical stimuli, thereby, releasing vasodilatory substances.(Wierzbowska et al., 2014) The factors that are known to

contribute to vasodilation are myogenic, neurogenic, and other local factors, such as potassium ions, hydrogen ions, carbon dioxide, catecholamines, prostaglandins, and adenosine (Wierzbowska et al., 2014; Lanting et al., 2017). Substances such as prostaglandins and endothelium-derived hyperpolarizing factors are known to be involved in the mechanism of PORH (Carasca et al., 2017; Marche et al., 2017; Wierzbowska et al., 2014). However, some researchers contend that nitric oxide and prostaglandins may not be contributing to the mechanism (Cracowski et al., 2011; Wierzbowska et al., 2014). It is argued that whilst nitric oxide is known to play a major role in the vasodilation of macrovessels, endothelium-derived hyperpolarising factors are found to play a substantial role in the dilation of microvessels (Quyyumi and Ozkor, 2006; Cracowski et al., 2011). Apart from these substances, the sensory nerves make a vital contribution to the PORH mechanism (Larkin and Williams, 1993; Lorenzo and Minson, 2007; Cracowski et al., 2011; Lanting et al., 2017; Marche et al., 2017). To summarise, various studies have shown that PORH response is elicited with temporary tissue hypoxia upon occlusion through the accumulation of vasodilators (substances that cause the blood vessels to dilate or expand) and other complex factors that are myogenic, endothelial, neurogenic and metabolic (Guyton and Hall, 2015; Klabunde, 2012; Lanting et al., 2017).

The PORH test has a wide range of applications. PORH has been used to assess microcirculatory function in people with arterial diseases, certain ophthalmologic conditions, cardiovascular disorders and in diabetes related complications (Morales *et al.*, 2005; Wierzbowska et al., 2014; Carasca *et al.*, 2017; Lanting et al., 2017). An impaired PORH response has been identified in people with peripheral arterial disease (Morales *et al.*, 2005). The test was observed to be useful as an early marker of cardiovascular damage and indicated a higher risk of cardiovascular risk (Morales *et al.*, 2005; Busila *et al.*, 2015). PORH test is also used to assess the altered microvascular reactivity in patients with advanced renal dysfunction (Busila *et al.*, 2015). A prolonged hyperaemic response and reduced response have been observed in people with normal-tension glaucoma (Wierzbowska et al., 2014). Besides these conditions, the use of the PORH test has also been explored in people with diabetes, both in type 1 and 2.

The impaired hyperaemic response to injury in people with diabetes was demonstrated for the first time in as early as 1986 (Rayman *et al.*, 1986). In patients with insulin-dependent diabetes and peripheral arterial disease, a prolongation of the hyperaemic reaction and a decrease in response have been observed (Wierzbowska et al., 2014). The PORH hyperaemic response that occurs due to vasodilation is significantly decreased in people with type 1 diabetes, both adults and children (Schlager et al., 2012; Marche et al., 2017). PORH is known to be impaired not only in adults but also in children with type 1 diabetes (Schlager et al., 2012) The findings in children are just as important as the studies in adults to apply the knowledge in diabetic foot complications for two reasons. Firstly,

although this segment of the population is less likely to be vulnerable to foot complications at a younger age, but they are likely to develop complications as they advance in age. Therefore, understanding the microvascular reactivity from an earlier period may prove to be useful. Secondly, this particular study explored other less commonly assessed variables such as biological zero and reperfusion time, which can shed more light on understanding PORH. It was identified that peak perfusion was higher and biological zero was lower in children with type 1 diabetes in comparison to the controls. A key implication from this study was that higher peak perfusion might reflect a decline in the vasoconstrictive ability of arteriolar smooth muscle cells upstream of capillary beds in children with type 1 diabetes (Schlager et al., 2012).

A few studies have explored PORH more specifically in diabetic foot complications. Impaired PORH responses were associated in the presence of peripheral vascular disease, sensory neuropathy in people with type 2 diabetes and history of foot complications (Cheng et al., 2004; Barwick et al., 2016; Lanting et al., 2017). It was observed that although PORH was impaired in the presence of history of foot complications (feet at risk for subsequent ulcers), it was not the case with existing (active) ulcers (Lanting et al., 2017). Considering this evidence, it seems that PORH may be a useful assessment method to identify a foot at risk as it reflects the microcirculatory mechanism during a physiological demand that may be useful to assess a foot at risk. In future, their application may be a useful indicator for determining the future risk of diabetic foot complications, especially in predicting ulcers and preventing amputations.

#### 1.2.4.10.2 Microcirculation in Response to Local Application of Pressure

In the foot, the areas prone to high pressure such as the heel, the great toe and areas under the metatarsal heads are at risk of ulceration (Veves *et al.*, 1992; Ledoux *et al.*, 2013). Based on this, many weight-bearing activities were considered to be a contraindication for people with neuropathy (Kluding *et al.*, 2017). However, this has recently changed as there is emerging evidence of positive adaptations of the musculoskeletal and integumentary system to overload stress (Kluding *et al.*, 2017). Literature suggests that peripheral neuropathy may no longer be a hindrance to promoting weight-bearing activity as it did not lead to significant increases in foot ulcers (LeMaster *et al.*, 2008). However, in people with diabetes various other factors may interplay with pressure such as increased stiffness of tissues, aging related changes, presence of other comorbidities, mobility and vascular issues. Studies show that the accumulated mechanical stimulus affected blood perfusion in the foot and should be considered when assessing the risk of developing ulcers (Ledoux *et al.*, 2013; Pu *et al.*, 2018). However, more understanding on the relationship between pressure stimulus and microvascular responses could shed more light on the effect of different levels of accumulated mechanical stimulus on microvascular response and their significance in an ulcer incident.

Responses to local mechanical stresses are mediated by a significant number and variety of cutaneous receptors and some of these receptors are connected to the small fibres (Abraham *et al.*, 2001). The vasodilation to pressure strains not only occur for noxious stimuli but also non-noxious stimuli applied over a period (Abraham *et al.*, 2001). Local pressure strain to the skin is recognised to play a vital role in cutaneous microcirculatory impairment (Fromy, Abraham and Saumet, 2000; Abraham et al., 2001). It is presumed that this may be linked to the development of cutaneous lesions such as pressure sores and diabetic foot ulcers (Abraham et al., 2001; Fromy et al., 2002). Two important microcirculatory responses to locally applied pressure identified through the literature review are discussed below.

#### 1.2.4.10.2.1 Pressure-induced vasodilation

The transient increase in cutaneous blood flow initially before it decreases in response to a progressive locally applied pressure strain is known as pressure-induced vasodilation (PIV). This microcirculatory response appears to be a protective cutaneous response that relies on the excitation of unmyelinated afferent nerve fibres (Fromy *et al.*, 2002; Klabunde, 2012; Koïtka, Abraham, et al., 2004; Körei et al., 2016). PIV is considered to be more than a transient phenomenon and an important physiological response allowing the skin to respond adequately to mechanical stimuli (Abraham et al., 2001). Cutaneous receptors in the skin respond to local mechanical stresses such as local pressure strain and these receptors are found to be of mechanothermal nature (Fromy *et al.*, 2002). This response is noted to be compromised in the ageing population (Fromy *et al.*, 2010; Fouchard *et al.*, 2019). In addition, it has been postulated that impairment of PIV contributes to the development of lesions such as pressure sores and diabetic foot ulcers (Abraham et al., 2001; Saumet, 2005; Vouillarmet et al., 2019).

The interplay between biomechanical factors and physiological responses is well-realised in the development of pressure ulcers, including in people with diabetes. Current studies shed light on PIV in connection with the development of decubitus or decubitus in the sacral area. As discussed above, one of the key implications from the studies on PIV is that it is a protective mechanism without which certain pressure-associated lesions may develop and plausibly this could explain the high risk of decubitus and plantar ulcers in people with diabetes (Abraham et al., 2001; Fromy et al., 2002; Bergstrand, 2014).

Although pressure ulcers and plantar ulcers may differ in many ways, one of the key causal pathways to foot ulceration is somatic motor neuropathy that leads to small muscle wasting, foot deformities, loss of sensation, increased plantar pressure and repetitive trauma resulting in neuropathic foot ulcer (Armstrong and Lavery, 2016). This suggests that local pressure strain (compressive loading) increases the vulnerability of the foot to ulcerate. Similar to pressure ulcer development, reduced physiological responses may induce local ischaemia and reperfusion injury in the foot (Flynn and Tooke, 1992;

Coleman et al., 2014). A similar role of reduced microcirculatory responses in foot ulcer development is widely discussed in the literature (Flynn and Tooke, 1992; Boulton et al., 2020; Korzon-Burakowska and Edmonds, 2006b). This knowledge can potentially be translated to diabetic foot ulcer prediction to see if the microcirculatory response to local pressure and plantar pressure has any association. This also accords with other observations, which showed that people with impaired or absent PIV are known to be at a higher risk to develop pressure ulcers (Fromy et al., 2002; Braden and Blanchard, 2007; Bergstrand, 2014). Decreased hyperaemic response and the absence of PIV are known to increase the risk of pressure ulcers (Bergstrand et al., 2014). However, very limited research is available on PIV in human hand and feet in relation to diabetes (Abraham et al., 2001; Klabunde, 2012; Koïtka, Abraham, et al., 2004).

A particular study by Koitka et al. (2004) observed PIV at the foot level in people type 1 diabetes (Klabunde, 2012Koïtka, Abraham, et al., 2004). Since low skin temperature in people with diabetes is known to interfere with microcirculation, this research was performed in warm conditions of 29.5 +/- 0.2°C (Klabunde, 2012; Koïtka, Abraham, et al., 2004). The cutaneous blood flow was studied at warm conditions using laser Doppler flowmetry on the first metatarsal head in response to applied pressure at 5.0 mmHg/min and PIV was found to be absent at foot level in people with type 1 diabetes whereas it existed in healthy participants at 29.5 +/- 0.2°C (Klabunde, 2012; Koïtka, Abraham, et al., 2004). These findings were attributed to an interaction between functional changes in C-fibres and the endothelium in people with diabetes (Klabunde, 2012; Koïtka, Abraham, et al., 2004). A similar study found PIV to be absent at low skin temperature even in healthy participants (28.7 +/- 0.4°C) (Fromy *et al.*, 2002). It was explained that a skin temperature close to 34°C was optimal for the assessing the vasomotor reflexes of the lower extremities and the nerve receptors involved in the PIV development are mechanothermal, and not only mechanical (Fromy *et al.*, 2002). The results from Koitka et al. (2004) revealed that in the same subjects the non-endothelial-mediated response to sodium nitroprusside was preserved, whereas the endothelial-mediated response to acetylcholine was impaired (Klabunde, 2012; Koïtka, Abraham, et al., 2004). Therefore, suggesting the relevance of endothelial dysfunction to PIV. Also, a previous study on PIV found that the absence of vasodilatory axon reflex response to local pressure strain when the capsaicin-sensitive nerve terminals were pre-treated with local anaesthetic or chronically applied capsaicin (Fromy et al., 1998). The capsaicin-sensitive nerve fibres are the small nerve fibres and their role in neuropathic pain and related complications, especially in people with diabetes is well-established (Boulton et al., 2020). Thus, the researchers speculated that the PIV, which is associated with the stimulation of small fibre nerves, could be a missing link between neuropathy and foot ulcers in diabetes (Klabunde, 2012; Koïtka, Abraham, et al., 2004). Several studies have shown that C-fibre damage has a major impact on the

skin, with impaired blood flow predisposing to foot ulcers (Vinik *et al.*, 2001; Caselli *et al.*, 2003; Boulton et al., 2020; Themistocleous *et al.*, 2014). As previously discussed, impaired microcirculatory response to local pressure strain may potentially make people with diabetes more vulnerable to pressure strains and explain the high prevalence of foot ulcer that occurs in diabetic patients (Klabunde, 2012; Koïtka, Abraham, et al., 2004).

Findings from the studies discussed above suggest that PIV is absent at the foot level in people with diabetes. Identifying the timing or stage of disappearance of PIV in the foot during disease progression through prospective studies may help to understand the progression of neurovascular dysfunction in the foot. On the other hand, since PIV can be absent at an earlier stage, its ability to indicate risk of ulceration is controversial and requires further research. In addition, previous studies have observed PIV in only two locations, namely the head of the first metatarsal and the area above the inner ankle in a small sample size. Further research is needed to examine different regions of the plantar aspect of the foot, particularly in areas subjected to increased plantar pressure. Findings from this research may help understand the link between PIV and plantar ulcers and identify a foot at risk. Additionally, it may help fill the research gap to understand the role of microcirculation in the development of diabetic foot ulcers.

#### 1.2.4.10.2.2 Reduced skin blood flow to locally applied pressure

As previously mentioned, PIV allows for an increase in response to locally applied pressure. In the absence of the transient PIV response, the cutaneous blood flow is observed to progressively decrease with the application of increasing local pressure (Fromy *et al.*, 2002). The observed cutaneous blood flow in response to locally applied pressure is found to be impaired in people with diabetes owing to the combined effects of low cutaneous temperature and alterations in microcirculatory function (Fromy *et al.*, 2002). Additionally, the presence of neuropathy may aggravate the condition (Fromy *et al.*, 2002). This study used a laser Doppler flowmetry system and applied local pressure using a specially designed apparatus at the internal anklebone allowing for a 5.0 mmHg/min rate of pressure increase (Fromy, Abraham and Saumet, 2000). The skin blood flow decreased significantly from baseline at much lower applied pressure of 7.5 mmHg in people with diabetes in groups without neuropathy and with subclinical or clinical neuropathy at 6.3 mmHg in comparison to the healthy controls at 48.8 mmHg (Fromy *et al.*, 2002). The large difference between these pressures reported within this study indicates a plausible association between decreased skin blood flow to local pressure and the development of decubitus and plantar ulcers (Fromy *et al.*, 2002). This hypothesis is consistent with the one proposed by Koitka et al. (2004) who suggested an association between microcirculatory dysfunction and the high prevalence of foot ulcers (Klabunde, 2012; Koïtka, Abraham, et al., 2004). They also postulate that the arterial wall and surrounding tissues are highly compressible in people

with diabetes, making them prone to developing foot ulcers and pressure ulcers (Fromy *et al.*, 2002; Coleman et al., 2014). The application of this knowledge to understand the role of microcirculation in foot ulceration may potentially be useful.

Although the results collected raise the possibility that reduced skin perfusion and PIV are associated with pressure ulcer development, further investigation is needed to understand the mechanism in relation to diabetic foot complications. The aetiology for pressure ulcers and plantar ulcers may differ, yet pressure remains a common contributing factor in both events. Studies suggest pressure-induced local ischaemia and reperfusion injuries in relation to both pressure ulcers and diabetic foot ulcers (Flynn and Tooke, 1992; Korzon-Burakowska and Edmonds, 2006b; Coleman et al., 2014; Shahwan, 2015). Understanding PIV, reduced skin flow and other microcirculatory responses in various regions prone to diabetic foot ulcers and with plantar pressure during standing or walking is important. The need for such a study is further supported by the evidence from a study that identified participants who lacked PIV and reactive hyperaemia in response to locally applied pressure, to be particularly vulnerable to pressure exposure (Bergstrand, 2014; Bergstrand et al., 2014). These participants were stratified to have a higher risk of developing pressure ulcers (Bergstrand, 2014; Bergstrand et al., 2014). Thereby, translating the knowledge generated from the studies on microcirculatory responses in the development of pressure ulcers into the areas of diabetic foot ulcers can prove to be useful.

#### 1.2.4.10.3 Interplay between microcirculation and temperature - Vasodilation in response to local heating

While specific literature on microcirculatory responses and temperature changes in response to injury and healing of plantar skin tissue is limited, previous studies have reviewed microcirculatory assessments in various organs in people with diabetes. Knowledge of the microcirculatory responses to temperature changes in other organs may indicate that external stimuli cause increased microvascular demand. This demonstrates the role of the cutaneous microcirculatory response in tissue injury and healing.

When injuries and repair occur, monitoring the conditions between the skin, soft tissues or even after skin grafts can help improve prognosis. A study explored the proposed theory that conducive interface conditions between soft tissue and prostheses are necessary for a better outcome with prosthodontic treatment. This study by Nakamoto et al. (2012) focused on the gingiva and mucosa surrounding anterior implants and both LDF and thermographs were concurrently used to elucidate the relationship between temperature and blood flow as peri-implant soft tissues are often portrayed to have decreased blood flow because of the lack of blood supply from the periodontal ligament. The study also analysed the morphological changes of the cutaneous microvasculature and temperature

changes between participants with and without bone grafting associated with implant placement. The results indicated that the soft tissues around the implants had reduced blood flow compared to the periodontal tissues of adjacent natural teeth, despite the absence of clinical signs such as chronic inflammation. The study also emphasised the importance of bone quality in maintaining soft tissue blood flow, as the area around implants with bone grafting showed significantly reduced blood flow. Many research studies suggest that microcirculatory blood flow is affected by thermal changes and has been reported to increase proportionally with temperature to an extent, which is not limited to dentistry but also in studies of cutaneous microcirculation in other areas (Molnár *et al.*, 2015). However, the observed results by Nakamoto et al. (2012) were contrary to this popular idea. The suggested explanation for this was the involvement of deeper structures that modified the thermal properties and the usually observed increase in temperature was often associated with inflammation due to infection such as periodontitis but not in case of tissue surrounding implants (Baab, Öberg and Lundström, 1990).

Although the skin and oral mucosa share certain similarities and differences anatomically, they share some comparable physiological properties. For instance, they play a crucial role in the prevention of infections and act as a barrier against exogenous or endogenous substances, pathogens, and mechanical stresses (Liu et al., n.d.). The dysfunction of these barriers can compromise the integrity of the underlying tissue as well. The combination of findings from the study provides some support for the conceptual premise that the simultaneous measurements of blood flow and temperature are useful to evaluate the microcirculation of soft tissue behaviour in injury and healing and its significance even in the absence of noticeable signs of chronic inflammation. A similar study compared the peripheral blood flow in the lower limbs during the local heating tests with different temperature protocols in people with diabetes mellitus and healthy participants (Filina *et al.*, 2017). The LDF was used to evaluate the adaptive changes of the microvascular bed during thermal tests and the detection of the preclinical stage of trophic disorders due to disruption of nutritional or nerve supply (Filina *et al.*, 2017). Research suggests that in the feet of patients with diabetic neuropathy, total skin blood flow is increased due to an increased shunt flow due to denervation (Harpuder, Stein and Byer, 1940; Schaper, Huijberts and Pickwell, 2008). Further study in the area has shown that the increased anastomotic shunt flow led to either under or over perfused nutritive capillaries (Netten *et al.*, 1996). Skin temperature measurements and LDF were performed to record mainly shunt flow and capillaroscopy to study nailfold capillary blood flow (Netten *et al.*, 1996). The study showed that in insulin-dependent diabetic patients with neuropathy, the baseline skin temperature and capillary blood-cell velocity were higher in comparison to those without neuropathy and healthy control participants (Netten *et al.*, 1996). The results of the study highlighted the presence of hyperperfused

nutritive capillary circulation in the feet of patients with diabetic neuropathy, favouring the previously discussed hyperdynamic hypothesis and contradicting the capillary steal phenomenon to explain the reduced healing potential in diabetic neuropathic foot ulcers. Significantly higher wound bed perfusion can be attributed to a local ‘‘rubor,’’ described as local hyperaemia and microcirculatory vasodilation in the presence of inflammation (Schreml *et al.*, 2010; Varetto *et al.*, 2020). An impaired venoarteriolar reflex (vasoconstrictive responses to various stimuli) leads to hyperperfusion on dependency, increased venous pressure, reduced skin capillary flow, increased fluid filtration and oedema (Korzon-Burakowska and Edmonds, 2006a). Hyperperfusion leads to venous distension and warmth in the lower calf and foot and higher oxygen concentration, suggesting an arteriovenal shunt (Ward *et al.*, 1983). Although no occlusion may be present, asymmetrical thickening of the tunica intima, sclerosis, and basement membrane thickening are in line with the hemodynamic hypothesis (Korzon-Burakowska and Edmonds, 2006a; Balasubramanian, Chockalingam and Naemi, 2021). This may be the result of an “injury response” by the endothelium, initially caused by increased blood flow in small arteries (Korzon-Burakowska and Edmonds, 2006a). The thickening of the endothelial basement membrane of the blood vessel wall can reduce the migration of inflammatory cells and nutrients and in addition to impaired function of the endothelium such as autoregulation, limited vasodilation, local ischemia and an inability to meet increased metabolic demands, resulting in a negative environment for wound healing and adverse outcomes (Dinh and Veves, 2005; Korzon-Burakowska and Edmonds, 2006a).

As previous research has suggested, microcirculation and temperature measurements could become useful techniques to assess healthy, infected, injured, inflamed and treated skin and soft tissues of the foot (Netten *et al.*, 1996; Gatt *et al.*, 2018, 2020). However, there is abundant room for further progress in determining if these two measurements may be useful for the diagnosis or prognosis of foot ulcers. Such research can help draw a boundary between the affected tissue and the surrounding healthy tissue when determining the course of treatment, surgery, or even amputation. Furthermore, comparative studies conducted on healthy vs inflamed/injured tissue in the foot can help to identify early signs of dysfunction, inflammation and injury in a foot in order to effectively manage the condition. For instance, Ren et al. (2021) explored the stimulation of microcirculation using simple thermal stimuli such as infrared and warm baths in healthy adults to explore the options in the hope to design interventions to promote better circulation in the lower extremities of the body in the geriatric population and those suffering from diabetes who are likely to have impaired microcirculation (Ren *et al.*, 2021).

The vasodilation in response to local heating and the neurogenic flare response to nociceptive stimuli is mediated by an axon reflex involving C-fibres. This is studied using the laser Doppler imager (LDI)

and the induced flare response is known as the LDI flare. The LDI flare area, which is the area of hyperaemic response, is known to reflect small fibre function. Therefore, the size of the LDI flare is known to be dependent on the C-fibre function and the underlying skin small fibre neural network and its extent (Green et al., 2010; Vas, Green and Rayman, 2012). Whereas the LDI max (perfusion) in the skin immediately beneath the heating probe is shown to be mediated by non-neurogenic means and to reflect the endothelial function (Green et al., 2010; Vas, Green and Rayman, 2012). Therefore, the intensity of the hyperaemic response depended on the microvascular ability to vasodilate. The site commonly studied is the dorsum of the feet because the underlying skin is less influenced by the thermoregulatory blood flow due to the absence of arteriovenous anastomoses (Braverman, 2000). The method used to assess this reflex involves local skin heating to 44°C for 20 minutes or 6 min in a stepwise fashion: 44°C for 2 min, 46°C for 1 min and finally 47°C for 3 min in a temperature-controlled room to evoke the flare followed by scanning the site using an LDI to measure the area (Krishnan and Rayman, 2004; Green et al., 2010; Vas, Green and Rayman, 2012). Another technique is also known to be used to observe the hyperaemic response to local heating. This involves the use of a skin-heating probe filled with deionized water and heating to 44°C to assess heat-induced vasodilation. In summary, the LDI flare test in subjects shows reduced microcirculatory response as well as a neurogenic flare in people with either type 1 or 2 diabetes (Krishnan and Rayman, 2004; Vas, Green and Rayman, 2012). It facilitates early diagnosis of C-fibre dysfunction even before its detection by other available methods such as quantitative sensory testing, which focuses on the testing of sensory abnormalities in the areas of temperature change sensation, vibration, and pain threshold testing (Example: Using equipment named Computer Aided Sensory Evaluator–IV - CASE IV) (Krishnan and Rayman, 2004). Therefore, the heat provocation or LDI flare test is commonly used with a focus on LDI flare for the assessment of C-fibre function rather than with a concentration on the LDI max for evaluating the microcirculatory function. However, the test can be used to assess not only C-fibre function but also microcirculation, and additionally investigate their association with neuropathy (Vas, Green and Rayman, 2012; Marche et al., 2017). This may further clarify the link between impaired microcirculation and tissue damage in the face of impaired sensitivity.

#### *1.2.4.11 Summary*

In summary, this review helped to gain an understanding of small fibre function and its role in mediating microcirculatory responses. Selected tests that are non-invasive and less time-consuming that allow the simultaneous assessment of small fibre functions and the respective microcirculatory response were summarised. The commonly used evaluation methods discussed in this review help to explore the neurovascular aspects simultaneously in routine practice. Besides, most of these tests facilitate isolating the neurogenic and vascular components of the responses. The in-depth results

from the review have been published as two different articles in a peer-reviewed journal (Appendix 1 and 2). In people with diabetes, especially those with neuropathy small fibre dysfunction is known to precede large fibre dysfunction. In such people, the small fibres mediated protective sensations such as pain, temperature perception and cutaneous superficial touch/pressure sensations may be impaired. Besides, the small fibre dysfunction may impact the microcirculation or vice-versa. The microcirculatory impairment may correspond to the sequential dysfunction of the small fibre nerves. However, more prospective studies are required to substantiate it. This will be valuable to understand disease progression as microcirculatory impairment and small fibre neuropathy are known to precede many other diabetic foot-related complications, outcomes of such research can aid in early diagnosis and better prognosis. Furthermore, the role of microcirculation is well-realised in wound healing, however, its role in ulceration remains speculative. Future research in this area may help to bridge the gaps in knowledge and aid in identifying a foot at risk.

## 1.3 Rationale for the current work

### 1.3.1 Current research gaps

From critically appraising the existing literature, it could be established that although microvascular disease alone may not be a primary cause of the pathogenesis of DFU, the co-existence of abnormal microcirculatory function with both PAD and neuropathy resulting in tissue damage cannot be denied (Flynn, Tooke and Flynn, Michael D, 1990; Boulton et al., 2020; Körei et al., 2016). This is supported by the evidence from studies that implicate the role of microcirculation in impaired tissue response to injury or trauma, the development of foot complications, the development of ulceration, gangrene, necrosis and poor wound healing (Flynn and Tooke, 1992; Boulton et al., 2020; Levin *et al.*, 2008; Lanting et al., 2017). Therefore, understanding functional abnormalities is important when studying the risks of DFU. Moreover, small fibre neuropathy may precede large fibre neuropathy and microcirculatory dysfunctions begin even in a prediabetes state (Körei et al., 2016). Hence, more research is required to understand the association between microcirculation and small fibre function, which dysfunction precedes the other, and their role in the development of DFU. This may give useful insights into the progression of diabetic foot-related complications and aid in device prognostic tools to prevent adverse events such as ulcers and amputation.

Risk Stratification Systems (RSS) are a cornerstone for the prevention of DFUs and related amputations and serve as clinical tools to assess the foot at risk. As the causes of DFU are complex and multifactorial, a variety of different parameters are needed to accurately assess risk. However, the current RSS do not take these multifactorial aspects into account and instead relies only on a few basic clinical measurements. Only five variables namely, diabetic neuropathy, peripheral vascular disease,

foot deformity, and history of previous foot ulcer and amputation are commonly included across most RSS (Monteiro-Soares *et al.*, 2011). None of these systems include microcirculation or specifically address small and large fibre neuropathy. However, these parameters may be potentially useful and aid early prediction. One of the main reasons for the RSS not including the investigations of these parameters is the unavailability of Non-invasive testing has been used for decades as a first-line investigatory tool in the diagnosis and classification of PAD. Non-invasive techniques are widely available and are low-cost options without the exposure of patients to ionising radiation or invasive techniques (Cooper *et al.*, 2018). However, a vast majority of current guidelines and recommendations for the diagnosis of PAD hinges on only select macrocirculatory measures. The commonly used measure for macrocirculation is ABI. However, it is unreliable in people with arterial calcification such as those with diabetes. Furthermore, ABI does not allow the localisation of the disease, the distinction between single-level and multilevel disease, or the characterisation of arterial occlusive lesions (Cooper *et al.*, 2018). In that case, the Toe Brachial Index (TBI) is measured as the subsidiary arteries are less likely to be calcified. Current guidelines rely on the use of taking measurements manually using a Doppler probe of suitable frequency in preference to an automated system (NICE, 2018). In many other countries, the manually operated sphygmomanometer is the standard choice. Through recent advancements in research technologies, there are automated systems that facilitate a quick and objective assessment of a wide range of measures that are not limited to ABI and TBI. Neither ABI nor TBI give a whole picture of the vascular complications, therefore, the use of automated systems in addition to conventional methods may aid thorough assessment of PAD. Optimising technology for early diagnosis and prevention of PAD is a vastly deliberated topic in research (Mani *et al.*, 2016). Not only with PAD, but also for the RSS to become comprehensive and include the assessments of microcirculation and small fibre functions, the need for using more simplistic, reliable, non-invasive, affordable and time-effective methods is essential.

Understanding the relationship between microcirculation and small fibre functions, microcirculatory hyperaemic responses, and their role in DFU may aid in early prediction and it remains an intriguing area of research. Firstly, research that collectively explores microvascular and small fibre nerve functions and their interplay is required. The findings from such research would help to enhance the understanding of the relationship between microvascular and small fibre nerve functions. Secondly, appropriate methods to measure the microcirculatory and small fibre nerve functions need to be identified. This, in turn, may throw more light to inform if these parameters could help to identify foot at risk of DFU. Such research would be to improve existing or build comprehensive RSS for early prediction and prevention of DFU.

### 1.3.2 Scope and boundaries of this PhD

This PhD intends to establish the relationship between microcirculation and small fibre nerve functions by identifying and utilising a set of tests that assesses microvascular and small fibre nerve parameters to determine their relationship. The derived insights can aid in mapping the variables that may be used to assess foot in diabetes. Consequently, practical implications from this PhD research can potentially be valuable for a comprehensive approach to the foot in diabetes to promote early diagnosis, treatment, enhancing prognosis by devising management and adapting prevention strategies that can change the paradigm of diabetic foot care in future. The main intention of this research was to develop clinical protocols and simplistic methods for microcirculatory assessment in the foot. However, in this work, no clinical interventions were involved and the testing in people with diabetes or diabetes foot-related complications will not be discussed as the main purpose was to gain a better understanding of the underpinning mechanisms between microcirculation and small fibre nerve functions in the foot of the healthy that may aid to develop a strategic approach to assess foot at risk in the future. Clinical recommendations are beyond the scope of this research.

### 1.3.3 State of the Art

#### *1.3.3.1 Overall Aim*

This PhD aims to determine the association between the cutaneous (skin) microcirculation and the small fibre functions in the foot.

#### *1.3.3.2 Objectives*

The objectives of this program of work are:

- To understand the neurovascular interactions by examining the existing body of literature
- To develop a clinically applicable method to quantify cutaneous microcirculation in the foot
- To develop a clinically applicable method to quantify small fibre nerve functions in the foot
- To establish the relationship between cutaneous microcirculation and small fibre functions in the foot

This research will contribute to a deeper understanding of cutaneous microcirculation assessment in the foot. The unique contributions of this research will be the profound knowledge of cutaneous microcirculation of the foot and the neurovascular interaction. In recent decades, it has been realised that complex health issues need to be broken down into simple problems and solved through integrated research. Therefore, this study uses a system thinking approach and aims to break down the complexity of neurovascular interactions in the foot by separately quantifying cutaneous microvascular and small fibre nerve functions and then determining their relationship.

#### *1.3.3.3 Research execution*

The systematic research execution and study flow are presented in different stages below. The study encompasses five stages, each with specific objectives and research questions. The overall step-by-step plan of work and studies undertaken to answer the questions in various stages are presented in Figure 1.12.

##### 1.3.3.3.1 Stage 1 Literature review

A literature review is conducted to identify the relationship between microcirculation and small fibres in the diabetic foot, establish their interactions, and identify non-invasive methods to study these functions.

Research question: Is there an interaction between microcirculation and small fibre functions and what are the relevant non-invasive methods to quantify microcirculation and small fibre nerve functions?

Objectives: Conduct a narrative review to:

- Identify the relationship between microcirculation and small fibres in diabetic foot
- Establish the interactions
- Identify various non-invasive methods to study microcirculation and small fibre functions

##### 1.3.3.3.2 Stage 2 Quantitative Study 1

It involves a quantitative study to systematically investigate cutaneous microcirculation of the foot in relation to sensory nerve fibres. This includes evaluating existing protocols, developing new investigative protocols, and quantifying microcirculation at specific sites on the foot.

Research question: How to systematically investigate the cutaneous microcirculation of the foot in relation to sensory nerve fibres?

Objectives:

1. Utilise the test identified in stage 1 that aids in assessing cutaneous microcirculation in minimal time
2. Evaluate existing protocols and identify key gaps
3. Develop various protocols to systematically investigate microcirculation.
4. Quantify microcirculation at specific sites at the plantar aspect of the foot
5. Finalise protocol for subsequent studies

#### 1.3.3.3.3 Stage 3 Quantitative Study 2

This focuses on assessing cutaneous microcirculation in relation to autonomic nerve fibres. This involves identifying sympathetic stimuli to quantify microcirculation and conducting measurements at the plantar aspect of the foot.

Research question: How to assess cutaneous microcirculation in relation to autonomic nerve fibres?

Objectives:

1. Based on the literature identify simple, easy to replicate and quick sympathetic stimuli to quantify microcirculation of the foot
2. Quantify microcirculation at the plantar aspect of the foot using sympathetic stimuli: deep inspiration and expiration

#### 1.3.3.3.4 Stage 4 Quantitative Study 2

This study aims to assess the functions of small fibres in the foot. This includes identifying tests to assess small fibre functions relevant to the clinical setting, developing protocols, and quantifying autonomic and sensory small fibre functions.

Research question: How to assess the functions of small fibre in the foot?

Objectives:

1. Based on the literature identify tests that assess small fibre functions in minimal time relevant to the clinical setting.
2. Evaluate existing protocols and identify key gaps
3. Develop various protocols to quantify small fibre nerve functions in the foot
4. Quantify autonomic small fibre functions:
    a. Heart rate
    b. Galvanic Skin Response
5. Quantify a sensory small fibre function, the temperature on the plantar aspect of the foot

#### 1.3.3.3.5 Stage 5 Quantitative Study 2

The final stage seeks to determine the relationship between cutaneous microcirculation and small fibre nerve functions in the foot. The data gathered from Stages 3 and 4 are utilised to analyse interactions and inter-relationships between various parameters, establish associations, and identify predictors of microcirculation.

Research question: Is there a relationship between cutaneous microcirculation and small fibre nerve functions in the foot and can microcirculation be predicted based on measures related to small fibre nerve function?

Objectives:

1. Utilise the data on quantifying the microcirculation at the plantar aspect in response to sympathetic activities: deep inspiration and expiration (Stage 3 data)
2. Utilise the data on quantifying small fibre functions (Stage 4 data):
    a. Skin temperature
    b. Heart rate
    c. Galvanic skin response
3. Analyse the interactions and inter-relationships between various parameters
4. Establish the association
5. Identify a predictor of microcirculation

Overall, the research spans from reviewing existing literature on microcirculation and small fibre functions to conducting quantitative studies to assess these functions. The ultimate goal is to establish the interplay between microcirculation and small fibre functions and to identify non-invasive methods for their quantification.

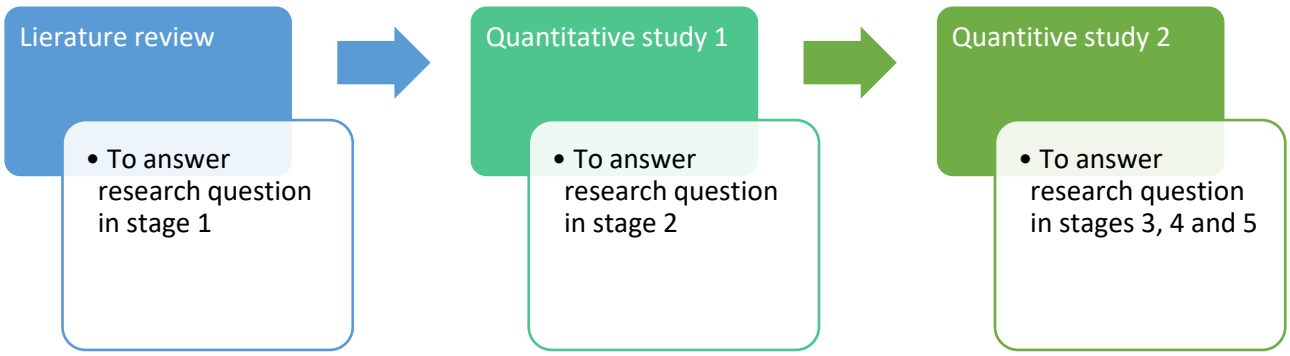



*Figure 1.12 Step by step research plan to answer research questions set out in stages 1 to 5*

## 1.4 Conclusion

This chapter provided an overview of the importance of continued research in the area of diabetic foot, research interests and gaps in existing literature. Given the rising prevalence of DFUs, there is an essential need to develop solutions for prevention and early diagnosis. To achieve this, a deeper understanding of neurovascular parameters is required. The previous research focus was broad, which primarily included the macrocirculatory aspect of peripheral vascular disease and peripheral

neuropathy. A closer look at the literature, reveals several gaps and shortcomings, especially in understanding the physiology and mechanisms of microcirculation and small fibre nerve functions. A more concise understanding of this concept is required to unravel their role in the intricate events that may lead to DFU. The aims, objectives, approach, scope and boundaries of this current research were also discussed in this chapter. The following chapters focus on the methodology and primary research undertaken to answer the research questions set out in this introductory chapter.

# CHAPTER II

METHODOLOGY

# Chapter 2 Methodology

## 2.1 Introduction

From the chapters (1-3), it is evident that the work to date in the field of the diabetic foot has two main sub-areas; first vascular and second neurological. However, most of these studies have focused extensively on the macrovascular (vascular) and large fibre functions (neurological). But none of the existing approaches seem comprehensive enough to include microvascular (vascular) and small nerve fibre functions (neurological). A deeper understanding of these parameters is required to identify an at-risk foot, prevent unwanted complications at an early stage, or implement preventive strategies. In the literature, as described in Chapters 2 and 3, there is ample theoretical and experimental evidence that microcirculation plays a central role in foot health. Nevertheless, there are research gaps to be closed. Several questions remain about the occurrence of foot complications, the factors that provoke ulceration, and whether microcirculatory impairment precedes small fibre dysfunction or vice-versa. All these questions led to the realisation that it is important not only to gain a deeper understanding of the microcirculation in the foot but also to study the neurovascular interactions.

As mentioned earlier in Chapter 1, this PhD aimed to determine the relationship between cutaneous microcirculation and small fibre functions in the foot. The research objectives were used to develop relevant research questions as seen in Table 2.1 below. While Chapter 1 outlined the research questions and generic objectives, following a thorough literature review (Chapters 2 and 3), the specific objectives are presented in Table 2.1. This chapter describes the methodology and methods for answering the research questions to adequately address the goal of this PhD. In addition, the details and theoretical insights into the method are given.

*Table 2.1 PhD research execution in stages*

| Stage | Study | Research question | Objectives |
|---|---|---|---|
| **Stage 1** | **Literature review** | **Is there an interaction between microcirculation and small fibre functions and what are the relevant non-invasive methods to quantify microcirculation and small fibre nerve functions?**<br>A literature review was performed and the synthesised information was published in peer-reviewed | Conducted an iterative literature review to:<br>1. Identify the relationship between microcirculation and small fibres in diabetic foot<br>2. Establish the interactions<br>3. Identify various non-invasive methods to study microcirculation and small fibre functions |

| | | | |
|---|---|---|---|
| | | journals. The Chapter 1 on neurovascular interactions in the foot was published as a synoptic review (Appendix 1). Additionally, Chapter 1 on the role of microcirculation in tissue injury and inflammation was published as a narrative review (Appendix 2). | |
| **Stage 2** | **Quantitative study 1** | **How to systematically investigate the cutaneous microcirculation of the foot using Post Occlusive Reactive Hyperaemia (PORH)?** Chapter 3 on the systematic investigation of the microcirculation on the foot using PORH was published as a primary research article (Appendix 3). Also, a poster presentation (Appendix 10). | 1. Utilise the test identified in stage 1 that aids to assess cutaneous microcirculation in relation to sensory nerve fibre functions in minimal time<br>2. Evaluate existing protocols and identify key gaps<br>3. Develop various protocols to systematically investigate PORH<br>4. Quantify microcirculation using PORH at specific sites at the plantar aspect of the foot<br>5. Finalise protocol for subsequent studies |
| **Stage 3** | **Quantitative study 2** | **How to assess cutaneous microcirculation in response to sympathetic stimuli?** Chapter 5 | 1. Based on the literature identify simple, easy to replicate and quick sympathetic stimuli to assess microcirculation of the foot in relation to autonomic fibre functions<br>2. Quantify microcirculation at the plantar aspect of the foot using sympathetic stimuli: deep inspiration and expiration |

| | | | |
|---|---|---|---|
| **Stage 4** | **Quantitative study 2** | **How to assess small fibre functions in the foot?**<br>Chapter 5 | 3. Based on the literature identify tests that to assess small fibre functions in minimal time<br>4. Evaluate existing protocols and identify key gaps<br>5. Develop various protocols to quantify small fibre nerve functions in the foot<br>6. Quantify autonomic small fibre functions:<br>a. Heart rate<br>b. Galvanic Skin Response<br>7. Quantify sensory small fibre function: the temperature on the plantar aspect of the foot |
| **Stage 5** | **Quantitative study 2** | **Is there a relationship between cutaneous microcirculation and small fibre nerve functions in the foot and which parameter is a predictor of microcirculation?**<br>Chapter 5 | 1. Utilise the data on quantifying the microcirculation at the plantar aspect in response to sympathetic activities: deep inspiration and expiration (Stage 3 data)<br>2. Utilise the data on quantifying small fibre functions (Stage 4 data):<br>a. Skin temperature<br>b. Heart rate<br>c. Galvanic skin response<br>3. Analyse the interactions and inter-relationships between various parameters<br>4. Establish the association<br>5. Identify a predictor of microcirculation |

## 2.2 Research Methodology

### 2.2.1 Research Strategy

Positivism is an epistemological position that advocates the use of scientific methods, which is objective in nature (Bryman, 2015). Positivism is associated with the hypothetico-deductive model of science (Park, Konge and Artino, 2020). The hypothetico-deductive method, also known as the scientific method, is a circular process as represented in Figure 2.1 below (Park, Konge and Artino, 2020). As seen in the Figure below, the hypothetico-deductive method consists of several steps: a phenomenon is observed, a hypothesis is proposed to explain the phenomenon, implications of the hypothesis are inferred, implications of the hypothesis are evaluated using empirical evidence, and a conclusion is either accepted or rejected /change the hypothesis is drawn (Reichardt, 2021). This hypothetico-deductive method was used in this research. As seen in Table 2.1 above, a review was conducted of studies showing the importance of microcirculation while highlighting the knowledge gaps related to neurovascular interactions, how to assess them to identify a foot at risk and the role of microcirculation in tissue injuries and inflammation. The work in this area of microcirculation is extensive but is primarily concerned with delayed wound healing. Therefore, this research investigated several questions related to microcirculation in the foot, neurovascular interactions and the relationship between microcirculation and small fibre nerve functions using experimental research. This, in turn, will help gain a deeper understanding of the role of microcirculation in diabetic foot-related complications, and the findings will be useful to develop a new strategy for the early detection and prevention of diabetic foot-related complications, thereby, contributing to the body of literature by generating new theories or perspectives.

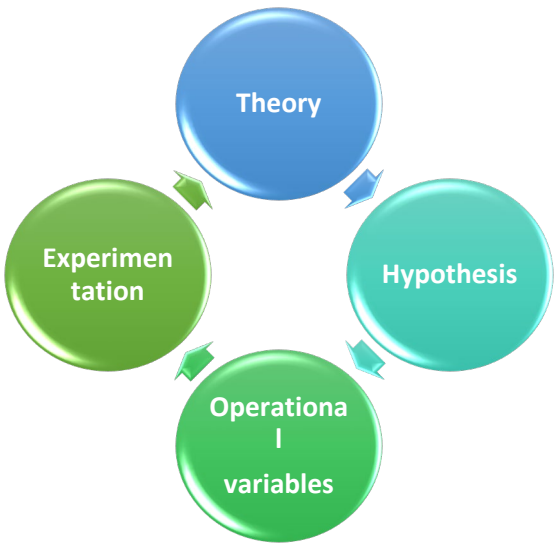



*Figure 2.1 Hypothetico-deductive method*

### 2.2.2 Research Method

In accordance with the hypothetico-deductive approach, the quantitative research method was used for this research. The quantitative method is accepted and widely used in clinical research and implementational research in health and social care (Curtis, 2013). The use of quantitative research methods has many advantages. First, this method uses numerical data and statistics to collect and interpret findings, which is time and resource-efficient (Bryman, 2015; Daniel, 2016). Secondly, it aids objectivity, since observations that are quantifiable and constitute real evidence are key to quantitative research methods (Balnaves and Caputi, 2001). The numerical data that are generated by quantitative research are 'objective' and exist independently of the researcher and are not the result of undue influence on the part of the researcher. The data are viewed as the product of research tools that have been tested for validity and reliability to ensure that the data accurately reflect the events and not the researcher's preferences (Denscombe, 2014). Hence, it can be considered as a "researcher detachment" research approach, one of the strengths of quantitative research (Denscombe, 2014; Daniel, 2016). Secondly, quantitative research is systematic and empirical, as theories and hypotheses are proposed and the research question is answered utilising various methodological approaches and techniques (Curtis, 2013). Hence, they are reproducible, and the interpretation of research findings does not have to be regarded as mere coincidence. Thirdly, quantitative research helps to answer more specific and narrow research questions (Curtis, 2013). Thus, quantitative research can be construed as a research strategy that lay emphasis on quantification in data collection and analysis (Bryman, 2015). It involves a deductive approach and is thus consistent with positivism and its norms (Bryman, 2015). In quantitative research, the steps usually involve a hypothesis that is derived from the theory and tested (Bryman, 2015). The Figure 2.2 below shows the main steps involved in quantitative research and how the current study was aligned with it.

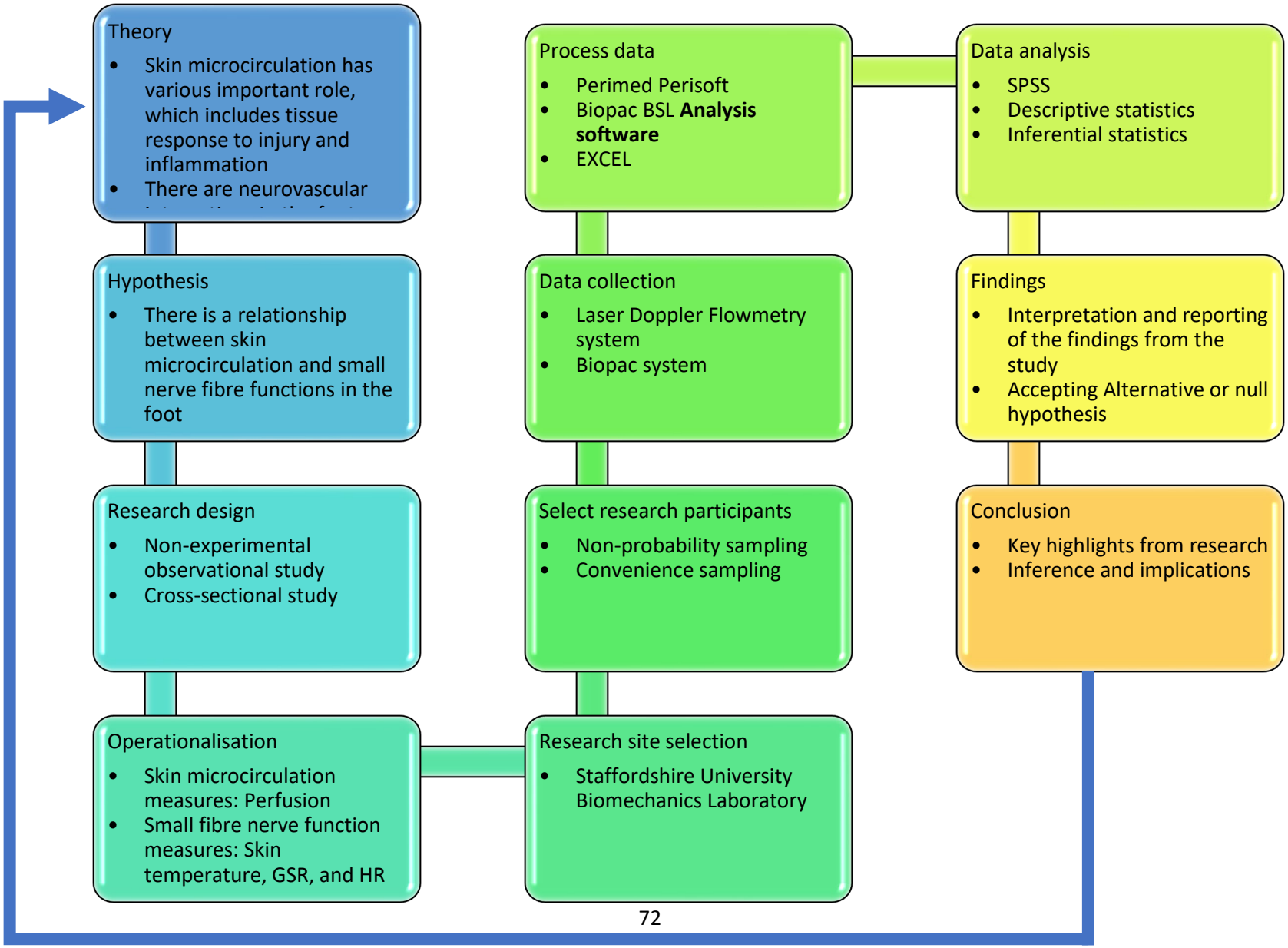





*Figure 2.2 Quantitative research process*

### 2.2.3 Research Design

Study design plays an important role in the quality, conduct, and interpretation of research in the healthcare sector. The current study was an observational study and the research design used was a cross-sectional design. In observational studies, the researcher simply observes the associations between factors and outcomes, and they remain important because many questions can be answered efficiently through these methods (Mann, 2003; Thiese, 2014). A cross-sectional study design is a type of observational study design. In a cross-sectional study, the investigator simultaneously measures outcome and exposures in study participants selected using inclusion and exclusion criteria (Setia, 2016). Only one group is used, data are collected only once, and multiple outcomes can be examined simultaneously, so it is less time-consuming and less expensive as it does not require follow-up (Mann, 2003). This type of study design is used to understand the association between variables and is also used to infer causality but not to determine cause and effect (Mann, 2003; Setia, 2016). However, the goal of distinguishing cause and effect from simple associations can then be further investigated using a cohort study or a randomised controlled study (Mann, 2003). Therefore, a cross-sectional study is a steppingstone to the next level of research, especially when it comes to establishing relationships between variables. The Figure 2.3 below shows the plan for the current study using cross-sectional study design.

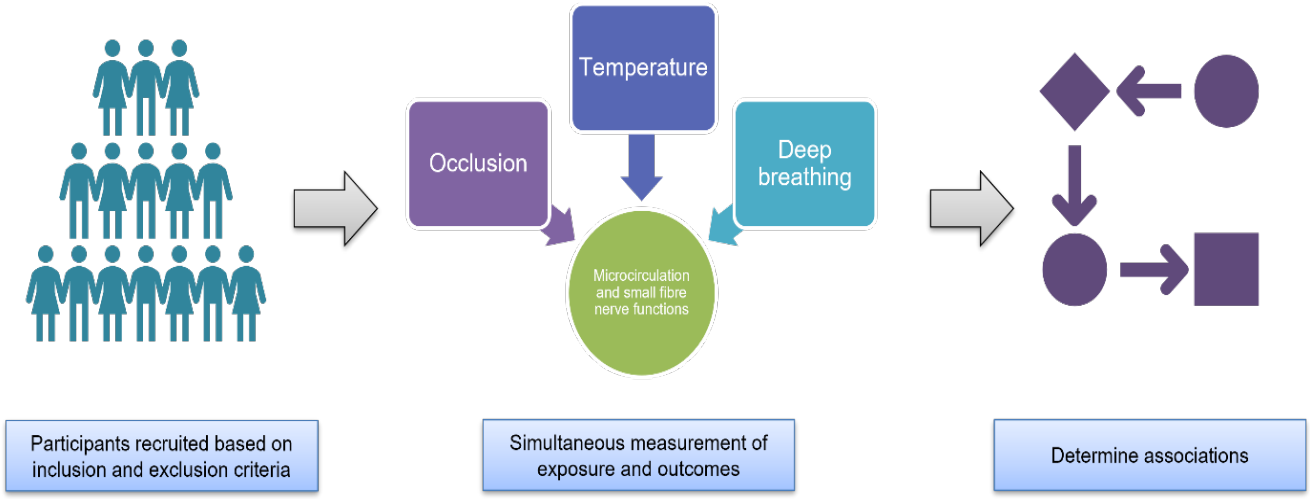



*Figure 2.3 Cross-sectional study plan*

### 2.2.4 Time Horizon

The PhD research was conducted over a time period of 4 years. The Figure 2.4 below shows the timeline for every stage of the research over four years. The data collection process for study 2 was impacted by the COVID-19 outbreak, which resulted in a smaller sample size. The data was collected and extracted from the current sample size of 10 between November 2019 and March 2020. However, the lab was subsequently closed due to lockdown regulations.

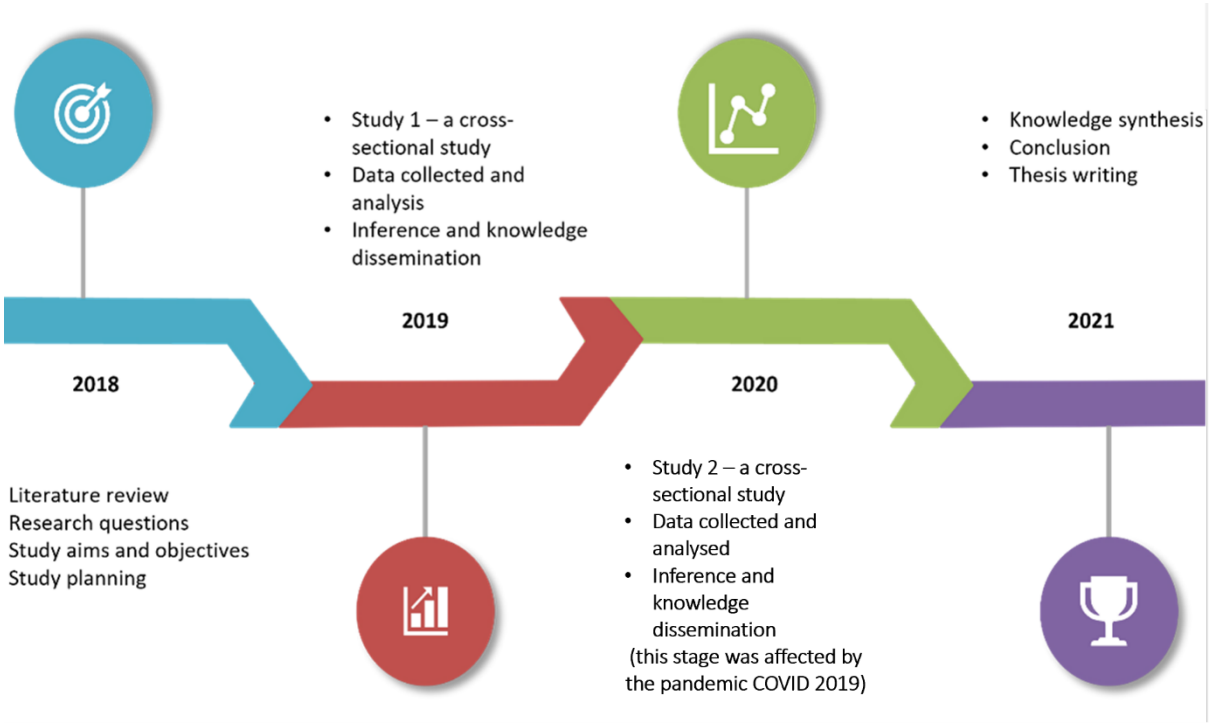



*Figure 2.4 Timeline for the PhD research*

### 2.2.5 Ethics

The ethics approval was sought from the University Ethics Board. The rationale for the study, risks assessment forms, information sheet, an advertisement for recruitment, consent form and other relevant forms along with the ethics application were submitted for review. The study commenced after obtaining ethical approval. These documents and the ethics approval from the committee are included in the Appendix (4, 5, 6, 7 and 8). A training was undertaken to operate the equipment Perimed® Periflux System with the manufacturers on site at Perimed®, Stockholm, Sweden. The Diploma awarded is attached as Appendix 9.

### 2.2.6 Sampling Strategy and recruitment

The sampling strategy used in this study was the non-probability sampling technique called convenience sampling. This sampling method is also referred as accidental sampling or grab sampling.

In convenience samples, participants who are accessible and readily available to the researcher are more likely to be included as shown in the Figure 2.5 below (Wu Suen, Huang and Lee, 2014; Setia, 2016). This method is relatively easy and is one of the common types of sampling methods used particularly in postgraduate dissertations and clinical studies (Setia, 2016). Some of the limitations of using a convenience sampling strategy are that the estimates from such a study may not necessarily be generalisable to the larger population as the participants who were accessible to the researcher may be different compared with others in other settings. In addition, low external validity can be a problem due to the selection bias that may arise. For convenience sampling in a clinical study, this could be an issue as not all patients may have access to a particular healthcare setting, their healthcare-seeking behaviours may vary and patients in tertiary care centres may be severe, complicated, or recalcitrant (Setia, 2016). As a result, research findings may differ among these compared to the general population (Setia, 2016). However, the current study was conducted on healthy adults. Thus, the above issue may not be a limitation of the current study. But there may be potential bias that can occur due to convenience sampling, which is also acknowledged in the final conclusion chapter along with other strengths and limitations of this thesis (Setia, 2016).

For the recruitment of participants for this study using convenience sampling, the recruitment materials for the study such as the information sheet and the advertisement, used in both paper and digital form (Appendix 6 and 7), were distributed within the university and the social circles. After the potential participants were approached, they were informed about the study. All individuals who consented to participate in the study were assessed for eligibility based on inclusion and exclusion criteria.

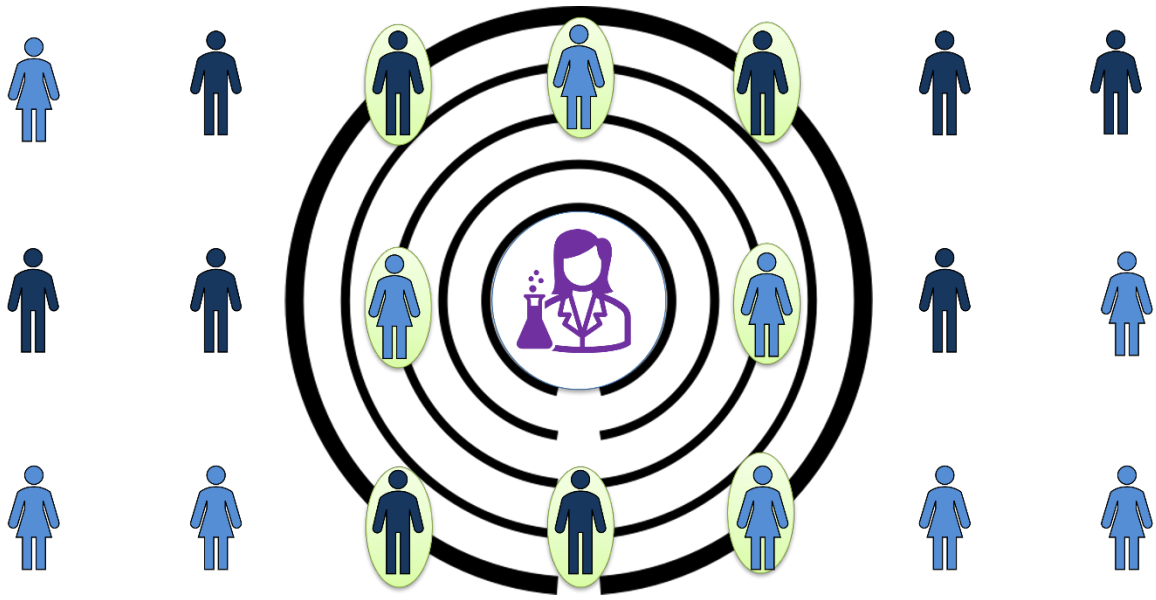


*Figure 2.5 Convenience sampling*

## 2.3 Data Collection Method

First, a thorough literature search was systematically performed to identify the main gaps in the literature, as described in the previous chapter 1. The literature review aided to explore the existing evidence available on neurovascular interactions. In addition, clinically applicable, less time-consuming, and convenient non-invasive tests to assess microvascular and small fibre nerve function were identified. Finally, a list of vascular and neurological measures was drawn up. The rationale for selecting these tests with evidence from the literature along with the data collection methods and equipment used are presented in the tables below. The focus of this research is not on utilising generic techniques, but on specific ones for neurovascular assessments. Therefore, some distinctive aspects of the combinations of tests included in this thesis are that they are non-invasive, less time-consuming and had the ability to assess neurovascular attributes. Although the main focus is on microcirculation and small fibre nerve functions, the most commonly used macrovascular measurements such as ABI and TBI and neurological tests such as QST for small fibre functions were also performed. These tests were performed as a part of standard assessment and not as the core of the research. The research process shown in Figure 2.6 below briefly outlines the stepwise execution of the research in line with the research questions, aims and objectives laid out in Table 2.1. The rationale for the use of specific data collection methods, the name of the assessments and the equipment used for vascular and neurological measures are presented in Tables 2.2 and 2.3, respectively. Both the quantitative studies were conducted within the Biomechanics laboratory at Staffordshire University. The study set up for each of these studies is included in the respective chapters (3-5).

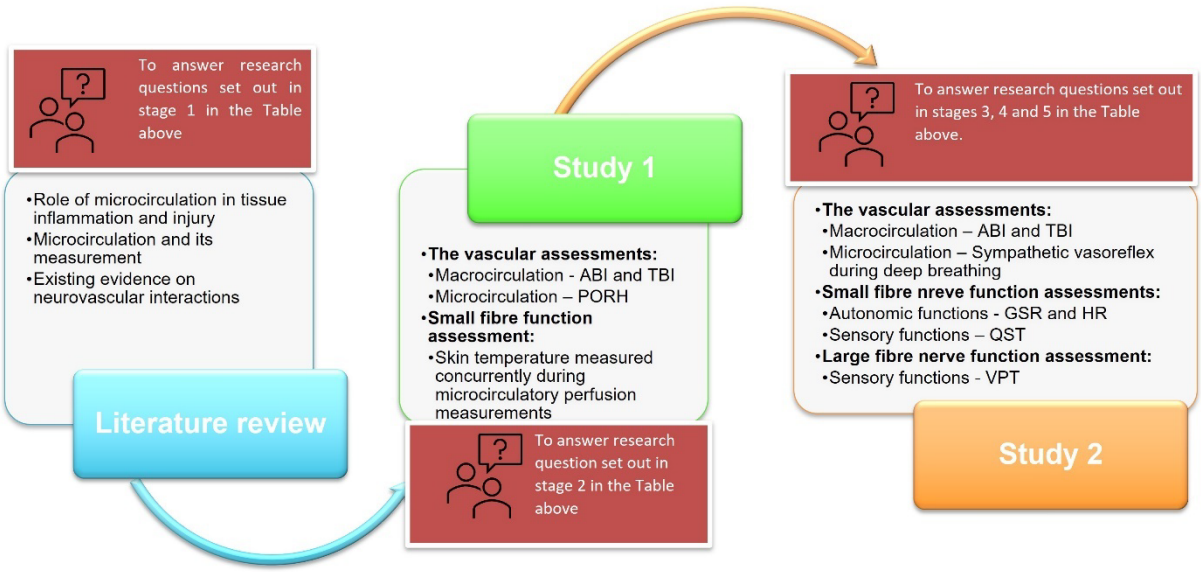



*Figure 2.6 An overview of the research process*

*Table 2.2 Vascular measures*

| S.No | Name of the test(s)/method(s) | The rationale with evidence from the literature | Method (a brief description of the methods is given here but elaborated in the subsequent chapters) | Equipment |
|---|---|---|---|---|
| 1 | ABI and TBI | ABI is the ratio between the highest systolic pressure measured from both arms and the systolic pressure in each leg. Also, TBI is the ratio between the highest systolic pressure measured from both arms and the systolic pressure in each toe. ABI is commonly used to measure blood pressure and to measure the extent of block or stiffening of the arteries. It is used to support the diagnosis of vascular disease by providing an objective indicator of arterial blood flow to a lower extremity (Høyer, Sandermann and Petersen, 2013; Hyun *et al.*, 2014). The ABI is known to be unreliable in patients with vascular stiffness or calcified blood vessels and fails to detect the early phase of arteriosclerotic development, in such cases, TBI is | To measure ABI and TBI blood pressure cuffs are placed on both arms followed by both ankles and then toes. There are different types of equipment to measure blood pressure and the most commonly used devices are sphygmomanometer (auscultatory and oscillatory methods), automated blood pressure monitors and Doppler wave pen. The Laser Doppler Flowmetry (LDF) system is one of the more recent equipment that is used for measuring microcirculation (described in detail below). However, this can also be used to measure the blood pressure in the arms, ankles and toes. To be | In this study, the LDF (Perimed® Periflux system 5000 by Perimed® Stockholm, Sweden) was used for data collection. Laser Doppler measures the total, local microvascular flow including capillaries, arterioles, venules and shunts and the results are given in perfusion units (Jeschonneck *et al.*, 2000). There can be several main systems, each equipped with four functional units to have the desired number of channels that facilitates simultaneous measurements of various vascular parameters. The system used for this study had a single main unit with 4 different functional units. These |

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| | | recommended as the smaller vessels are less likely to be affected by calcification (Høyer, Sandermann and Petersen, 2013). | consistent with the equipment used for vascular measures, LDF was used. The measurements from the LDF technique are comparable to that derived from the oscillometric sphygmomanometer (Hu *et al.*, 2012). | function units were: two PF 5010 – Laser Doppler Perfusion Monitoring (LDPM) units used for blood perfusion measurements based on laser Doppler technology, a PF 5020 – Temperature unit used to perform local heat provocation tests and/or for temperature measurements and PF 5050 – Pressure unit used to control linear or instant deflation of the pressure cuffs. The pressure unit helps to simplify and standardise tests such as peripheral pressure measurements and PORH. The thermostatic laser Doppler probes (capable of measuring perfusion and temperature) have a contact area of 1 $cm^2$ each. They were secured to the participants' skin on the location of interest using double-sided adhesive tape. The system was attached to a laptop with exclusive software, PeriSoft that helps to run |

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| | | | | the protocols, record data and perform analysis. |
| **2** | PORH | PORH is the transient increase in blood flow in the organ or tissue that occurs following a brief period of arterial occlusion (Klabunde, 2012).<br>It is known that PORH is primarily an endothelial-dependent process, however, both endothelial-dependent and independent mechanisms are involved (Wierzbowska et al., 2014; Lanting et al., 2017). Upon application of an arterial occlusion using a cuff, a hyperaemic response is induced as a result of the shear stress, the tangential frictional force acting at the endothelial cell surface. PORH is reduced in people with peripheral arterial disease and has been associated with increased cardiovascular risk (Morales *et al.*, 2005). Besides, it is decreased PORH response is associated with diabetic foot complications | Based on this study's interest to assess the microcirculatory response to occlusion in the plantar aspect of the foot, the cuff will be placed on both the ankles or both the toes. The laser probes will be placed on the distal end of the toes. The non-invasive Laser probes which are $1cm^2$ discs will be placed at the distal end of the toes on the plantar aspect and will be secured using double-sided tape. The pressure in the cuff will be elevated above physiological arterial blood pressures, 200-220 mmHg, which will cause a mechanical arterial occlusion at the site. The occlusion time will be either 60, 30 or 10 seconds with and without temperature control of 33°C at the site. The protocols for both studies 1 | The same equipment as described above, the LDF system (Perimed® Periflux system 5000 by Perimed® Stockholm, Sweden) was used for data collection. The system was connected to a laptop running the exclusive PeriSoft software that helps run the protocols, record data and perform analysis. With PeriSoft most of the tests were automated and set protocols based on the current research were entered into the system. |

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| | | (Barwick *et al.*, 2016). A study by Lanting et al (2017) showed that an increase in time to Peak, which is a variable that shows the time taken for a maximum flux post occlusion, increased the likelihood of a participant having a history of foot complications by 2%. These findings in a cohort with type 2 diabetes with previous history or existing foot related complications support the need for further investigation into the relationship between measures of microvascular function and the development of diabetic foot complications, prospectively. Hence, this test is used in the current study as the core of the study focuses on understanding microcirculation of the foot for application in the area of diabetic foot-related complications. The current study will aid in further expounding the knowledge of microcirculation in the vascular domain. | and 2 are discussed later in the subsequent chapters. | |

| | | | | |
|---|---|---|---|---|
| **3** | Sympathetic vasoreflex during deep inspiratory gasps | Although impairment of autonomic function and microcirculation does not necessarily imply a causative role in ulcer formation, as discussed in Section 1 of the literature review, their potential role in ulcer development cannot be ignored (Deanfield, Daggett and Harrison, 1980; Delis *et al.*, 2001). Damage to the sympathetic nervous system is an important characteristic of diabetic neuropathy. This in turn impairs vasomotor control and increases skin capillary permeability (Deanfield, Daggett and Harrison, 1980; Delis *et al.*, 2001). Therefore, it is important to understand the microcirculation in response to sympathetic vasoconstriction in healthy individuals to gain an understanding of it for application in people with diabetes to use as a prognostic tool for diabetic foot ulceration. One of the stimuli that induce a vasoconstrictive reflex is a deep inspiration. This has been demonstrated as early as 1939 | The LDF's laser probes were attached to the distal aspect of the toes in the same manner as above. Measurement of microcirculation was performed during a baseline of 10 minutes under normal breathing conditions and three deep inspiratory gasps, which included deep inspiration and deep expiration. | The same equipment as described above, the LDF system (Perimed® Periflux system 5000 by Perimed® Stockholm, Sweden) was used for data collection. The system was connected to a laptop running the exclusive PeriSoft software that helps run the protocols, record data and perform analysis. |

| | | | | |
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| | | (Mulinos and Shulman, 1939). The deep inspiration vasoconstriction reflex is mediated by the sympathetic nervous system (Khoo and Chalacheva, 2019). In people with diabetes, autonomic neuropathy is a result of impairment within the autonomic nervous system, which includes the sympathetic and parasympathetic divisions. Autonomic neuropathy causes microcirculatory disturbances (Deanfield et al., 1980). Although the existing literature emphasises this association, little is known about the autonomic functions in the foot and their impact on skin microcirculation. | | |

*Table 2.3 Small fibre function measures*

| S.no | Name of the test(s)/method(s) | The rationale with evidence from the literature | Method (a brief description of the methods is given here but elaborated in the subsequent chapters) | Equipment |
|---|---|---|---|---|
| 1 | GSR and HR | Autonomic functions mediated by the small fibre nerves play a vital role in the maintenance of the homeostasis within the body and understanding these functions is essential to underpin the mechanisms behind neurovascular interactions (Ewing *et al.*, 1985; Themistocleous *et al.*, 2014; Balasubramanian *et al.*, 2020). One of the non-invasive testing that can be used is measuring HR and GSR during deep breathing, which uses the sympathetic vasoreflex (Ewing *et al.*, 1985; Allen, Frame and Murray, 2002). Understanding the autonomic functions is vital to underpin the mechanisms that underlie a diabetic foot ulcer incident. Autonomic neuropathy causes decreased HR and sympathetic denervation in the skin. This | The BIOPAC system has leads and pre-gelled electrodes with adhesives were secured on the hands and foot of the subject. There were 1 HR channel and 2 channels that recorded the Electrodermal activity (EDA) on right and left feet. Measurements of GSR and HR were taken during a baseline of 10 minutes under normal breathing conditions and three deep inspiratory gasps, which included deep inspiration and deep expiration. | The BIOPAC wired physiology measurement kit was connected to a laptop with the BSL software used for data acquisition. There are various hardware units available to collect a range of physiological parameters. For this study, the MP systems were used for the acquisition of EDA also known as Galvanic Skin Response (GSR) and heart rate (HR). The system has leads and pre-gelled electrodes with adhesives were secured on the hands and foot of the subject. There were 1 HR channel and 2 channels that recorded the EDA on right and left feet. Measurement of microcirculation was performed |

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| | | results in 'capillary steal syndrome' and arteriovenous (AV) shunting in people with diabetes, which deprives skin nutrition (Chao and Cheing, 2009; Kaufmann and Biaggioni, 2010; Armstrong and Lavery, 2016). When skin is deprived of nutrition, it results in and dry feet that is prone to cracks, injury and infections. understanding the interactions between autonomic functions of the small fibre nerves and skin microcirculation at the foot can be useful to establish the neurovascular interactions that may potentially have application in the diagnosis and management of various pathological conditions such as this can be specifically relevant to diabetic foot complications. | | during a baseline of 10 minutes under normal breathing conditions and three deep inspiratory gasps, which included deep inspiration and deep expiration. |
| **2** | Skin temperature (Sensory functions) | The small fibre nerves mediate the temperature sensation, the Aδ (warm perception and pain) and C (cold perception and pain). Apart from this, the microvasculature aids thermoregulation. In people with diabetes, both sensory and | The laser probes of the LDF were attached to the distal aspect of the toes as described above. The skin temperature was recorded concurrently with perfusion measurement using thermostatic | The same equipment as described above, the LDF system (Perimed® Periflux system 5000 by Perimed® Stockholm, Sweden) was used for data collection. The system was connected to a laptop with PeriSoft |

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| | | autonomic capabilities of the nerves may be compromised. In addition, the microcirculatory component may also be impaired. One of the cardinal inflammatory signs in a tissue is the temperature rise. Studies have shown that skin temperature varies in people with diabetes-related complications such as neuropathy (Papanas *et al.*, 2009; Bagavathiappan *et al.*, 2010; Gatt *et al.*, 2018).<br>It has also been suggested that skin temperature may rise when the foot is at risk of ulceration (Bus *et al.*, 2021). However, most of these studies have examined the skin temperature of macrovascular complications or neuropathy but not microvascular ones (Papanas et al., 2009; Shapiro and Nouvong, 2011a; Chatchawan et al., 2018). However, it is evident from the literature that microcirculation plays a vital role in regulating temperature and hyperaemic responses. Furthermore, the role played in | probes 457 which captures both of these measures. Skin temperature was measured in both study 1 while assessing PORH and study 2 while examining sympathetic vasoreflex during deep breathing. | software that helps run the protocols, record data and perform analysis. The PF 5020 – Temperature unit recorded and displayed temperature measurements for both feet. |

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| | | microcirculation in inflammation and injury response is well-realised (Balasubramanian, Chockalingam and Naemi, 2021b). Therefore, it is important to study the relationship between skin temperature and microcirculation. | | |
| **3** | Sensory functions | Quantitative Sensory Testing (QST) allows us to measure changes in sensitivity to different types of sensations that can include temperature, touch or pressure. QST is used to assess the Aδ (warm perception and pain) and C (cold perception and pain) small nerve fibres. The QST has been used to assess the small nerve functions for various diabetes and other conditions involving sensory neuropathy (Ziegler, Mayer and Arnold Gries, 1988; Siao and Cros, 2003; Themistocleous *et al.*, 2016). Although the core part of this research was to use neurovascular assessments, the QST was tested as it is one of the common assessments for small fibre | The participant was requested to touch a square electrode on the plantar aspect of the feet. The temperature will be gradually increased from 32ºC to 45ºC (The heat sensitivity temperature and maximum threshold will be noted). The temperature will be gradually decreased from 32ºC to 10ºC (The cold sensitivity temperature and maximum threshold will be noted). However, under no circumstance will the temperature supersede the safety limits. | The Medoc® TSA2 QST machine by Medoc®, Ramat Yishay, Israel was used in conjunction with a laptop with software uploaded to it. The software came with different protocols to test the 4 sensations:<br>1. Heat perception<br>2. Cold perception<br>3. Heat-induced pain perception<br>4. Cold-induced pain perception.<br>The thermode was attached to the dorsal and plantar side of the foot to understand vibration perception The thermode was secured on the dorsal |

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| | | functions similar to ABI/TBI measured for the vascular assessment. | | and plantar aspect of the foot, This is to understand the vibration perception in glabrous and non-glabrous skin. The testing was performed for the right and left feet. |

## 2.4 Data analysis

Data extraction was performed using the software provided with each device. The details of the software that accompanied each device are provided in the tables above. The software Perimed's PeriSoft for vascular measures and BSL software for Biopac were set to run automated protocols and add events/markers. The in-built software in Medoc TSA II Neuroanalyser was used to analyse the QST data. Although with PeriSoft most of the tests and analysis were automated, the tests were performed under close supervision and some manual input. In certain cases, such as drawing a PORH graph the automated tools in the software were used. On the other hand, for BSL software the baseline, inspiratory and expiratory areas needed to be manually marked using the markers inserted during data collection. This process has been discussed separately in the methodology sections of each of the forthcoming chapters (3-5) based on the research study. After the data was extracted, it was saved in an EXCEL format for cleaning and sorting. The data was then imported into SPSS® 26 to 28 versions (depending on the version available for the particular year when analysis was performed) were used for further analysis using appropriate descriptive and inferential statistics. The report was then written on a Word document.

## 2.5 Conclusion

This chapter provided an overview of the methodology used throughout this doctoral research. There were sections, which provided the outline, rationale and main strategies used in this thesis. The chapter discussed the key parameters of interest consistent with the research question, ethical approval, and data collection and analysis steps. In addition, the research plan and the steps of its implementation were discussed. In the following chapters, the quantitative studies are discussed individually in detail, including the specific methodology used in each case.

# CHAPTER III

## A SYSTEMATIC EVALUATION OF CUTANEOUS MICROCIRCULATION IN THE FOOT USING POST-OCCLUSIVE REACTIVE HYPERAEMIA

A part of this chapter has been published in the following paper:

Balasubramanian, G., Chockalingam, N. and Naemi, R. (2021) 'A systematic evaluation of cutaneous microcirculation in the foot using post-occlusive reactive hyperemia.', *Microcirculation (New York: 1994)*, 28(5), p. e12692. Available at: https://doi.org/10.1111/micc.12692.

A part of this chapter has been presented as:

A Poster Presentation on "Systematic evaluation of skin microcirculation in the foot using PORH (Updated)" for poster presentation at the 71th Meeting of the British Microcirculation Society", UK, 2021

A Poster Presentation on "Systematic evaluation of skin microcirculation in the foot using PORH (Updated)" accepted[1] for poster presentation at the 70th Meeting of the British Microcirculation Society", UK, 2021

An Oral presentation on "Systematic and swift investigation of microcirculation of the foot using PORH", Research Seminar Series and meeting of Visiting Professors/Fellows, Staffordshire University, UK, 2019

An Oral presentation on "Systematic Investigation of skin microcirculation of the foot", Staffordshire Clinical Biomechanics Conference, Staffordshire University, UK, 2019

A Poster presentation on "Systematic evaluation of PORH in the foot", 14th International Symposium on Biomechanics in Vascular Biology and Cardiovascular Disease, Imperial College, UK, 2019

[1] The accepted poster was not presented in this case because of COVID-related issues

# Chapter 3 A systematic evaluation of cutaneous microcirculation in the foot using post-occlusive reactive hyperaemia

## 3.1 Introduction

This chapter comprises work concerned with developing a convenient and time-efficient protocol for measuring microcirculatory responses in the foot using Post-Occlusive Reactive Hyperaemia (PORH). Cutaneous microcirculatory impairments are associated with skin injury to the foot. Post Occlusive Reactive Hyperemia (PORH) is one of the quick and easy methods to assess microcirculatory function. However, there are variations in the protocols currently used. Hence, this study aimed to systematically investigate the reproducibility of PORH protocols with minimal occlusion time in the foot. PORH was measured using 12 different protocols (3 occlusion times, 2 occlusion sites and with or without temperature control) in 25 healthy adults. Each of the 12 different protocols was tested 3 times and the Intraclass correlation coefficient (ICC) was calculated. The results in this chapter suggest that microcirculatory assessment is feasible in routine practice and can potentially be included for routine assessment of foot in people with diabetes.

## 3.2 Background

Assessments of peripheral vascular function have long relied on macrocirculatory measurements such as the ankle-brachial index (ABI) and toe-brachial index (TBI). Even assessment of peripheral vascular function in individuals at risk for complications such as foot ulcers, clinical decisions, and risk stratification are based on guidelines that have been traditionally limited to measures such as ABI and TBI (Crawford *et al.*, 2006; Monteiro-Soares *et al.*, 2012; Hinchliffe *et al.*, 2016; Gerhard-Herman *et al.*, 2017; Schaper *et al.*, 2020). However, in recent times the measurement of microcirculation has gained importance, especially in the field of diabetes. Previous research shows that microcirculation plays a major role in diabetic foot-related complications (Tooke, 1989; Dinh *et al.*, 2012; A E Körei *et al.*, 2016). The recent development in medical technologies has facilitated the non-invasive assessment of the microcirculation. Various provocation tests such as heat provocation, cold provocation, postural changes and application of pressure stimuli are used to assess the cutaneous microcirculatory responses (Van den Brande, De Coninck and Lievens, no date; Roustit and Cracowski, 2012; Vouillarmet *et al.*, 2019). One such test is PORH, which is the transient increase in blood flow in the organ or tissue that occurs following a brief period of arterial occlusion (Klabunde, 2012; Balasubramanian *et al.*, 2020). A thorough literature review identified the knowledge gaps in the measurement of PORH in the foot and the aims and objectives of this research were to fill in some of the key gaps in order to strengthen the existing evidence base to enable its future application in diabetic foot syndrome.

PORH is known to be primarily an endothelial-dependent process, however it involves both endothelial-dependent and independent mechanisms (Wierzbowska et al., 2014; Lanting et al., 2017). The hyperemic response is a result of the shear stress, the tangential frictional force acting at the endothelial cell surface caused by arterial occlusion. In response to the mechanical stimulus, the endothelium releases vasodilating substances (Wierzbowska et al., 2014). Various factors are known to contribute to vasodilation which are myogenic, neurogenic, humoral and other local factors such as potassium ions, hydrogen ions, carbon dioxide, catecholamines, prostaglandins, and adenosine (Wierzbowska et al., 2014; Lanting et al., 2017). It is particularly well known that endothelial nitric oxide and other endothelium-derived agents such as prostaglandins and endothelium-derived hyperpolarizing factors play a role in the mechanism of PORH. Apart from these substances, the sensory nerves contribute to the PORH mechanism (Larkin and Williams, 1993; Lorenzo and Minson, 2007; Cracowski et al., 2011; Barwick et al., 2016; Lanting et al., 2017; Marche et al., 2017). PORH is a quick, easy and useful method to assess microcirculation in the foot. However, the methods and equipment used to measure PORH vary widely. There were three distinctive variations in the protocols: occlusion time, use of temperature control at the probe site and occlusion site.

A review of the existing literature identified a range of equipment that has been used to measure PORH. Recently, there are various types of equipment available that measure the change in blood perfusion proficiently such as the commonly used Laser Doppler flowmetry or fluxmetry system with a pressure unit (Morales *et al.*, 2005; Lanting et al., 2017) or Laser Speckle Contrast Imager (LSCI) which helps to visualize the reactive hyperemia in real-time (Mennes *et al.*, 2019). The Laser Doppler flowmetry or fluxmetry system have exclusive software programs that facilitate running automated protocols and monitoring various measures of interest continually. So far there is little evidence for the reliability of the measurement of PORH (Barwick, Lanting and Chuter, 2015). The study by Barwick et al. (2015) compared the use of various techniques but no attempt was made to compare the protocols. Therefore, there is a need for more studies to enable the reliable and practical measurement of PORH in a clinical setting (Barwick, Lanting and Chuter, 2015).

As highlighted earlier, the protocols used to measure PORH varies widely and there is no standard protocol. Firstly, in terms of occlusion time, studies have used occlusion times ranging from 30- to 300-seconds in people with peripheral arterial disease, diabetic foot ulcers and history of ulcers (Morales et al., 2005; Barwick et al., 2016; Lanting et al., 2017; Mennes et al., 2019). Whilst these studies pave a way to understand the importance of measuring PORH and its relevance to various complications, there remains a need for more evidence to support the selection of protocols in order to systematically investigate PORH in minimal time. Morales et al. (2005) recommended a protocol to measure PORH in people with peripheral arterial obstructive disease (Morales *et al.*, 2005). According

to that study conducted in people without diabetes, the PORH measurements were obtained with an occlusion that lasted for a maximum of 3 minutes or 1 minute in case of strong leg pain and the entire (both acclimatisation and measurement) session lasted for 33 minutes without stops (Morales *et al.*, 2005). But these long provocation tests in people with diabetes might trigger tissue damage, discomfort and pain, especially when they present with various complications. In such conditions, tests that can be conducted in a minimal time may be useful in order to decrease the risk of tissue damage, discomfort and pain. Also, if the test can be performed in a few minutes it can easily be combined with ABI or TBI measures in a clinical setting.

Secondly, recommendations regarding the use of temperature control at the probe site vary. Some studies recommend temperature control at the probe site, while others advise against it (Morales *et al.*, 2005; Barwick, Lanting and Chuter, 2015; Lanting et al., 2017). However, it needs to be noted that the study population varied in these studies, especially in terms of including people with diabetes (Morales *et al.*, 2005; Barwick, Lanting and Chuter, 2015; Lanting et al., 2017). Temperature is known to influence microcirculation, hence an optimal temperature of 32-33℃ is recommended in order to standardize the methods for cutaneous vascular evaluation. However, this may not help to identify other physiological differences (Aso, Inukai and Takemura, 1997; Morales *et al.*, 2005; Cracowski *et al.*, 2006; Barwick, Lanting and Chuter, 2015). A previous study has demonstrated the relationship between PORH measure (time to peak) and sensory neuropathy, which is the involvement of small sensory nerve fibers (Barwick et al., 2016). This suggests the role of sensory nerves in mediating cutaneous microcirculation. The foot temperature in people with diabetes varies incredibly. People with diabetes may either present with warm or cold feet depending on the presence of neuropathy and its type, based on which the protocols for PORH needs to be customised (Armstrong and Lavery, 2016). Therefore, the reproducibility of protocols with and without temperature control must be evaluated.

Thirdly, different studies have used different occlusion sites (Morales *et al.*, 2005; Barwick, Lanting and Chuter, 2015; Lanting et al., 2017). Thigh occlusion may be better whilst assessing PORH in people with complications such as arteriosclerosis, diabetes, renal insufficiency and such as it may be more reliable than ankle (del Guercio, Leonardo and Arpaia, 1986; Morales *et al.*, 2005). However, people with diabetes tend to be overweight/obese and often present with various complications (predominantly vascular and neuropathic). Hence, it may not be convenient and always possible to perform thigh occlusion. ABI is a common macrovascular measure in people with diabetes. TBI is measured in people with arterial calcification where ABI could be unreliable (Potier *et al.*, 2011; Rac-Albu *et al.*, 2014). Considering how these measures of macrocirculation are measured, similar strategies can be applied whilst measuring PORH by occluding the blood flow at either the ankle or

hallux site. Furthermore, in people with digital amputations, hallux occlusion is impossible. This requires evaluating and comparing the reproducibility of PORH protocols measured with occlusion at the ankle and hallux sites.

In summary, considering the three key gaps in the literature, this study aimed at investigating the reproducibility of PORH protocols in the microvascular assessment of feet in healthy young adults. The objective was to investigate a combination of occlusion time, occlusion site and the skin temperature control protocols that can be reproducible.

## 3.3 Methodology

### 3.3.1 Participants and setting

There were 25 healthy participants (15 females and 10 males) who participated in this study, which was conducted upon obtaining University Ethics Committee's approval. The participants were recruited through convenience sampling. Any adult over the 18 years with no severe neurological or vascular issues and no major vascular trauma or injury (bleeding, bruising, hematoma and fractured bones) that affects circulatory measurements could participate in the study. The participants were requested not to consume any caffeinated or alcoholic beverages 2 hours before the study as this is known to affect vascular measures (Kudo *et al.*, 2015; Noguchi *et al.*, 2015; Piano, 2017). Besides, the participants were requested not to engage in any strenuous exercise of any form 2 hours prior to the study (Oh, Hong and Lee, 2016). The participants upon arrival to the Biomechanics laboratory were familiarized to the settings and the protocol. The test was performed by a single observer with a medical and clinical research background.

### 3.3.2 Data collection

#### *3.3.2.1 Protocol*

The participant was requested to lie supine on the couch. The nature of the tests required the participants to be as still as possible during the recording as even minor movements cause artefacts. The study commenced after a minimum of 15 minutes of acclimatisation (Barwick, Lanting and Chuter, 2015; Moreira-Marconi, E. *et al.* 2019). The laser Doppler probes (contact area 1 $cm^2$ each) were secured using a double-sided adhesive tape from on the distal/plantar aspect of the hallux for ankle and toe pressure and PORH measurements or the pulp of the index finger for the arm pressure measurements. The ABI and TBI were measured as they are common macrocirculatory measures to confirm healthy peripheral vascular status of participants. The PORH occlusion was at the ankle and hallux levels (one followed by the other), while the cuffs were inflated to a supra systolic pressure (~200 mmHg). There was a thermometer that measured the room temperature to ensure that the temperature was maintained throughout the data collection period.

Firstly, the PORH protocols included the use of three different occlusion times 10, 30 and 60 seconds. The occlusion time selection rationale was based on the objective of finding the shortest occlusion time that would produce the post-occlusive reactive hyperemia (PORH) response. The literature review indicated that the lowest occlusion time found was 60 seconds as per a study conducted by Morales et al. (2005) in people with peripheral arterial disease who developed pain during data collection. To determine a more precise time, preliminary testing was conducted, starting with half of the lowest time (30 seconds) and gradually decreasing to 10 seconds. However, it was observed that occlusion times below 10 seconds did not produce a significant response. The reason for selecting a minimal occlusion time of 10 seconds was to facilitate the combination of PORH testing with ABI or TBI measures in a clinical setting. This point has been added in the revised version of the thesis. Additionally, considering that individuals with diabetes often present with various existing complications, the intention was to assess the occlusion times that allow hyperamic response, but at the same time do not exacerbate any other complications. This rationale is mentioned in the thesis and the published article, providing a comprehensive explanation for the selection of the minimal occlusion time.

The recording for 60 seconds protocol consisted of two minutes baseline, occlusion for 60 seconds and two minutes of post-occlusion. The recording for 30 seconds protocol consisted of one-minute baseline, occlusion for 30 seconds and one minute of post-occlusion. The recording for 10 seconds protocol consisted of 30 seconds baseline, occlusion for 10 seconds and 30 seconds of post-occlusion. Secondly, the same protocols were tested under two different conditions without and with temperature control of 33°C at the probe site. Finally, the same set of protocols were tested with occlusion at two different sites, ankle and hallux. Both right and left feet were evaluated simultaneously. On the whole, there were 12 protocols (Figure 3.1) tested over 2.5 to 3 hours in a sequential manner and the rest between each testing was 60 seconds (Eguchi et al., 2009).

During the phase where protocols were being developed, to determine the appropriate ratio of times for the different phases, statistical tests were conducted to assess if the perfusion measures varied significantly. If the results indicated that the selected times were not sufficient to produce the desired post-occlusive reactive hyperemia (PORH) response, adjustments were made during the protocol development stage. By carefully considering the previous research examples and conducting preliminary testing, the baseline and post-occlusion times were determined to create a protocol that would effectively elicit the PORH response (Morales et al., 2005; Wierzbowska et al., 2014; Lanting et al., 2017; Rašić, L. *et al.,* 2014). This approach ensured that the selected times were appropriate for capturing the desired physiological changes.

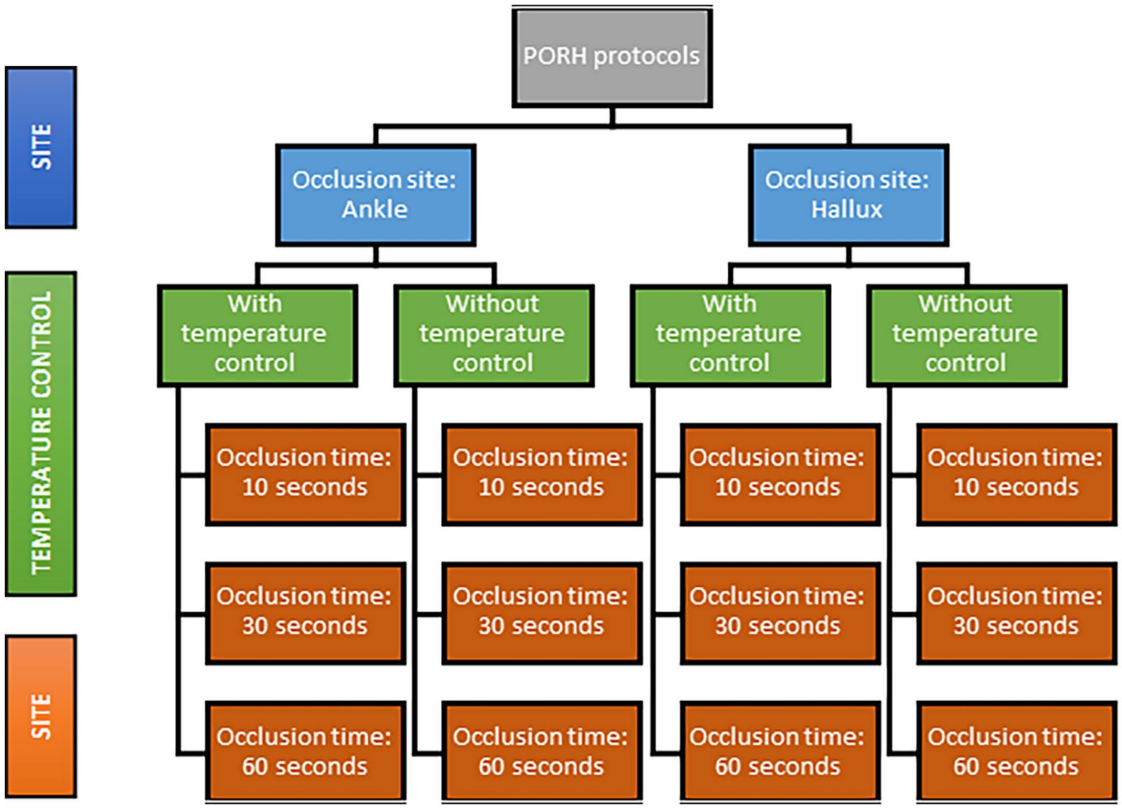



*Figure 3.1 Protocols used in the current study*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). The image shows the 12 different protocols used in the current study based on 3 different occlusion times, 2 different occlusion sites and use of temperature control.

#### *3.3.2.2 Equipment*

The laser Doppler flowmetry (Periflux system 5000 manufactured by Perimed, Stockholm, Sweden) system was used for data collection. There can be several main systems, each equipped with four functional units to have the desired number of channels that facilitate simultaneous measurements of various vascular parameters. The system was a single main unit with 4 different functional units including: 2 perfusion units, 1 temperature unit and 1 pressure unit. The pressure unit helps to simplify and standardize tests such as peripheral vascular pressure measurements and PORH. The thermostatic laser Doppler probes were used to allow temperature monitoring for protocols without temperature control and to precisely maintain a temperature of 33C for protocols with temperature control at the measurement site. The kit with two Laser Doppler Perfusion Monitoring (LDPM) units allowed simultaneous blood pressure measurements at both arms followed by both ankles and halluces. The system was connected to a laptop with exclusive software, PeriSoft that helps to run the protocols in a sequential manner and record data. The steps for the protocols and the sequence of events were defined in order to facilitate effective, precise and replicable data collection across participants. The

inflation of the pressure cuffs was manual; however, the deflation was automatic. The data for ABI, TBI and the PORH measures were collected using the same equipment.

### 3.3.3 Data analysis

For every participant, in each of the 12 protocols, three trials were performed. Upon completion of each test, the PORH area, which included a baseline, occlusion trough and a hyperemic area was marked (Figure 3.2) for the system to autogenerate the results with values for PORH measure. This was done for every trial. Then, the reproducibility for the 14 parameters in Table 3.1: Rest Flow (RF), Biological Zero (BZ), Peak Flow (PF), Time to Latency (TL), Time to Recovery (TR), Time to Half Before Hyperemia (TH1), Time to Max (TM), Time to Half After Hyperemia (TH2), Area of Occlusion (AO), Area of Hyperemia (AH), Area of Hyperemia/Area of Occlusion and Hyperemia repayment ratio (AH/AO) was analyzed through SPSS and Microsoft EXCEL was used for collating information. For every protocol, for each of these 14 measures, the ICC between the three trials was statistically analyzed. Regarding ICC values, >0.75, 0.50-0.75, <0.50 were considered to be excellent, moderate (fair-to-good) and poor reproducibility (Portney and Watkins, 2009; Rašić *et al.*, 2014; Barwick, Lanting and Chuter, 2015). All these statistical tests were conducted for right and left foot data separately.

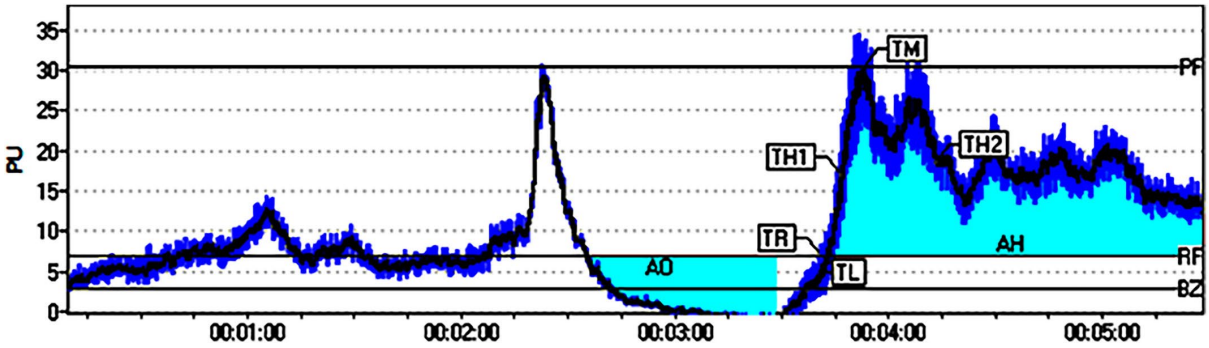



*Figure 3.2 PORH graph showing various measures*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Rest Flow (RF), Biological Zero (BZ), Peak Flow (PF), Time to Latency (TL), Time to Recovery (TR), Time to Half Before Hyperemia (TH1), Time to Max (TM), Time to Half After Hyperemia (TH2), Area of Occlusion (AO), Area of Hyperemia (AH), Area of Hyperemia/Area of Occlusion and Hyperemia repayment ratio (AH/AO).

*Table 3.1 Various PORH Measures**

| **Perfusion measures (PU)** | **Percentage change measures (%)** | **Time measures (milliseconds)** | **The area under the curve** |
|---|---|---|---|
| **RF - Rest flow Baseline blood perfusion** | RF - BZ: Percent change | TL - Time to latency Time taken to reach baseline flow | AO - Occlusion area (Unit*seconds) |

| | | | |
|---|---|---|---|
| | | | The area under the occlusion curve |
| **BZ - Biological zero**<br>**Temporary cessation of the blood flow during occlusion** | BZ - PF: Percent change | TR - Time to recovery<br>Time taken to recover baseline level after the occlusion is released | AH - Hyperemia Area (Unit*seconds)<br>The area under the hyperemic curve |
| **PF - Peak flow**<br>**Maximum perfusion after the release of occlusion** | RF - PF: Percent change | TH1 - Time to half before hyperemia<br>Time taken after the release of the occlusion for perfusion to reach the midpoint between no-flow and peak flow | AH/AO - Hyperemia repayment (ratio) |
| | | TM - Time to max<br>Time taken after the release of the occlusion for perfusion to reach peak flow | |
| | | TH2 - Time to half after hyperemia<br>Time taken after the occlusion release for perfusion to reach the midpoint between peak flow and baseline | |
| *The table shows various measures of PORH in the report generated by the Laser Doppler Flowmetry/Fluxmetry system. | | | |

## 3.4 Results

The mean (standard deviation) age of the participants was 26.9 (9.2). The average height, weight and BMI were 168.1cm (14.2) and 72.9 kg (16), and 26.3.kg/$m^2$ (5.6), respectively. There were 14 different PORH measures recorded by the equipment (Figure 3.2). The mean with error bars (95% Confidence intervals) for 3 different measures of PORH across 12 protocols is provided in Figures 3.3 and 3.4. For the right and left foot, the mean and the trend across protocols for temporal parameters TM and TR

are presented in Figures in 3.5 and 3.6 and Figures 3.7 and 3.8, respectively. The ICC values, along with the mean and standard deviation for key parameters of interest are presented in Table 3.2.

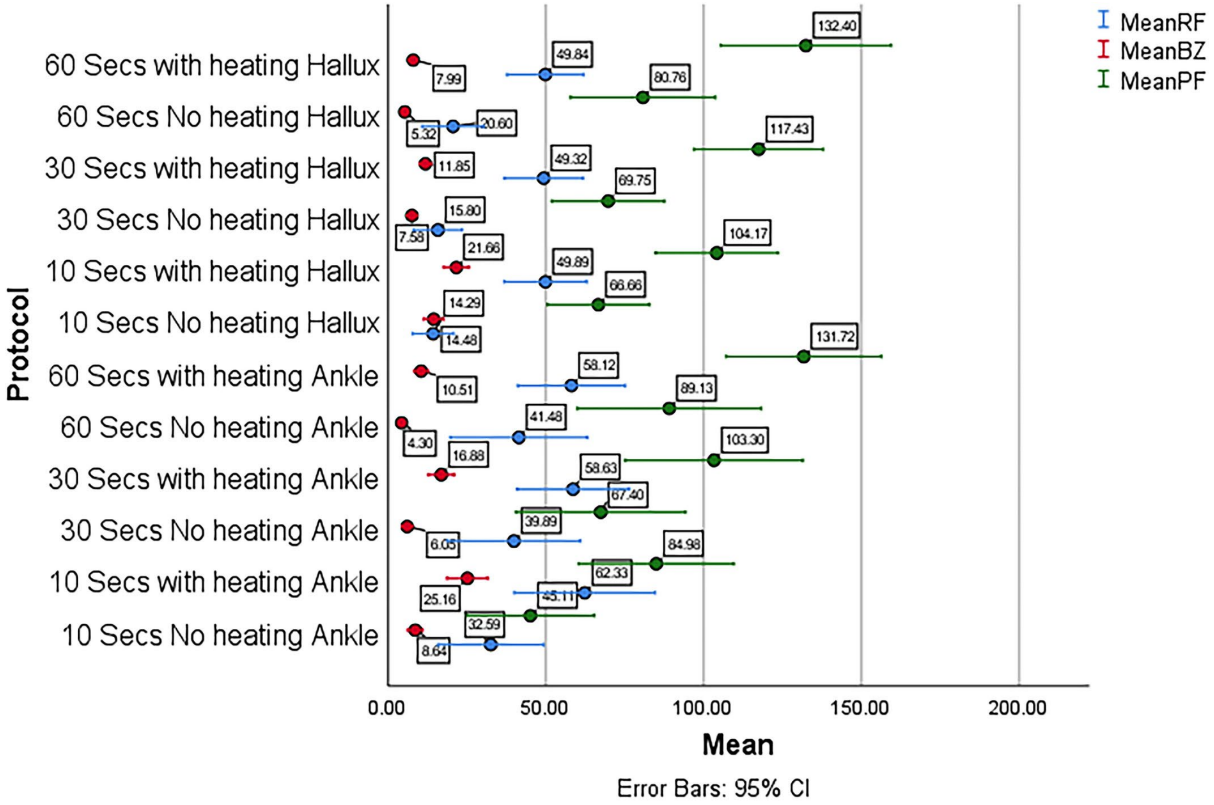



*Figure 3.3 Right Foot: Mean of perfusion measures RF, BZ and PF (PU) across 12 protocols*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Secs = Seconds; TC = Temperature Control.

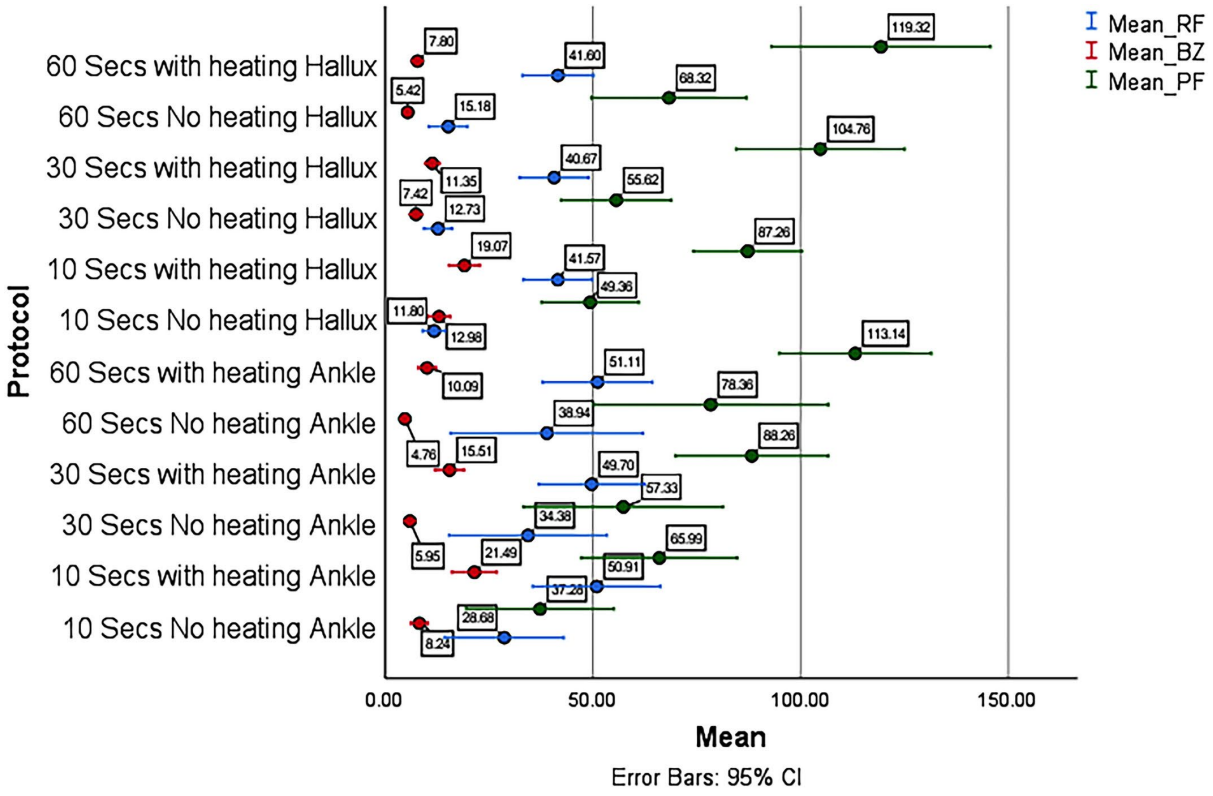



*Figure 3.4 Left foot: mean of perfusion measures RF, BZ, and PF (PU) across 12 protocols. TC, temperature control*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Secs = Seconds; TC = Temperature Control.

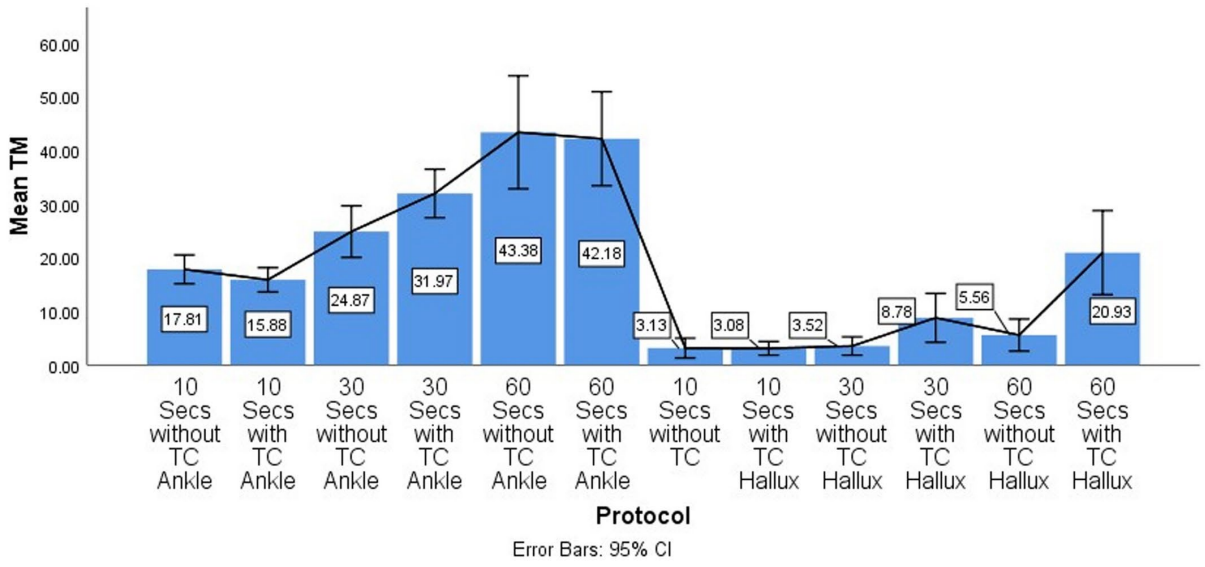



*Figure 3.5 Right foot: Mean Time to max (seconds) categorized based on 12 protocols*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Secs = Seconds; TC = Temperature Control.

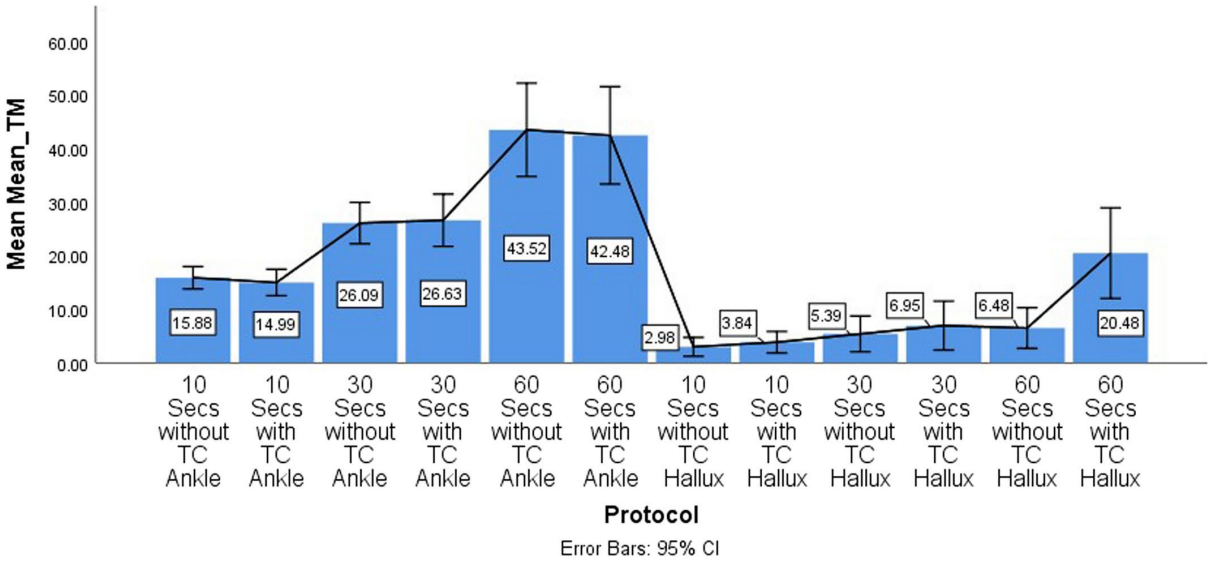



*Figure 3.6 Left foot: Mean Time to max (seconds) categorized based on 12 protocols*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Secs = Seconds; TC = Temperature Control.

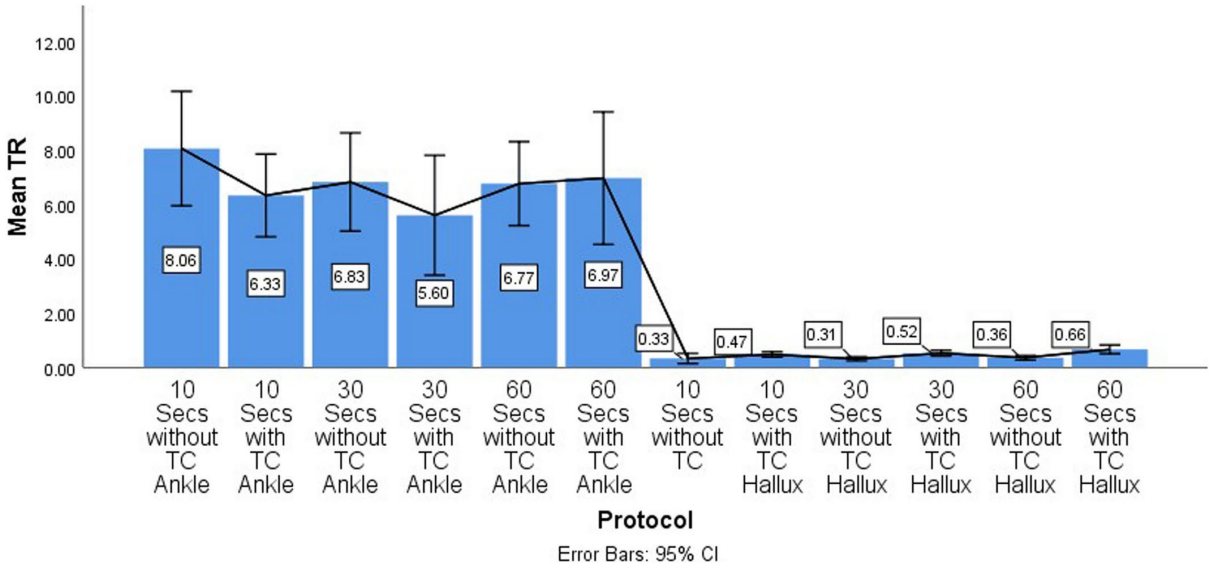



*Figure 3.7 Right foot: Mean Time to Recovery (seconds) categorized based on 12 protocols*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Secs = Seconds; TC = Temperature Control

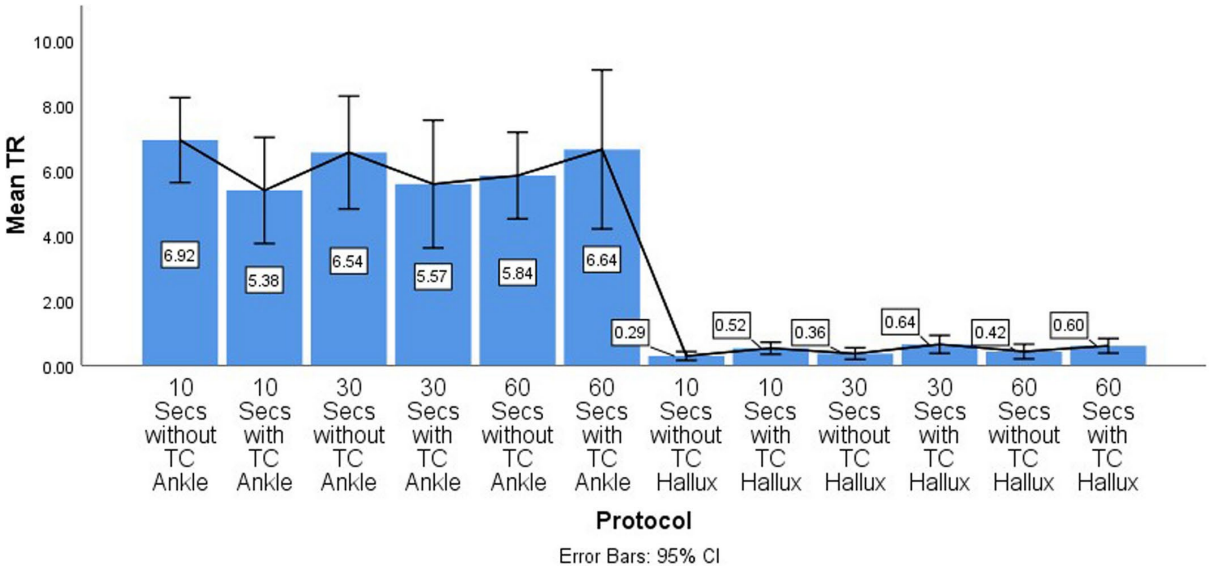



*Figure 3.8 Left foot: Mean Time to Recovery (seconds) categorized based on 12 protocols*

Microcirculation, Volume: 28, Issue: 5, First published: 02 March 2021, DOI: (10.1111/micc.12692). Secs = Seconds; TC = Temperature Control

Overall, ICC showed moderate to excellent reproducibility for most PORH measures with ankle-level occlusion. The perfusion parameters (RF, BZ and PF) showed excellent reproducibility with all protocols. Closer inspection of the results showed that the 30- and 60-seconds without temperature control protocols showed moderate to excellent reproducibility for most PORH measures. In the right foot, 30 seconds ankle level occlusion without temperature control showed ICC of >0.50 for all parameters except AH (ICC = -0.36) and AH/AO (ICC = 0.48). More specifically, the ICC Values were > 0.75 for RF, BZ, PF, RF-BZ, BZ-PF, TL, TH, TH2 and AO parameters (p value <0.05). The 60 seconds ankle level occlusion without temperature control in the right foot showed ICC of >0.50 for all parameters except BZ-PF percent change (ICC = -0.46). The ICC values were > 0.75 for RF, BZ, PF, RF-BZ, RF-PF, TL and TM (p value< 0.05).

Similarly, in the left foot, 30 seconds ankle level occlusion without temperature control showed ICC of >0.50 for all parameters except three temporal parameters (ICC values TL= 0.29, TH2 = 0.37, TM = -0.01) and AH (ICC =-0.36). With the 60- seconds protocol showed ICC >0.50 for all except TM (ICC = 0.38).

In the hallux protocols, all three 10-, 30- and 60-seconds protocols without temperature control fared well as they had a minimum of 11 out of 14 (78.57%) PORH measures that showed moderate to excellent reproducibility (ICC >0.50). The ICC for select parameters are presented in the Table 3.2 below.

When temperature control was used at the probe site, be it with the ankle or hallux level occlusion, in most cases the temporal and area under the perfusion-time curve parameters showed poor reproducibility with ICC values <0.50.

In addition to the PORH measures, the ABI and TBI was measured. The mean ± standard deviation for ABI was 1 ± 0.13 and for TBI was 0.66 ± 0.14.

*Table 3.2 ICC for PORH parameters in the Foot*

| Right Foot | | | | Left Foot | | | |
|---|---|---|---|---|---|---|---|
| PF | | | | PF | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.96 | 0.00 | ANKLE | 30-seconds OT without TC | 0.96 | 0.00 |
| | 30-seconds OT with TC | 0.98 | 0.00 | | 30-seconds OT with TC | 0.96 | 0.00 |
| | 60-seconds OT without TC | 0.98 | 0.00 | | 60-seconds OT without TC | 0.98 | 0.00 |
| | 60-seconds OT with TC | 0.96 | 0.00 | | 60-seconds OT with TC | 0.92 | 0.00 |
| HALLUX | 10-seconds OT without TC | 0.99 | 0.00 | HALLUX | 10-seconds OT without TC | 0.97 | 0.00 |
| | 10-seconds OT with TC | 0.97 | 0.00 | | 10-seconds OT with TC | 0.93 | 0.00 |
| | 30-seconds OT without TC | 0.97 | 0.00 | | 30-seconds OT without TC | 0.99 | 0.00 |
| | 30-seconds OT with TC | 0.97 | 0.00 | | 30-seconds OT with TC | 0.97 | 0.00 |
| | 60-seconds OT without TC | 0.98 | 0.00 | | 60-seconds OT without TC | 0.98 | 0.00 |
| | 60-seconds OT with TC | 0.95 | 0.00 | | 60-seconds OT with TC | 0.95 | 0.00 |
| RF-PF Percent Change | | | | RF-PF Percent Change | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.65 | 0.00 | ANKLE | 30-seconds OT without TC | 0.85 | 0.00 |
| | 30-seconds OT with TC | 0.73 | 0.00 | | 30-seconds OT with TC | 0.54 | 0.01 |
| | 60-seconds OT without TC | 0.92 | 0.00 | | 60-seconds OT without TC | 0.88 | 0.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 60-seconds OT with TC | 0.61 | 0.00 | | 60-seconds OT with TC | 0.52 | 0.01 |
| HALLUX | 10-seconds OT without TC | 0.90 | 0.00 | HALLUX | 10-seconds OT without TC | 0.94 | 0.00 |
| | 10-seconds OT with TC | 0.95 | 0.00 | | 10-seconds OT with TC | 0.89 | 0.00 |
| | 30-seconds OT without TC | 0.94 | 0.00 | | 30-seconds OT without TC | 0.94 | 0.00 |
| | 30-seconds OT with TC | 0.87 | 0.00 | | 30-seconds OT with TC | 0.86 | 0.00 |
| | 60-seconds OT without TC | 0.89 | 0.00 | | 60-seconds OT without TC | 0.93 | 0.00 |
| | 60-seconds OT with TC | 0.63 | 0.00 | | 60-seconds OT with TC | 0.64 | 0.00 |
| TM | | | | TM | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.58 | 0.01 | ANKLE | 30-seconds OT without TC | -0.01 | 0.49 |
| | 30-seconds OT with TC | -0.28 | 0.73 | | 30-seconds OT with TC | 0.33 | 0.12 |
| | 60-seconds OT without TC | 0.76 | 0.00 | | 60-seconds OT without TC | 0.38 | 0.08 |
| | 60-seconds OT with TC | 0.45 | 0.04 | | 60-seconds OT with TC | 0.31 | 0.15 |
| HALLUX | 10-seconds OT without TC | 0.83 | 0.00 | HALLUX | 10-seconds OT without TC | 0.72 | 0.00 |
| | 10-seconds OT with TC | 0.21 | 0.24 | | 10-seconds OT with TC | 0.47 | 0.03 |
| | 30-seconds OT without TC | 0.71 | 0.00 | | 30-seconds OT without TC | 0.75 | 0.00 |
| | 30-seconds OT with TC | 0.60 | 0.00 | | 30-seconds OT with TC | 0.77 | 0.00 |
| | 60-seconds OT without TC | 0.52 | 0.02 | | 60-seconds OT without TC | 0.02 | 0.47 |
| | 60-seconds OT with TC | 0.40 | 0.08 | | 60-seconds OT with TC | 0.31 | 0.09 |
| TR | | | | TR | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.63 | 0.00 | ANKLE | 30-seconds OT without TC | 0.60 | 0.00 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 30-seconds OT with TC | -0.09 | 0.58 | | 30-seconds OT with TC | 0.35 | 0.11 |
| | 60-seconds OT without TC | 0.67 | 0.00 | | 60-seconds OT without TC | 0.50 | 0.02 |
| | 60-seconds OT with TC | -0.13 | 0.61 | | 60-seconds OT with TC | -0.24 | 0.70 |
| HALLUX | 10-seconds OT without TC | 0.98 | 0.00 | HALLUX | 10-seconds OT without TC | 0.90 | 0.00 |
| | 10-seconds OT with TC | 0.94 | 0.00 | | 10-seconds OT with TC | 0.98 | 0.00 |
| | 30-seconds OT without TC | 0.95 | 0.00 | | 30-seconds OT without TC | 0.96 | 0.00 |
| | 30-seconds OT with TC | 0.82 | 0.00 | | 30-seconds OT with TC | 0.60 | 0.00 |
| | 60-seconds OT without TC | 0.91 | 0.00 | | 60-seconds OT without TC | 0.77 | 0.00 |
| | 60-seconds OT with TC | 0.77 | 0.00 | | 60-seconds OT with TC | 0.93 | 0.00 |

OT = Occlusion Time; TC = Temperature Control at probe site

| | | | | | |
|---|---|---|---|---|---|
| | >0.75 Excellent reproducibility | | 0.50 – 0.75 Moderate reproducibility | | <0.50 Poor reproducibility |

## 3.5 Discussion

### 3.5.1 Key discussion points

The use of software aided protocols helps to pre-define the steps and abled to run tests systematically and time-effectively across participants. Each of the 12 protocols was performed at ease in minimal time. For instance, the 60 seconds protocol took only a maximum of 5 minutes and 10 seconds in total: 10 seconds preparation, 2 minutes baseline, 1 minute occlusion and 2 minutes hyperemia recording.

This study was conducted in healthy participants to assess the feasibility of conducting the test and reproducibility of various protocols. This study showed that the output for 30- and 60-seconds protocols produced more consistent results in the ankle as the output missed at least one parameter for a minimum of one of three trials for the 10 seconds protocol. A possible explanation for this might be that the 10 seconds occlusion time was insufficient to produce a hyperemic response. This is in line with the existing literature which suggests that the hyperemic response corresponds to the occlusion time (Larkin and Williams, 1993). The greater the occlusion time greater the hyperemic response; this is because longer the period of occlusion, the greater the metabolic stimulus for vasodilation resulting

in an increase in peak flow and prolonged duration of hyperemia (Larkin and Williams, 1993; Klabunde, 2012). Depending on the time taken to occlude the tissue blood supply, the reactive hyperemia increases four to seven times the baseline in the tissue and lasts from a few seconds to hours in relation to the initial occlusion time. In this instance, the use of 10 seconds of occlusion time at the ankle did not sufficiently provoke the vasodilation for a decent hyperemic response. However, this was not the case with hallux-level occlusion. This may be due to the smaller surface area and the type of vessels in the hallux as compared to the ankle region.

Another key observation was that there was increased perfusion when protocols with temperature control were used, which suggests the role played by the thermoreceptors on the microcirculation and the effect of foot temperature on the cutaneous microcirculation. This can be observed in Figure 3.3 and Figure 3.4, where the perfusion measures RF, BZ and PF are higher in protocols with temperature control of 33℃ compared to the corresponding values when the temperature was not controlled. This is consistent with the suggestions from a previous study on the influence of temperature on cutaneous microcirculatory responses such as pressure-induced vasodilation (Aso, Inukai and Takemura, 1997; Fromy *et al.*, 2002). Certain microcirculatory responses such as pressure-induced vasodilation are known to be absent even in healthy subjects due to low foot temperature and the role of mechanothermal receptors in such instances is highlighted (Aso, Inukai and Takemura, 1997; Fromy *et al.*, 2002; Balasubramanian *et al.*, 2020). These observations suggest an association between the small fibre nerve function and skin microcirculation. These findings generate interesting questions regarding the nature and extent of microcirculatory changes influenced by temperature variation. Further comparative analysis would be useful to understand the differences. While this was not within the scope of this study, it could be an area for further work.

The key measures of interest were the PF, RF to PF percent change, TM and TR as these measures are similar to the common parameters discussed in previous literature (Morales *et al.*, 2005; Rašić *et al.*, 2014; Lanting et al., 2017). The PF parameter showed excellent reproducibility (ICC > 0.75; p-value < 0.05) across all 12 protocols. Similarly, RF to PF percent change showed either moderate or excellent reproducibility (ICC > 0.50; p-value < 0.05) across all 12 protocols, except with 10-second occlusion at the right and left ankles without temperature control at the probe site where it showed poor reproducibility (ICC = 0.47 and 0.15, respectively; p-value = 0.03 and 0.3, respectively). But it is important to note that as highlighted earlier, 10-second protocols at the ankle never generated consistent reports for any parameters.

The ICC values for TM and TR showed variations across protocols (Table 3.2). Also, it can be observed from Figure 3.5 and Figure 3.6 that the TM was higher with ankle occlusion protocols than in the hallux

occlusion ones in both feet. Furthermore, as seen in Figure 3.7 and Figure 3.8, the TR shows a similar trend. This implies an existing relationship between the occlusion site and the time taken for a hyperemic reaction. The reperfusion of the entire foot may have taken longer compared to the hallux. These findings help to realize the importance of understanding the use of different protocols and their influence on PORH measures. Further comparative analysis may aid in understanding these differences.

Previous research has indicated mixed results on the reliability of PORH in the upper limb (Roustit *et al.*, 2010; Roustit and Cracowski, 2012; Rašić *et al.*, 2014). As indicated by Barwick et al. (2015) there is a wide variation within the literature on the reliability of PORH. A possible reason being the Laser Doppler Flowmetry system is extremely sensitive and the measurement varies depending on the probe location i.e. being directly over an arteriovenous anastomosis. Furthermore, PORH measures are sensitive to various factors such as temperature (Barwick, Lanting and Chuter, 2015). But Barwick et al. (2015), found that PORH can be measured reliably, especially with the use of temperature control. Similarly, the current study found that PORH can be measured reliably. However, the current study found moderate to excellent reproducibility when using no temperature control in contrast to the findings from the previous study with hallux level occlusion (Barwick, Lanting and Chuter, 2015). A potential reason for this could be the fact that the current study was conducted in healthy participants whereas the other study was conducted in people at risk of peripheral arterial disease. As suggested in the literature, temperature control at the probe site may aid in minimising the variations in the perfusion measures as mere control for room temperature may not be adequate (Barwick, Lanting and Chuter, 2015). However, measurements obtained using temperature control at the probe site are criticized to be less physiologically relevant (Morales *et al.*, 2005; Cracowski *et al.*, 2006; Barwick, Lanting and Chuter, 2015). Microcirculatory measurements are influenced by the underlying pathological conditions, therefore, a deeper understanding of variations with protocols may aid in obtaining PORH measures that are methodologically sound and also physiologically relevant.

### 3.5.2 Future Implications

The comparison of 12 different protocols in this study helped to assess the feasibility and reproducibility of the results in the systematic investigation of PORH in the foot. The study is a stepping-stone to proposing the incorporation of less-time-consuming microvascular assessments in routine practice. According to these data, we can infer that the 30-and 60-second protocols produced consistent results. Thus, the present study raises the possibility that PORH can be measured using minimal time as little as 30 seconds in the foot. These protocols can be easily replicated in a clinical or research setting.

### 3.5.3 Future application in diabetic foot syndrome

In people with type 2 diabetes, it was found that certain PORH measures such as Time to peak and per cent change from baseline were associated with the presence of peripheral sensory neuropathy, cardiac autonomic deficits, critical ischaemia in the feet and previous history of ulcers/amputations (Barwick et al., 2016; Lanting et al., 2017; Mennes et al., 2019). These findings from previous research support the need for incorporating microcirculatory investigation in people at risk for diabetic foot complications. People with diabetes can present with a plethora of symptoms based on several foot complications, which makes it challenging to perform microvascular assessments. For example, ABI has been reported to be less reliable in people with arterial calcification and in such populations, TBI is more reliable (Potier *et al.*, 2011; Rac-Albu *et al.*, 2014). The results of the current study showed that PORH assessment was reproducible in both ankle and hallux occlusion, which can be useful when a decision must be made to select an occlusion level. This may have practical implications for determining the risk of diabetic foot complications, especially with ulcer prediction, wound healing and prevention of amputation.

## 3.6 Strengths and Limitations

This study tested the same session reproducibility. An advantage of this same session testing (similar to a real-world setting) is that the factors that influence variations in physiological measures are restricted. In contrast, the limitation is that the day-to-day variations in reproducibility and the potential factors influencing the measures were not studied. The test was performed by a single observer. The study examined reproducibility in healthy subjects. Although the findings from the study hold value from a methodological standpoint, their application to detect dysfunction in people with pathological conditions needs to be determined. As mentioned earlier, PORH assessment using the Laser Doppler Flowmetry system is relatively quick and easy to perform in comparison to thermal provocation tests. However, the equipment is expensive. For this reason, it is used more for research purposes rather than clinical use at present. If the assessment aids with early diagnosis and prevention of adverse complications such as ulcers and amputations, it may prove to be beneficial in the long term. This needs further investigation. Future studies that explore the cost-effective options and perform a cost-benefit analysis for microcirculatory assessments for clinical use for a comprehensive assessment of the foot can be useful.

## 3.7 Conclusion

This study evaluated the reproducibility of PORH parameters measured on the foot in young healthy adults using 12 different combinations of PORH protocols. The main highlights are that PORH can be tested reliably and in a very short time. The 10-second occlusion time was sufficient to induce a hyperemic response with occlusion at the hallux but not the ankle, which did affect the reproducibility. The protocol using 30- and 60-second occlusion time fared well in comparison to the 10-second protocol and can be considered the most minimal time for ankle occlusion.

# CHAPTER IV

## UNDERSTANDING THE DIFFERENCES IN POST-OCCLUSIVE REACTIVE HYPERAEMIA MEASURES: A COMPARISON BETWEEN WITH AND WITHOUT TEMPERATURE CONTROL PROTOCOLS

A part of this chapter was presented as:

An Oral presentation on “The effect of Controlling Plantar Skin Temperature on the Measures of PORH”, Staffordshire Clinical Biomechanics Conference, Staffordshire University, UK, 2020

# Chapter 4 Understanding the Differences in Post-Occlusive Reactive Hyperaemia Measures: A Comparison between with and without Temperature Control Protocols

## 4.1 Introduction

In the previous Chapter 3, it was established that Post-Occlusive Reactive Hyperaemia (PORH) is a simple and reliable method to assess the microcirculation of the foot's skin. There may be benefits of using this assessment of the foot at risk in routine practice. The main reason for testing the reliability of several protocols was that the protocols in the existing literature varied greatly. Although the current study in Chapter 3 showed that the protocols using 30-second occlusion time at hallux or ankle level with or without probe site temperature control were found to have good reliability, an understanding of how probe site temperature control affects PORH measures is still lacking. Therefore, this study aimed to compare the difference between PORH measures assessed with a total of 6 protocols that differed in occlusion time and site using with and without temperature control of 33℃ at the probe site. PORH parameters measured with and without temperature control were compared pairwise using the Wilcoxon Sign rank test. The results of this study help to get deeper insights into the effect of temperature on PORH measures, and the findings reiterate that temperature at the probe site plays a vital role in cutaneous microcirculation and this needs to be accounted for when studying microcirculation.

## 4.2 Background

This section provides an overview of vascular measurements and their role in assessing peripheral vascular complications. Peripheral vascular complications can be either macrovascular or microvascular. Common macrovascular complications include coronary artery disease, peripheral arterial disease and stroke, while microvascular complications include diabetic nephropathy, neuropathy, and retinopathy (Fowler, 2008). In the foreground, however, are microcirculatory complications of the foot. Although the role of microcirculation in ulcer and delayed wound healing is well-established (Flynn and Tooke, 1992; Tooke and Brash, 1996; Dinh and Veves, 2005; Boulton, 2013), its role in early diagnosis and prevention needs further exploration. This can help inform practice for effective diabetic foot management as current guidelines, recommendations and risk stratification systems are limited to macrovascular measurements such as ABI and Toe Brachial Index TBI. Even clinical decisions and risk stratification for complications such as ulcers are based on guidelines that are limited to these measurements (Bus et al., 2016; Hinchliffe et al., 2016; Monteiro-Soares et al., 2012).

Recent developments have facilitated the non-invasive assessment of the microcirculation in the foot using different types of provocation tests such as heat provocation, cold provocation, postural changes and application of contact pressure. An easy-to-perform microcirculation test in addition to measuring ABI and TBI for macrovascular assessment of the lower extremities is PORH. PORH is the measure of the reactive response of the tissue following a brief period of arterial occlusion. It is considered to be predominantly endothelial-dependent; however, both endothelial-dependent and independent mechanisms are involved (Wierzbowska et al., 2014; Lanting et al., 2017; Marche et al., 2017).

In people with type 2 diabetes, specific PORH measures such as time to peak and percent change from baseline to peak flow are known to be associated with the presence of peripheral sensory neuropathy, critical-ischaemia and history of ulcers and amputations (Barwick et al., 2016; Lanting et al., 2017; Mennes et al., 2019). The findings from these studies have established an association between microcirculatory reactivity and the presence of a pathological condition. This, in turn, instigates the need for further investigation of the relationship between PORH measures to improve understanding of microvascular function and development of diabetic foot complications and to explore their value in early detection and prevention of ulcer/amputation. Studies to date have examined a wide range of occlusion times ranging from 30 seconds to 300 seconds (Morales *et al.*, 2005; Mennes *et al.*, 2019). For a quick assessment, especially in people with existing complications, below 60-second protocols can potentially be useful as prolonged occlusion time may lead to pain or discomfort (Morales *et al.*, 2005). Although PORH is a quick, simple and non-invasive method for assessing microcirculation, protocols vary widely, particularly with regard to controlling probe temperature during measurements (Morales *et al.*, 2005; Barwick, Lanting and Chuter, 2015; Lanting et al., 2017; Marche et al., 2017; Mennes *et al.*, 2019). Despite the importance of PORH in the microcirculatory assessment of the foot, a systematic understanding of how temperature control affects the measures of PORH is still lacking. Although different studies have used different protocols and the reliability of using various methods (Morales *et al.*, 2005; Barwick, Lanting and Chuter, 2015; Lanting et al., 2017; Mennes *et al.*, 2019), there is a lack of consensus in the use of temperature control and their effect. Therefore, this study aimed to investigate the differences between PORH parameters when measured with and without temperature control using different protocols with different minimum occlusion times (10, 30 and 60 seconds) at two occlusion sites, ankle and hallux.

## 4.3 Methodology

### 4.3.1 Participants and setting

For this study, 25 participants (15 females and 10 males) who were healthy adults over the age of 18, with no severe neurological or vascular issues and no major trauma or injury impairing circulation were recruited through convenience sampling and used a sample size similar to previous methodological studies in the area i.e. (Rašić *et al.*, 2014). The study was conducted after receiving approval from the University Ethics Committee. All study participants were requested not to consume any caffeinated or alcoholic beverages and not engage in any strenuous exercises 2 hours prior to the study as this might affect the vascular measures (Kudo *et al.*, 2015; Noguchi *et al.*, 2015; Oh, Hong and Lee, 2016; Piano, 2017). Each participant was made familiar with the settings and the protocol. the study commenced after 15 minutes of acclimatisation to room temperature (Barwick, Lanting and Chuter, 2015; Moreira-Marconi, E. *et al.* 2019). The mean age of the participants was 26.9 years and the mean (SD) height, weight and BMI were 1.68 (0.14) m and 72.9 (16.0) kg, and 26.2 (5.6) kg/$m^2$, respectively.

### 4.3.2 Data collection

#### *4.3.2.1 Protocol*

The occlusion was performed at the ankle and hallux levels (one followed by the other) where the cuffs were inflated to a supra systolic pressure (~200 mmHg). The PORH recording consisted of baseline, occlusion and post-occlusion period. This yielded a range of measures for every protocol tested. Overall, there were 6 protocols tested with and without temperature control at the probe site for over 2.5 to 3 hours. The protocols were tested sequentially; where without temperature protocols were used first, followed by the use of temperature control. Occlusion was first at the ankle level followed by the hallux. The right and the left foot were assessed simultaneously. Also, the rest between each protocol was 60 seconds to ensure that the baseline condition was achieved prior to the start of the subsequent test. Data were collected from each participant in one session in the same controlled environment to limit confounding factors such as influence of walking, changes in posture, differences in temperature (between the lab and the outside), hormonal variations in menstruating females, potential injuries sustained by participants and other physiological changes. There was a thermometer that measured the room temperature measurement to ensure the temperature was maintained throughout the data collection period. For every participant, three trials per protocol were performed, and the average of three measures for each parameter was calculated.

#### 4.3.2.2 *Equipment*

Protocols with and without temperature control were compared for 10-, 30- and 60-second occlusion at ankle and hallux levels. Figure 4.1 shows an overview of all protocols. The Perimed Periflux® laser Doppler flowmetry system with two laser Doppler perfusion monitoring systems, one temperature unit and a pressure unit was used for this study. A single-point laser Doppler probes with a contact area of 1 $cm^2$ were secured using double-sided adhesive tape on the distal/plantar aspect of the hallux for ankle and toe pressure and PORH measurements or the pulp of the index finger for the arm pressure measurements. The probes were thermostatic, facilitating the simultaneous measurement of the perfusion and temperature. Furthermore, the probes aid the use of temperature control (33̊C) at the probe site for temperature control protocols.

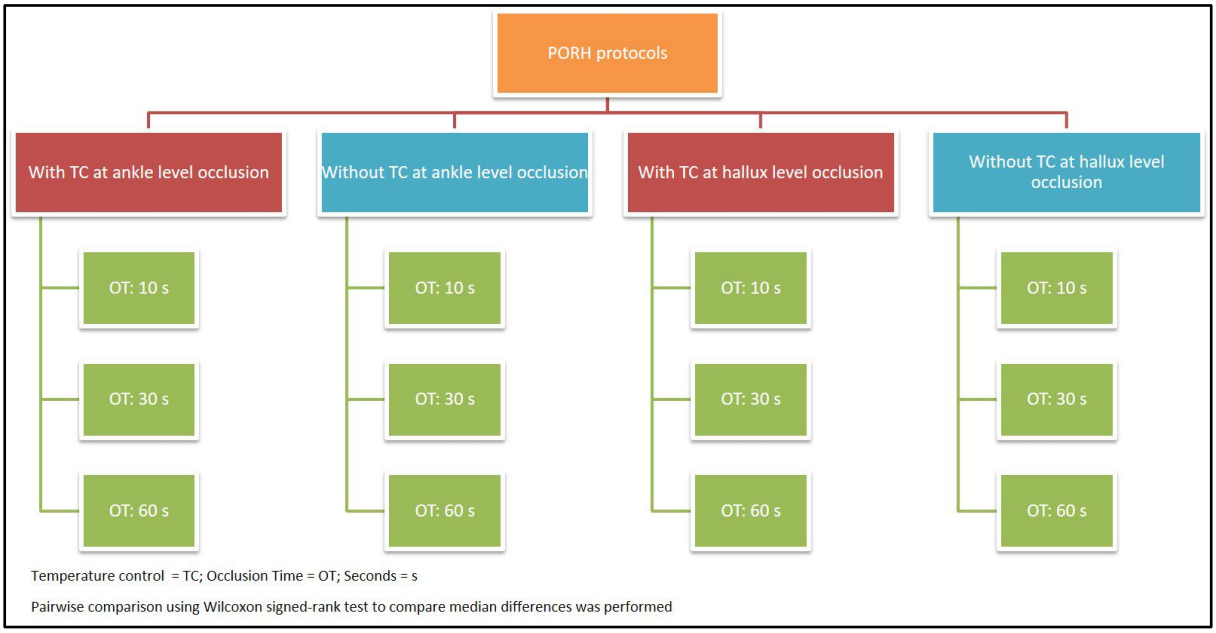



*Figure 4.1 PORH Protocols Used in the Study*

### 4.3.3 Data analysis

The study data was extracted using the laser Doppler Flowmetry software (PeriSoft). After carefully highlighting the PORH area, the automated report generates the values for different PORH measurements in three different phases: baseline, occlusion and hyperaemia. The data of the individual participants were collected separately for the right and left foot. The descriptive analysis and normality testing using Shapiro-Wilk's test did not show a normal distribution of the data. Therefore, to test the within-subject differences for the PORH measurements with and without temperature control per protocol, the non-parametric version of the paired t-test, which is the Wilcoxon signed-rank test, was used to compare median differences. The test was chosen because

the data set contained repeated measures and these independent variables were obtained from related groups (indicating that the same subjects were present in both groups). For each of the PORH parameters, the pairwise comparison was performed with and without the temperature control protocol. Data were analysed using SPSS version 26 (2019).

## 4.4 Results

The mean (SD) for ABI and TBI were 1 (0.13) and 0.66 (0.14), respectively. The most commonly discussed parameters such as the perfusion (RF, BZ and PF), the percent change (RFBZ, BZPF and RFPF) and the temporal (TL, TM and TR) parameters are presented in the results and discussed in this study. As the PORH measures did not show a normal distribution, the differences in the median between the perfusion parameters (RF, BZ and PF) for the right and left foot are presented in Figures 4.2 and 4.3, respectively. It was observed that the perfusion values were significantly higher when measured with temperature control than without the use of temperature control at the probe site ($p < 0.05$; the effect size (r) ranged from 0.32 to 0.62 for RF, 0.52 to 0.62 BZ and 0.42 to 0.62 PF).

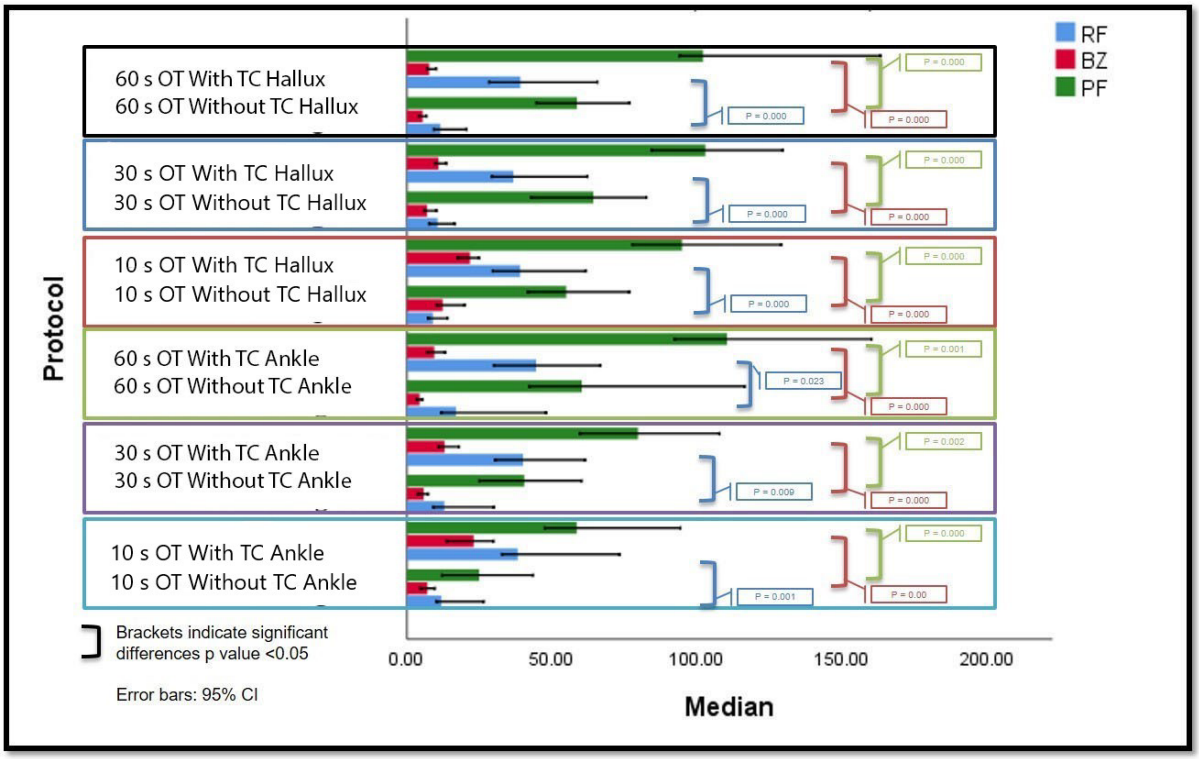



*Figure 4.2 Right Foot - Differences in Perfusion Measures Measured Using With and Without TC at Probe Site*

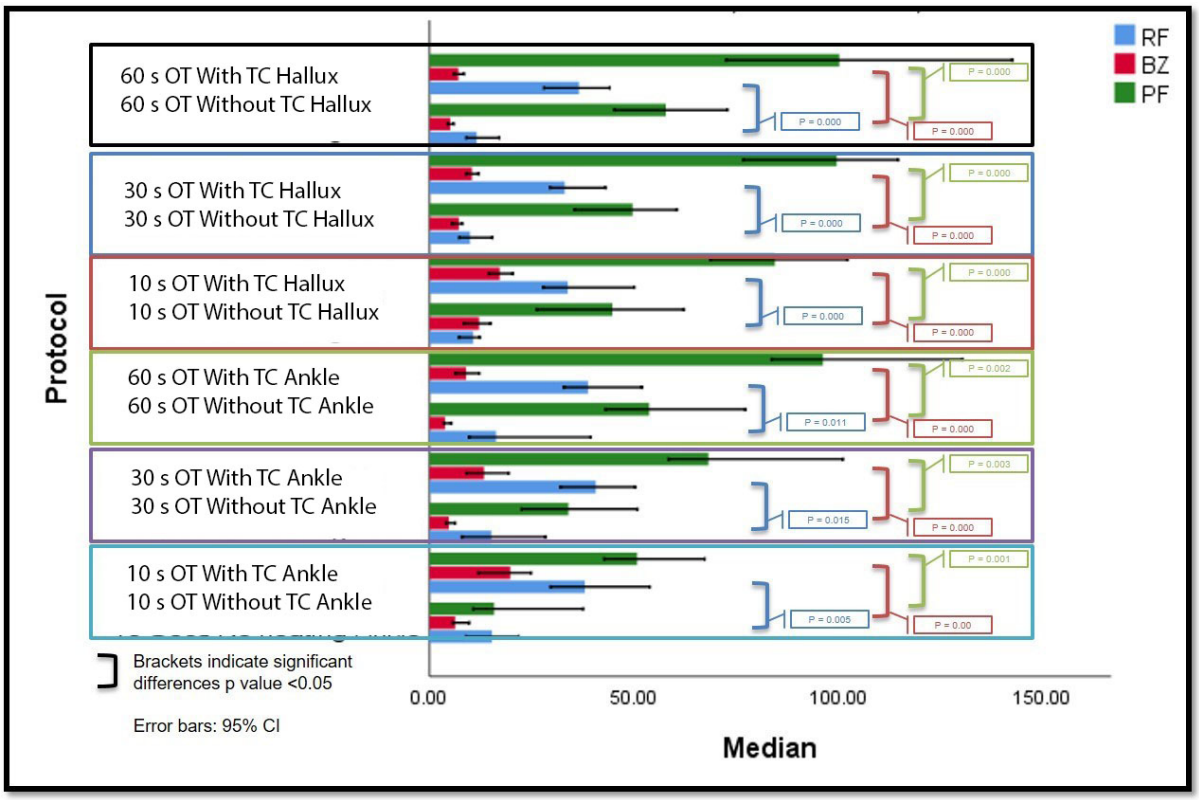



*Figure 4.3 Left Foot - Differences in Perfusion Measures Measured Using with and Without TC at Probe Site*

The median differences for percent change measures are shown in Figure 4.4 for the right foot and Figure 4.5 for the left foot. When observing the percentage change results in Table 4.1, in the right foot, the RFBZ percent change (z = -4.292, r = -0.607, p=0.00) for 10 s occlusion; z = -3.861, r = -0.546, p = 0.00 30 s occlusion; z = -3.807, r = -0.538, p = 0.00 60 s occlusion) and percentage changes (z = -4.211, r = -0.596; p = 0.00 for 10 s occlusion; z = -3.807, r = -0.538, p=0.00 30 s occlusion; z = -4.103, r = -0.580, p = 0.00 60 s occlusion) showed statistically significant differences for PORH measured with occlusion at the hallux level, independent of the occlusion time ranging from 10 to 60 s in right foot. Likewise, in the left foot the RFBZ percentage changes (z = -4.345, r = -0.614, p = 0.00 for 10 occlusion z = -4.076, r = -0.576, p = 0.00 30 s occlusion; z = -4.345, r = -0.614, p = 0.00 60 s occlusion) and RFPF percent change (z = -4.157, r = -0.588, p = 0.00 for 10 occlusion; z = -3.942, r = -0.557, p = 0.00 30 s occlusion; z = -3.888, r = -0.550, p = 0.00 60 s occlusion) showed statistically significant differences for PORH measured with occlusion at hallux level independent of the occlusion time. The BZPF percentage changes, on the other hand, showed statistically significant difference only for the 30 s occlusion time protocols at ankle and hallux level occlusion on the right foot, but for all protocols except 10 s occlusion at the ankle in the left foot.

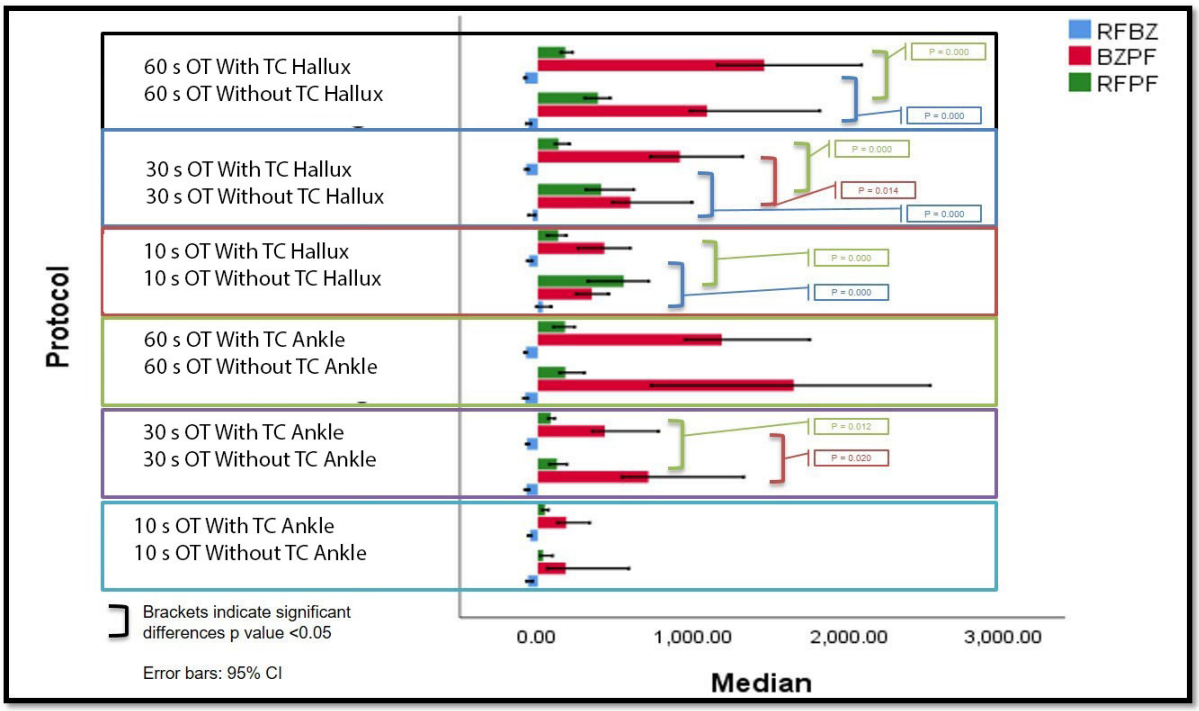



*Figure 4.4 Right Foot - Differences in Percent Change Measures Measured Using with and Without TC at Probe Site*

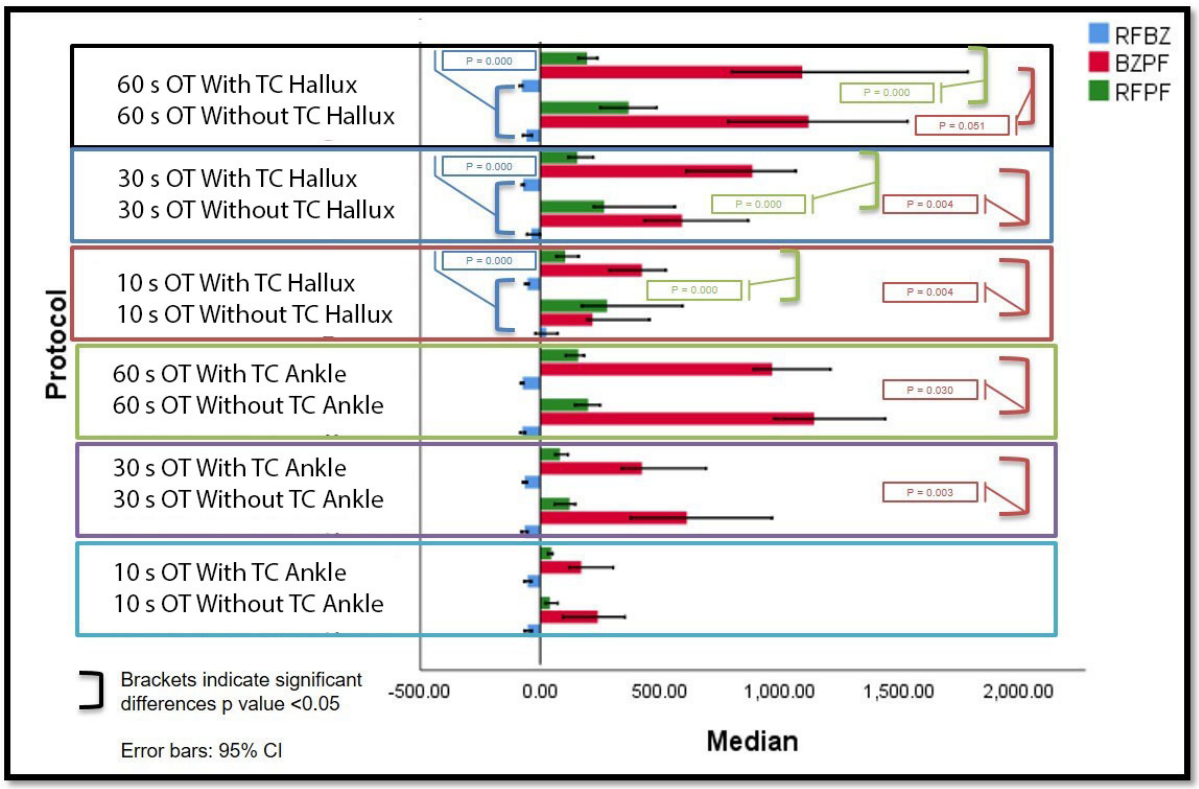



*Figure 4.5 Left Foot - Differences in Percent Change Measures Measured Using with and Without TC at Probe Site*

The temporal parameters TL, and TM did not show a consistent pattern of statistically significant median differences in both limbs (Figures 4.6 and 4.7). The TL showed statistically significant differences in the 10 seconds and 30-second ankle and 60 seconds hallux level occlusion at the right foot (Figure 4.6) and 10 seconds occlusion at the left ankle (Figure 4.7). But TR was found to demonstrate a consistent pattern of statistically significant differences at least with the hallux level occlusion protocols was TR (Figures 4.6 and 4.7).

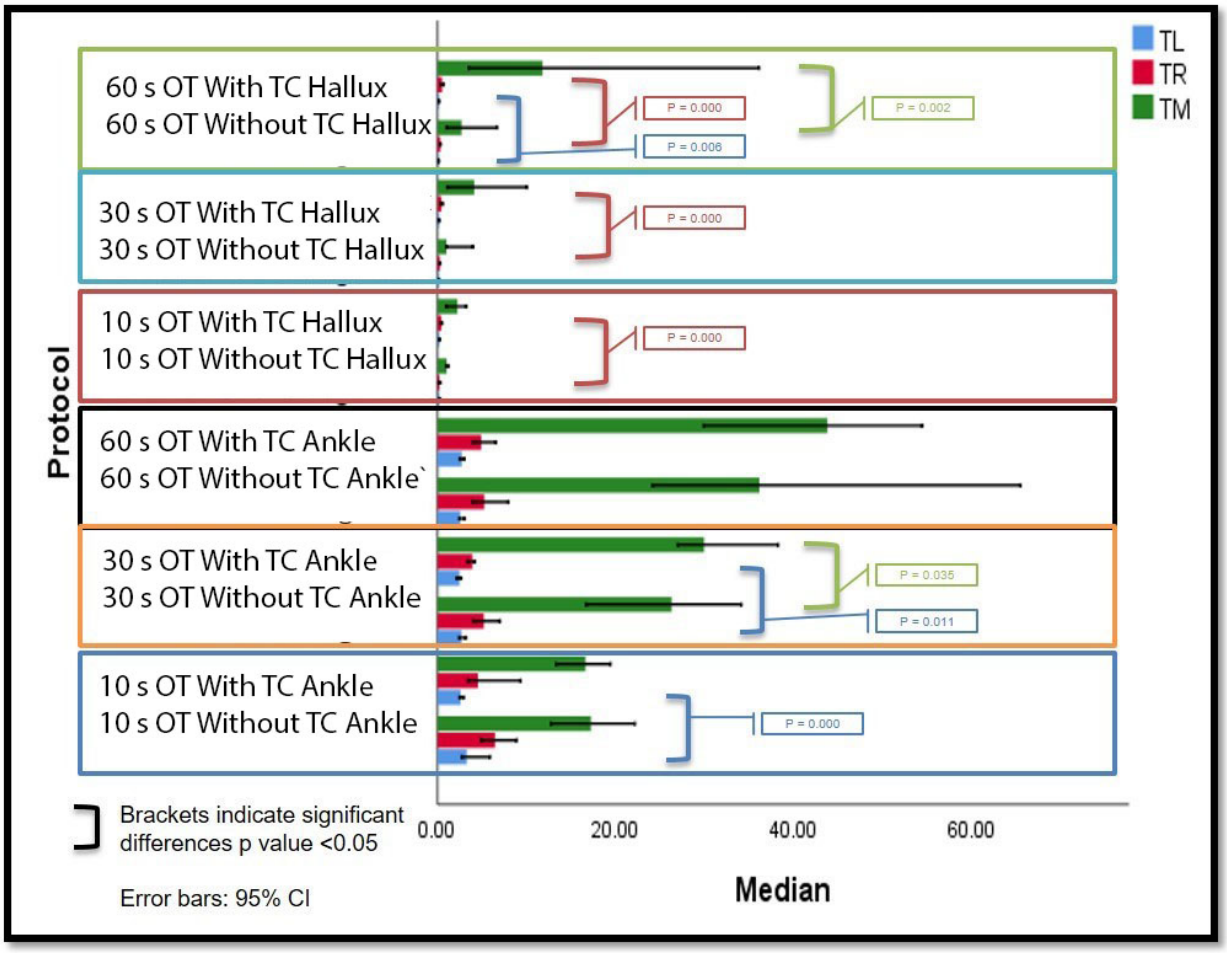



*Figure 4.6 Right Foot - Differences in Temporal Measures Measured Using with and Without TC at Probe Site*

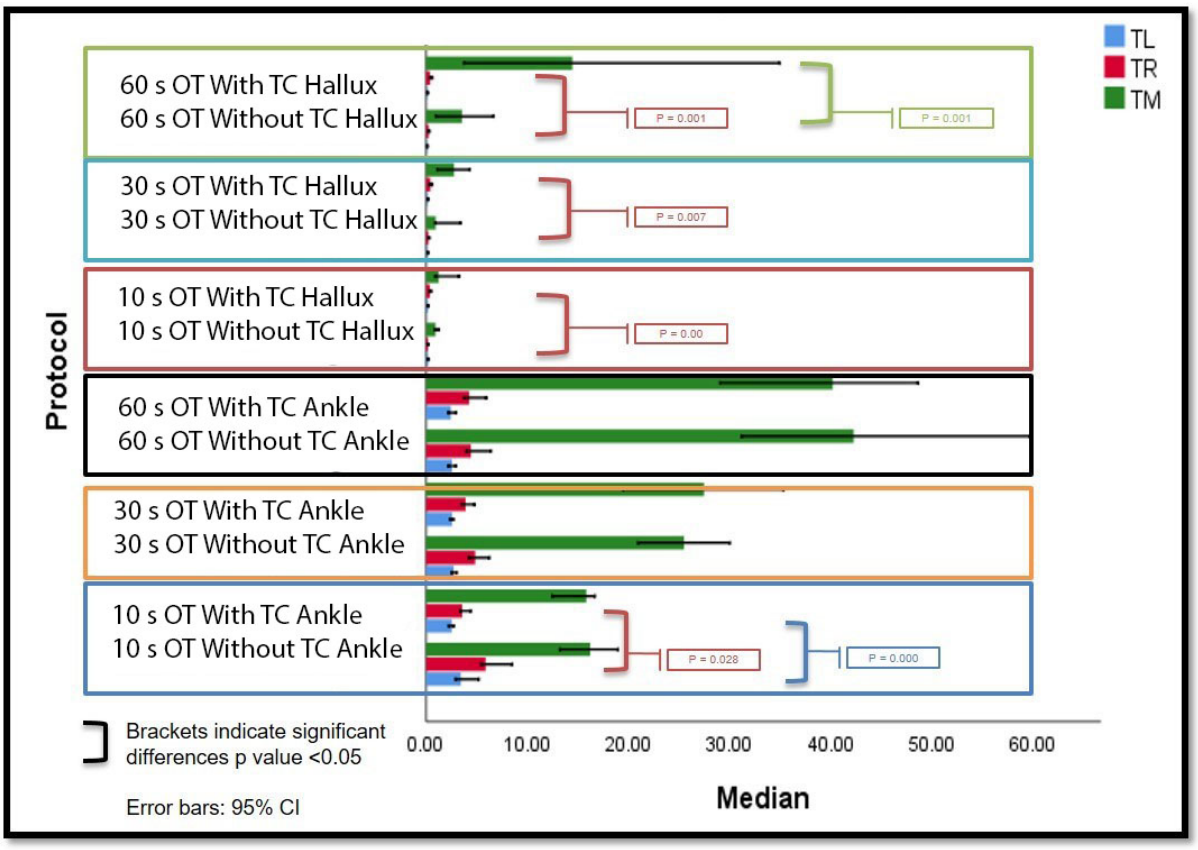



*Figure 4.7 Left Foot - Differences in Temporal Measures Measured Using with and Without TC at Probe Site*

The results of the within group pairwise comparison of with and without temperature control protocols are presented in Table 4.1 below.

*Table 4.1 The significance value, z and effect size for each pair (without and with temperature control) of protocols categorised according to occlusion time and site*

| | | Right Foot | | | | | | Left Foot | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Protocols | | | | | | Protocols | | | | | |
| Parameters | Values | 10 s OT at Ankle | 30 s OT at Ankle | 60 s OT at Ankle | 10 s OT at Hallux | 30 s OT at Hallux | 60 s OT at Hallux | 10 s OT at Ankle | 30 s OT at Ankle | 60 s OT at Ankle | 10 s OT at Hallux | 30 s OT at Hallux | 60 s OT at Hallux |
| RF | z | 3.188 | 2.597 | 2.274 | 4.372 | 4.345 | 4.372 | 2.785 | 2.435 | 2.543 | 4.372 | 4.372 | 4.372 |
| | r | 0.451 | 0.367 | 0.322 | 0.618 | 0.614 | 0.618 | 0.394 | 0.344 | 0.360 | 0.618 | 0.618 | 0.618 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | p | 0.001 | 0.009 | 0.023 | 0.000 | 0.000 | 0.000 | 0.005 | 0.015 | 0.011 | 0.000 | 0.000 | 0.000 |
| BZ | z | 4.345 | 4.372 | 4.373 | 3.700 | 4.130 | 4.372 | 4.265 | 4.372 | 3.861 | 4.049 | 4.345 | 3.969 |
| | r | 0.614 | 0.618 | 0.618 | 0.523 | 0.584 | 0.618 | 0.603 | 0.618 | 0.546 | 0.573 | 0.614 | 0.561 |
| | p | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| PF | z | 3.780 | 3.081 | 3.242 | 4.319 | 4.372 | 4.372 | 3.188 | 3.000 | 3.135 | 4.372 | 4.372 | 4.372 |
| | r | 0.535 | 0.436 | 0.458 | 0.611 | 0.618 | 0.618 | 0.451 | 0.424 | 0.443 | 0.618 | 0.618 | 0.618 |
| | p | 0.000 | 0.002 | 0.001 | 0.000 | 0.000 | 0.000 | 0.001 | 0.003 | 0.002 | 0.000 | 0.000 | 0.000 |
| RFBZ | z | 0.229 | 1.520 | 0.256 | -4.292 | -3.861 | -3.807 | -0.256 | 0.336 | -0.714 | -4.345 | -4.076 | -4.345 |
| | r | 0.032 | 0.215 | 0.036 | -0.607 | -0.546 | -0.538 | -0.036 | 0.048 | -0.101 | -0.614 | -0.576 | -0.614 |
| | p | 0.819 | 0.128 | 0.798 | 0.000 | 0.000 | 0.000 | 0.798 | 0.737 | 0.475 | 0.000 | 0.000 | 0.000 |
| BZPF | z | -0.256 | -2.327 | -1.493 | 0.632 | 2.462 | 1.278 | -0.982 | -2.946 | -2.166 | 2.866 | 2.892 | 1.951 |
| | r | -0.036 | -0.329 | -0.211 | 0.089 | 0.348 | 0.181 | -0.139 | -0.417 | -0.306 | 0.405 | 0.409 | 0.276 |
| | p | 0.798 | 0.020 | 0.135 | 0.527 | 0.014 | 0.201 | 0.326 | 0.003 | 0.030 | 0.004 | 0.004 | 0.051 |
| RFPF | z | -0.202 | -2.516 | -1.117 | -4.211 | -3.807 | -4.103 | 0.094 | -1.762 | -1.144 | -4.157 | -3.942 | -3.888 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | r | -0.029 | -0.356 | -0.158 | -0.596 | -0.538 | -0.580 | 0.013 | -0.249 | -0.162 | -0.588 | -0.557 | -0.550 |
| | p | 0.840 | 0.012 | 0.264 | 0.000 | 0.000 | 0.000 | 0.925 | 0.078 | 0.253 | 0.000 | 0.000 | 0.000 |
| TL | z | -3.619 | -2.543 | -0.829 | 0.471 | 1.102 | 2.762 | -3.538 | -1.224 | -0.350 | -0.431 | 1.144 | 1.446 |
| | r | -0.724 | -0.509 | -0.166 | 0.094 | 0.220 | 0.552 | -0.708 | -0.245 | -0.070 | -0.086 | 0.229 | 0.289 |
| | p | 0.000 | 0.011 | 0.407 | 0.638 | 0.271 | 0.006 | 0.000 | 0.221 | 0.726 | 0.667 | 0.253 | 0.148 |
| TR | z | -1.063 | -1.800 | -1.520 | 3.646 | 4.265 | 4.319 | -2.193 | -1.386 | -0.928 | 3.808 | 2.707 | 3.431 |
| | r | -0.150 | -0.255 | -0.215 | 0.516 | 0.603 | 0.611 | -0.310 | -0.196 | -0.131 | 0.539 | 0.383 | 0.485 |
| | p | 0.288 | 0.072 | 0.128 | 0.000 | 0.000 | 0.000 | 0.028 | 0.166 | 0.353 | 0.000 | 0.007 | 0.001 |
| TM | z | -0.982 | 2.112 | -0.444 | 0.843 | 1.125 | 3.081 | -0.955 | 0.417 | -0.498 | 0.928 | 1.036 | 3.431 |
| | r | -0.139 | 0.299 | -0.063 | 0.119 | 0.159 | 0.436 | -0.135 | 0.059 | -0.070 | 0.131 | 0.147 | 0.485 |
| | p | 0.326 | 0.035 | 0.657 | 0.399 | 0.211 | 0.002 | 0.339 | 0.677 | 0.619 | 0.353 | 0.300 | 0.001 |
| Key: | Significant p values | | | Effect size > 0.30 | | | The effect size for temporal measures, when compared between with and without temperature control protocols, were small. | | | | | | |

## 4.5 Discussion

The current literature shows ambiguous views on the techniques and reliability of measures related to PORH for both clinical and research purposes. The skin in general plays an important thermoregulatory role and this property influences cutaneous blood flow. Especially, the blood flow of glabrous skin such as plantar skin, where the arteriovenous anastomosis is dense, is highly influenced by temperature changes (Cracowski *et al.*, 2006; Jan *et al.*, 2013; Barwick, Lanting and Chuter, 2015). Therefore, the literature suggests that room temperature control may not be sufficient to minimise temperature-induced flow variations and controlling probe temperature is known to improve the reliability of PORH measurement (Barwick et al., 2015). Although controlling the probe temperature is considered to be useful to standardise protocols, methodologically it is criticised and it is deemed to be physiologically less accurate (Charkoudian, 2003; Cracowski *et al.*, 2006). However, there is no evidence in the current literature to demonstrate the physiological variations in PORH measures when measured without and with temperature control at the probe site. Furthermore, for this reason, and additional factors such as probe placement and variability in the basal skin perfusion has challenged the use of LDF methods for microcirculatory assessments. But, given that it is a non-invasive procedure, feasible to be replicated and can be performed in minimal time, it may have potential applications in the management of diabetic foot-related complications. However, this requires a better understanding to facilitate the use of PORH assessment for clinical or research purposes. Hence, this study aimed to address this knowledge gap by comparing the PORH parameters measured without vs with temperature control in healthy adults.

The results of the current study showed that the perfusion measurements were generally higher under controlled temperature conditions. For each occlusion time and site, pair-wise comparison between with and without temperature control protocols was made for each of the PORH measures generated by the Laser Doppler Flowmetry system. The median of the perfusion parameters across all protocols for the right foot and left foot is shown in figures 4.2 and 4.3, respectively. The Wilcoxon signed-rank test showed significant differences between the two protocols for the perfusion measures RF, BZ and PF. As observed in Table 4.1, there was a statistically significant increase in perfusion with temperature control for RF, BZ and PF ($p < 0.05$) across all protocols in both feet. These results highlight the role of the mechanothermal receptors on the foot and the effect of foot temperature on cutaneous microcirculation. The temperature is an important influencing factor for cutaneous microcirculation. The literature shows the impact of certain protective microcirculatory responses such as PIV, which is a transient increase in cutaneous microcirculation upon the application of sustained non-noxious stimuli (Koïtka, Legrand-Fernandez, *et al.*, 2004). PIV was discussed in detail in Chapter 1. PIV is impaired at low foot temperature even in a healthy individual; however, it was present when the foot

temperature was higher than 33C, which was achieved through maintaining a higher room temperature in the study by Koïtka et al. (2004). This is consistent with the results of this study, the PORH perfusion measures were significantly less at low foot temperature (without temperature control protocols), but when the temperature was controlled at the probe site they were significantly increased (Figures 4.2 and 4.3; Table 4.1). An important observation is that at the hallux for all three occlusion times, the RFPF percent changes were significantly higher ($p < 0.001$; effect size > 0.50) when measured without vs with temperature control (Figures 4.4 and 4.5; Table 4.1). A possible explanation for this could be that the lack of temperature control allowed greater reactivity to the pressure stress; however, with temperature control, thermoregulatory mechanisms may have influenced percent change in RFPF. This accords with the literature that states that peak as a percent of baseline measurements had higher reliability when temperature control (vs no temperature control) was used at the probe site (in addition to controlling room temperature), but the disadvantage of controlling the skin temperature at the probe site could be that it is a lesser physiological measurement (Cracowski *et al.*, 2006; Barwick, Lanting and Chuter, 2015). While differences in percent change measurements were observed between, with and without temperature protocols at the hallux, the results at the ankle were not always significantly different. This could be due to the differences in the way the skin microcirculation responds to vascular occlusion at the ankle and hallux levels, which becomes evident when more sensitive measures are quantified. This is consistent with the observations made by researchers in the past, where it is known that small variations in baseline values are known to contribute to large differences in percent changes and several factors (the nerve conduction speed and compensatory myogenic activity in case of reduced nerve function) were considered to influence the measures of percent change (Cracowski *et al.*, 2006; Lanting et al., 2017).

The temporal parameters TL and TM did not show a consistent pattern of significant differences, especially when the outcomes of both limbs were observed (Table 4.1 and Figures 4.6 and 4.7). However, one temporal parameter that was found to show a consistent pattern of statistically significant differences at least in the hallux-level occlusion protocols was TR (Figures 4.6 and 4.7). The TR, which is similar to the time to resting flux ($T_{RF}$) in the previous literature, is considered to be more of a physiologically interesting variable reflecting pathological conditions because it was able to distinguish between people with and without the disease (Bongard and Fagrell, 1990; Morales *et al.*, 2005; Yamamoto-Suganuma and Aso, 2009; Barwick, Lanting and Chuter, 2015). In the current study, the TR showed a statistically significant difference at the hallux level occlusion; it was higher when temperature control was used at the probe site in comparison to when it was not used (Table 4.1 showing medium to large effect size ranging from 0.38 to 0.61). This may be because the time to return rest flow may be longer when there is increased perfusion. Also, TR showed a consistent pattern

(significant difference) in the hallux level occlusion but not in the ankle level occlusion. A possible explanation for this observation could be that it takes a significantly longer time for the blood flow to return to baseline level with ankle occlusion protocols in comparison to the hallux occlusion protocols. This, in turn, could be a plausible explanation for the fact that the temporal parameters do not show consistent patterns (with significant differences) across the protocols, as they can be influenced by occlusion time and location. As previously mentioned, temporal parameters are considered to have the ability to discriminate between groups with and without pathological conditions, suggesting that they are more sensitive measures (Bongard and Fagrell, 1990; Morales *et al.*, 2005).

As highlighted earlier, while temperature control at the probe site helps to standardise the procedure and obtain reliable measures, the use of the method to standardise the measurement has been criticized as being less physiologically accurate and monitoring of skin temperature is recommended instead (Charkoudian, 2003; Cracowski *et al.*, 2006; Barwick, Lanting and Chuter, 2015). Furthermore, Morales et al. (2005) also recommended not using temperature control whilst performing PORH testing. When carefully selecting the protocol based on the participant characteristics or the parameter of interest, it is vital to have an understanding of the microcirculatory responses influenced by the temperature sensitivity. This study was conducted in healthy adults and has served as a stepping stone to understanding the effects of controlling the temperature at the probe site on microcirculation. This serves as a reference and supports further studies on the use of quick and simple methods such as PORH for microcirculatory assessment of the foot in routine practice. The results of the study reinforce the value of understanding the dynamics of cutaneous microcirculatory responses with respect to temperature. This knowledge can be translated when using PORH assessment in the feet of people with diabetes as their foot temperature varies incredibly; they may present with warm or cold feet depending on the presence and nature of the neuropathy (Armstrong and Lavery, 2016). In addition, their thermal sensitivity can vary depending on the presence or absence of neuropathy, which in turn can affect microcirculation. The literature shows that in people with diabetes the cutaneous microcirculatory responses are impaired due to ageing and underlying complications that may be neuropathic, vascular or both in nature (Fromy *et al.*, 2002; Morales *et al.*, 2005; Fromy *et al.*, 2010; Lanting et al., 2017; Fouchard *et al.*, 2019). Therefore, the assessment of microcirculation should be based on a thorough understanding of microvascular dynamics in relation to temperature changes. This can be useful in predicting certain complications, such as ulcers. This is consistent with existing literature suggesting that microcirculatory responses such as PIV may have clinical implications and their absence may increase the risk of ulcer development (Fromy *et al.*, 2002; Klabunde, 2012; Koïtka, Abraham, et al., 2004; Fromy *et al.*, 2012). Since PIV in the foot was also impaired in healthy participants and disappeared at low skin temperature, Koïtka et al. (2004) recommended high room

temperatures for bedridden patients as this mechanism could be one of the explanations for the risk of developing pressure ulcers (Koïtka, Legrand-Fernandez, *et al.*, 2004). This underscores the importance of understanding skin perfusion as temperature changes, as this can be beneficial for real-world applications in clinical populations. Similarly, PORH is a complex multifactorial microvascular response and a deeper understanding is required to determine the measures of diagnostic or prognostic relevance. Thus, the findings from this study bridge the knowledge gap on the effects of temperature control at the probe site on PORH measures and provide an understanding of the influence of temperature on key perfusion measures that may be relevant to foot assessment in people with diabetes in the future.

## 4.6 Conclusion

The findings from the study showed that perfusion measurements in both feet were significantly higher when measured with temperature control than without temperature control at the probe site across all protocols with either ankle or hallux occlusion. A few percent change measures (RFBZ and RFPF percent changes) and temporal (TR) measures consistently showed significant differences between protocols without temperature control and with temperature control protocols at hallux-level occlusion independent of time. While these findings reiterate that temperature plays a vital role in cutaneous microcirculation, they may have practical implications. For instance, this test can be incorporated in clinical settings as it is a less time-consuming microvascular assessment methods for a comprehensive foot assessment in people with diabetes, especially to study the neurovascular responses. More potential uses of these tests have been discussed in the conclusion chapter. Since the results of this study help to understand fluctuations in cutaneous perfusion and their potential influencing factors, these results may serve as stepping stones for future assessment of microcirculatory dysfunction in people with diseases such as diabetes.

# CHAPTER V

## UNDERSTANDING THE RELATIONSHIP BETWEEN MICROCIRCULATION AND AUTONOMIC FUNCTIONS IN THE FOOT

A part of this chapter was presented as:

A Poster Presentation on "The relationship between skin microcirculation and temperature in light of the autonomic functions of small nerve fibres in the foot" was accepted for poster presentation at the 22nd International Vascular Biology Meeting, UK, 2022

A Poster Presentation on "An evaluation of the association between skin microcirculation and temperature in light of the autonomic functions of the small nerve fibres in the foot" was accepted[2] for poster presentation at the Joint Meeting of the German Society for Microcirculation and Vascular Biology Society (GfMVB) and the British Microcirculation and Vascular Biology Society (BMVBS)", UK, 2022

An Oral presentation on "Relationship between skin microcirculation and the autonomic nerve functions in the foot and implications to diabetic foot neurovascular assessment", Staffordshire Clinical Biomechanics Conference, Staffordshire University, UK, 2022

An Oral presentation on "Association between microcirculation and small nerve fibre functions in the foot", Research Seminar Series and meeting of Visiting Professors/Fellows, Staffordshire University, UK, 2021

[2] The accepted poster was not presented in this case because of COVID-related issues

# Chapter 5 Understanding the relationship between microcirculation and autonomic functions in the foot

## 5.1 Introduction

In the previous Chapter 4, it was established that temperature has a significant impact on skin microcirculation in the foot by testing PORH with and without temperature control at the probe site with the hallux-level occlusion, regardless of duration (10, 30, or 60 seconds). PORH is a neurovascular response controlled by vascular, neurogenic and myogenic mechanisms. The mechanoreceptors and sensory receptors play a crucial role in this response (Balasubramanian *et al.*, 2020). The question of how skin microcirculation and sympathetic nerve fibres are related is the area to which this thesis now turns and Chapter 5 deals with the quantitative study 2 of this thesis. This study examined the sympathetic activity of the small nerve functions, heart rate (HR) and electrodermal activity (EDA), which is also known as Galvanic Skin Response (GSR) or Skin Conductance (SC) in response to deep inspiration and expiration using slow breathing technique. Simultaneously, the skin microcirculation/perfusion (PU) was also measured to gain an understanding of the microcirculatory responses during sympathetic activity in order to establish the relationship between skin microcirculation and the autonomic component of the small fibre nerves. As the previous study (Chapter 4) demonstrated the important role temperature (T) played on microcirculation, it was measured at the probe site where perfusion was measured. All measurements were performed in an environment with controlled conditions. The main results were that skin the skin PU decreased with inspiration and increased during expiration, there were significant correlations (Spearman Rho) between skin PU and skin T in both feet during inspiration and expiration ($p < 0.05$). Furthermore, the partial correlation results showed that there was a significant strong to very strong correlation between PU and T in both the feet and the HR and EDA had very little effect on their relationship ($p < 0.05$). As a final step, when a multiple regression analysis was performed separately for both feet, only T statistically significantly predicted PU in both feet and significantly contributed to the model. This provides a very convenient way to measure skin microcirculation using the skin temperature of the foot as a surrogate measure and finds many applications in the area of diabetic foot. Temperature measurements may be potentially used to indicate microcirculatory dysfunction or small fibre nerve dysfunctions. This current study is a valuable contribution to the field as the use of thermal techniques to determine foot at risk is an ongoing area of research. This is valuable as it does not always require expensive equipment such as perfusion measurements, which are convenient to adopt for mass screening and in resource-limited settings.

## 5.2 Background

Skin microcirculation is known to contribute to the pathogenesis of neuropathy and an association between the degree of microvascular dysfunction and the severity of neuropathy has been identified in diabetic foot-related complications (Veves, Giurini and LoGerfo, 2006; Vas, Green and Rayman, 2012). Thus far, the presence of microvascular complications is directly linked to small fibre dysfunction (Vas, Green and Rayman, 2012; Körei et al., 2016). Neuropathy with small fibre involvement results in altered pain perception impaired thermal sensation to both cold and heat stimuli and impaired autonomic functions. Peripheral autonomic nerve dysfunction at a sympathetic level results in dry skin (Khalfallah et al., 2012; Körei et al., 2016; Armstrong and Lavery, 2016). Furthermore, autonomic dysfunction co-existing with microcirculatory dysfunction resulting in decreased activity of sweat glands gives dry feet (Armstrong and Lavery, 2016). Consequently, dry skin along with cracks and fissures paves the way for infections (Veves, Giurini and LoGerfo, 2006). In summary, structural microcirculatory disease can affect the blood supply to nerves promoting neuropathy and on the other hand small fibres play a major role in the microcirculatory responses. Therefore, it can be concluded that there is a relationship between microcirculation and small fibre dysfunction relating to diabetic foot complications. Whilst there is evidence in the current literature to describe their association, there is a paucity of data in terms of based on non-invasive ways to assess microcirculatory and autonomic functions of small nerve fibres, which may potentially be relevant in the management of clinical conditions such as diabetic foot complications. Furthermore, the mechanisms that underpin the interactions between small fibre functions and microcirculation still need to be understood.

Skin microcirculation is a vascular mechanism by which the skin or tissue is perfused with oxygen and nutrients and waste products are removed via a network of small blood vessels according to the instantaneous tissue demands (Ovadia-Blechman *et al.*, 2017). Therefore, skin microcirculation or skin perfusion (the term is vastly used interchangeably in literature) is vital to maintain peripheral vascular homeostasis.

Microcirculation is not just a passive conduit for red blood cell transport, and nutrient and gas exchange, but a dynamic participant that actively contributes to the numerous processes involved in maintaining metabolic homeostasis and optimal end-organ function (Somani et al., 2010). Microcirculation is responsible for adjusting vascular tone to local tissue perfusion in response to oxygen demand (Gutterman *et al.*, 2016). In vital organs such as the heart, this regulation occurs every second to optimise cardiac output and prevent ischemia (Miller et al., 1997). Tissue-specific responses that are appropriate to variable stimuli are also required. For instance, hypoxic vasodilatation is needed in systemic circulation to maintain blood flow and sustain metabolic homeostasis at both

cellular and tissue levels (Somani et al., 2010). Not only does microcirculation respond to metabolic stimuli from local tissues to regulate flow, but local tissues also act in response to various factors such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarization via factors (EDHFs) and others that are released from the microvasculature in response to mechanical and chemical stimuli (Giles *et al.*, 2012; Gutterman *et al.*, 2016). Finally, microcirculation contributes to maintaining homeostasis by playing a vital role in thermoregulation. In the extremities, there are two types of skin, non-glabrous (hairy) and glabrous (non-hairy). The non-glabrous skin is innervated by both a sympathetic vasoconstrictor and a vasodilator system (Anderson, Pekas and Park, 2021). The non-glabrous microcirculation has a limited capacity for autoregulation, but it has a unique regulatory feature, namely the venoarteriolar reflex (VAR), a local sympathetic reflex, which combats blood pressure fluctuations and compensates for the limited autoregulatory capabilities (Wilson *et al.*, 2005; Anderson, Pekas and Park, 2021). For instance, when the orthostatic pressure is altered due to postural changes, the VAR is activated and causes vasoconstriction of the arterioles, protecting the capillary structures from blood flux due to the effects of gravity (Cisek *et al.*, 1997). On the other hand, glabrous skin lacks VAR. However, the microcirculation of glabrous skin has autoregulatory capabilities and is an integral part of core temperature regulation. A unique structure, the arteriovenous anastomoses (AVA), which are direct conduits between dermal arterioles and venules aid in autoregulation (Walløe, 2016; Anderson, Pekas and Park, 2021). However, AVAs do not contribute to nutrient circulation like the capillary networks and loops. AVAs contain thick concentric layers of smooth muscle and are densely innervated by sympathetic adrenergic nerves but there is no active vasodilator system (Walløe, 2016). Thus, AVAs are not responsive to metabolic vasodilators (Midttun and Sejrsen, 1996). Under thermoneutral conditions, the AVAs remain mostly closed, but during times of extreme heat stress, the sympathetic tone is released and the AVAs become fully dilated to allow for an increase in blood volume closer to the external environment (Walløe, 2016).

Literature states that this aspect renders skin microcirculation an important marker for the pathogenesis of numerous diseases but it is often faint and can be barely recognised (Ovadia-Blechman *et al.*, 2017). Literature suggests that Skin microcirculation monitoring has been demonstrated to provide information and alerts on disease progress and therapy effectiveness and may also have diagnostic capability (Morales et al., 2005; Lanting et al., 2017; Ovadia-Blechman et al., 2017). Evidence suggests that microcirculation interacts with systemic regulatory mechanisms such as heart rate (HR), respiration, electrodermal activity and thermoregulation (Ovadia *et al.*, 1995; Volynsky *et al.*, 2019; Ren *et al.*, 2021). These variables are relatively easy to measure, less time-consuming and non-invasive but at the same time, they may potentially provide some information about the microcirculatory and small fibre nerve functional aspects and the relationship between each

other (HR, respiration, perfusion, skin temperature and electrodermal activity) (Ovadia-Blechman *et al.*, 2017).

As mentioned earlier, autonomic functions mediated by the small fibre nerves play a vital role in the maintenance of homeostasis within the body and understanding these functions is essential to underpin the mechanisms behind neurovascular interactions (Balasubramanian *et al.*, 2020). The autonomic regulation depends on three main components, which are baroreceptors, chemoreceptors and pain receptors. The afferent fibres continuously perceive changes in blood pressure (baroreceptors), blood oxygen levels and other chemical signals (chemoreceptors), pain (sensory afferents) and cortical stimulation (Kaufmann and Biaggioni, 2010). These signals are integrated into brainstem centres that ultimately modulate sympathetic and parasympathetic outflows that are relayed to target organs via efferent fibres (Kaufmann and Biaggioni, 2010). These interactions ensure the maintenance of cardiovascular regulation. Current literature shows that there are physiological implications to different breathing techniques such as slow breathing (the effects of breathing at 4–10 breaths per min or 0.07–0.16 Hz in humans) on the respiratory, cardiovascular, cardiorespiratory and autonomic nervous systems, with a particular focus on diaphragm activity, ventilation efficiency, haemodynamics, HR variability, cardiorespiratory coupling, respiratory sinus arrhythmia and sympathovagal balance are connected (McCrimmon, Alheid and Zuperku, 2006; Kaufmann and Biaggioni, 2010; Russo, Santarelli and O'Rourke, 2017). However, the various underlying mechanisms behind these, especially their influence on microcirculation warrant further research, understanding and discussion. Investigations into the physiological effects of slow breathing have uncovered significant effects on the respiratory, cardiovascular, cardiorespiratory and autonomic nervous systems. Some of the findings suggest the effects of slow breathing on respiratory muscle activity, ventilation efficiency, chemoreflex and baroreflex sensitivity, HR variability, blood flow dynamics, respiratory sinus arrhythmia (RSA), cardiorespiratory coupling, and sympathovagal balance (Russo, Santarelli and O'Rourke, 2017). The changes in respiratory phases based on controlled breathing or varying techniques instigate fluctuations in venous filling, stroke volume, cardiac output and peripheral blood flow, which in turn contribute to fluctuations in HR and blood pressure. Under steady-state conditions, these variations may be undetectable (Kaufmann and Biaggioni, 2010; Ovadia-Blechman *et al.*, 2017). Literature suggests that HR increases during inspiration while arterial blood pressure decreases, and HR decreases during expiration while arterial blood pressure increases (Kaufmann and Biaggioni, 2010). Whilst the relationship between blood pressure, respiratory phases and HR has been extensively studied and evidenced. Little is known about its interaction with skin microcirculation. But, understanding the interactions between autonomic functions of the small fibre nerves and skin microcirculation at the foot can be useful to establish the neurovascular interactions

that may potentially have application in the diagnosis and management of various pathological conditions such as this can be specifically relevant to diabetic foot complications. This could shed further light on the impairment of both autonomic functions of small fibre nerves as well as microcirculation. The aim and objectives of the study are as follows:

### 5.2.1 Aim

This study aimed to investigate the relationship between microcirculation and autonomic functions of the small nerve fibres in the foot

### 5.2.2 Objectives

- to identify the changes in HR dynamics during different states of activity during baseline (normal breathing) and deep inspiration and expiration (achieved through slow breathing technique 6 breaths/minute)
- to identify the changes in EDA in the plantar surface of the foot during different states of activity baseline (normal breathing) and deep inspiration and expiration (achieved through slow breathing technique 6 breaths/minute)
- to monitor skin temperature changes at the site of perfusion measurement in the plantar surface of the foot during different states of activity baseline (normal breathing) and deep inspiration and expiration (achieved through slow breathing technique 6 breaths/minute)
- to assess the changes in skin microcirculation or perfusion PU in the foot simultaneously during different states of activity baseline (normal breathing) and deep inspiration and expiration (achieved through slow breathing technique 6 breaths/minute)
- to understand the interactions and relationship between skin perfusion, skin temperature, HR and EDA and their interdependence on one another.

## 5.3 Methodology

### 5.3.1 Participants and setting

In this study, the inclusion criteria were that any adult over 18 years old with no severe neurological or vascular issues and no major vascular trauma or injury (bleeding, bruising, hematoma and fractured bones) that affects circulatory measurements could participate in the study. The study was conducted upon obtaining the University Ethics Committee's approval. Through the information sheet, the participants were requested not to consume any caffeinated or alcoholic beverages 2 hours before the study as this is known to affect vascular measures (Kudo *et al.*, 2015; Noguchi *et al.*, 2015; Piano, 2017). Besides, they were requested not to engage in any strenuous exercise of any form 2 hours before the study for the same reason (Oh, Hong and Lee, 2016). The participants upon arrival to the Biomechanics laboratory were familiarised with the settings and the protocol. An ambient room

temperature was maintained at 23℃ +/- 1 throughout the experiments. There were 10 participants (6 females and 5 males) whose mean (SD) age was 23.8 (4.37) years and BMI was 24.53 (7.63). The ABI was 1.12 (0.14) and 1.11 (0.13) for the right and left foot, respectively. The Quantitative Sensory Test (QST) results are included in Appendix 11, Figures 0.1 to 0.4.

### 5.3.2 Data collection

#### *5.3.2.1 Protocol*

*Simultaneous measurement of microcirculation and autonomic functions during deep inspiratory gasps:*

The participant was requested to lie supine on the couch as still as possible because of the nature of the tests, which resulted in artefacts even with minor movements. The study commenced after a minimum of 15 minutes of acclimatisation (Barwick, Lanting and Chuter, 2015; Moreira-Marconi, E. *et al.* 2019). The laser Doppler probes (contact area 1 $cm^2$ each) were secured using a double-sided adhesive tape on the distal/plantar aspect of the hallux. Additionally, the leads with adhesives were secured on the hands and ankles of both feet to measure the HR. The two pre-gelled electrodes/sensors were placed on the medial and inner side of the plantar aspect of the foot as shown in Figure 5.1, which were used for the acquisition of EDA/GSR. Upon completion of the set-up, the recording using the PeriSoft for LDF system and BIOPAC for GSR were started simultaneously by hitting the start button based on the cue "1, 2, 3, start…". Following an initial 10 secs of preparation time, the recording was done for 10 minutes without any interruption. After the 10 minutes, the participant was requested to do slow breathing, a deep inspiration for 10 seconds followed by a deep expiration for 10 seconds paced to get 6 breaths per minute as recommended for sinus arrhythmia assessment (Mulinos and Shulman, 1939; Gilliatt, 1947; Allen, Frame and Murray, 2002). There was a thermometer that measured the room temperature to ensure that the temperature was maintained throughout the data collection period. Three readings of both were taken before the completion of the test.

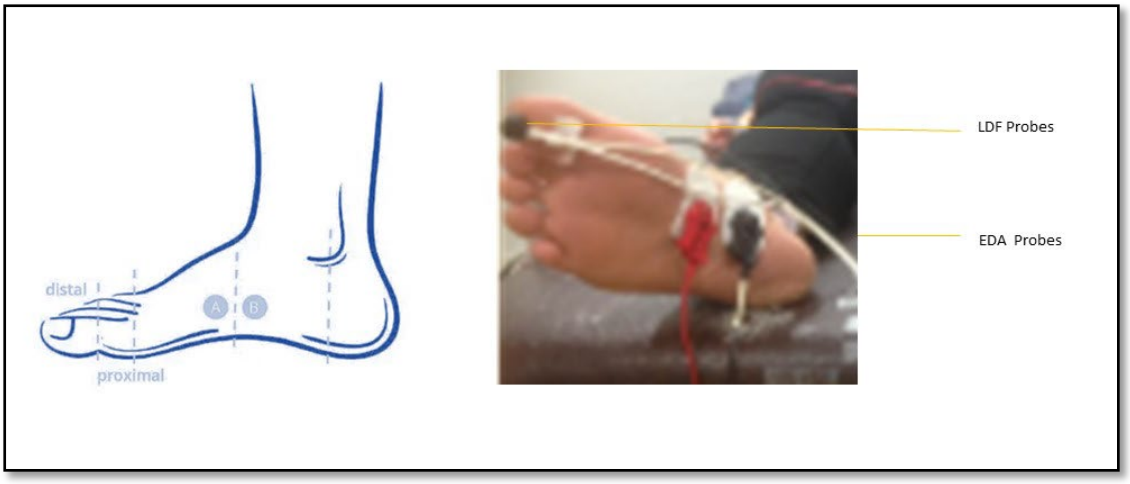



*Figure 5.1 Placement of electrodes for EDA and probes for microcirculatory measurements*

#### 5.3.2.2 *Equipment*

The nature of the study required the use of multiple devices that were operated by the main researcher and the research assistant. Figure 5.2 below shows the study setup.

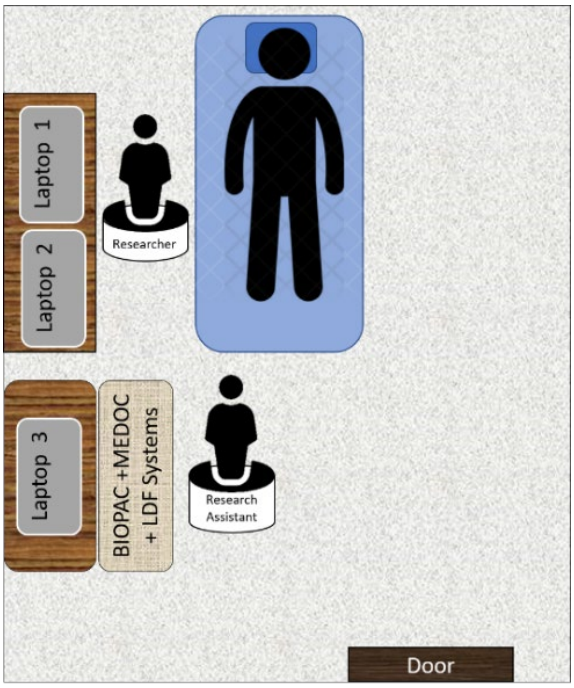



*Figure 5.2 Study set-up*

##### 5.3.2.2.1 Small fibre nerve functions:

The BIOPAC wired physiology measurement system connected to a laptop with the BSL software was used for data acquisition. There are various hardware units available to collect a range of physiological parameters. For the purpose of this study, the MP systems were used for the acquisition of EDA also GSR and HR. The system has leads and pre-gelled electrodes with adhesives were secured on the

hands and foot of the subject. There were 1 HR channel and 2 channels that recorded the EDA on the right and left feet.

#### 5.3.2.2.2 Microcirculatory measurement:

For the cutaneous microcirculatory assessment, the laser Doppler flowmetry (LDF) Perimed Periflux® system 5000 system was used. One main system equipped with 2 functional units that facilitated the simultaneous measurements of cutaneous perfusion in Perfusion Units (PU), 1 functional unit that recorded the temperature in Degree Celsius (̊C) and 1 pressure unit for occlusion was used for the study. The device with two LDPM units allowed simultaneous blood pressure measurements at both arms followed by both ankles and halluces. There were 2 thermostatic laser Doppler probes measuring $1cm^2$, which facilitated the measurement of both perfusion and temperature. An ambient room temperature was maintained at 23℃ +/- 1. The system was attached to a laptop with exclusive software, PeriSoft that helps to run the protocols in a sequential manner and record data. The steps for the protocols and the sequence of events were defined to facilitate effective, precise and replicable data collection across participants. The inflation of the pressure cuffs was manual; however, the deflation was set to be automatic. The data for ABI and perfusion measures during deep inspiration and expiration challenges and skin temperature at the probe site were collected using the same equipment.

### 5.3.3 Data analysis:

The Biopac software was used to extract average HR measured in beats per minute (bpm) during baseline, inspiration for 10 seconds and expiration for 10 seconds. Also, the baseline of EDA was measured in micro-Siemens, EDA during inspiration that lasted for 10 seconds and EDA during expiration that lasted for 10 seconds in the right and left foot separately. The software programmes used were: Perisoft for LDF parameters.

The data was initially populated on EXCEL and then analysed using SPSS version 27.0 (June 19, 2020). The data analysis involved 4 major steps. Figure 5.3 summarises the data analysis approach. Initially, as step one, an exploratory descriptive statistical test was run to assess the distribution and normality for all data. The normality tests using Shapiro Wilk's test and on observation of the stem and leaf diagram as well as histogram indicated a non-normal distribution of the data. Therefore, in step two, to compare the variables measured within participants (related sample) a pairwise Wilcoxon Signed rank test was used to compare the measurements during baseline, deep inspiration and deep expiration. These were done for the EDA and HR measured using Biopac. Similarly, a pairwise comparison was performed to compare the variables measured during baseline, deep inspiration and deep expiration within participants (related sample) using a pairwise Wilcoxon Signed rank test for

the perfusion (PU) and temperature (T ˚C) measured using LDF in the foot. The variables were compared as illustrated in Figure 5.4. In step 3, a Correlation matrix (Spearman's Rho) was used to calculate the correlation strengths between one variable to another and for mapping interrelationships between variables. As step 4, Partial correlation tests were performed to understand the relationship between variables whilst controlling for the confounding parameters. For instance, when testing the relationship between PU and T the confounding parameters were EDA and HR. Similarly-, when testing the interrelationship between EDA and T, the confounding parameters were set to be PU and T. Thus, a better understanding of the relationship between parameters and to what extent the confounding parameters influenced the strength of their relationships was gained. Finally, as step 5, Regression analysis to identify the best predictor of PU. These statistical tests were performed based on the existing literature of similar studies (Balbinot et al., 2012; Krishnan and Rayman, 2004).

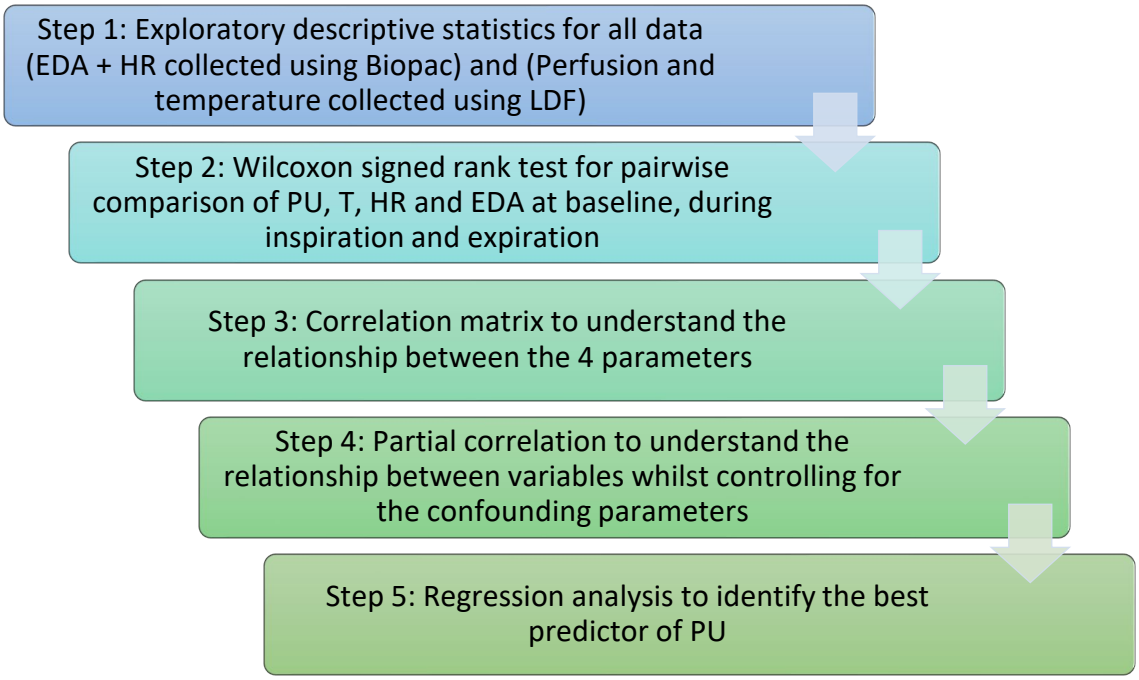



*Figure 5.3 Data analysis approach*

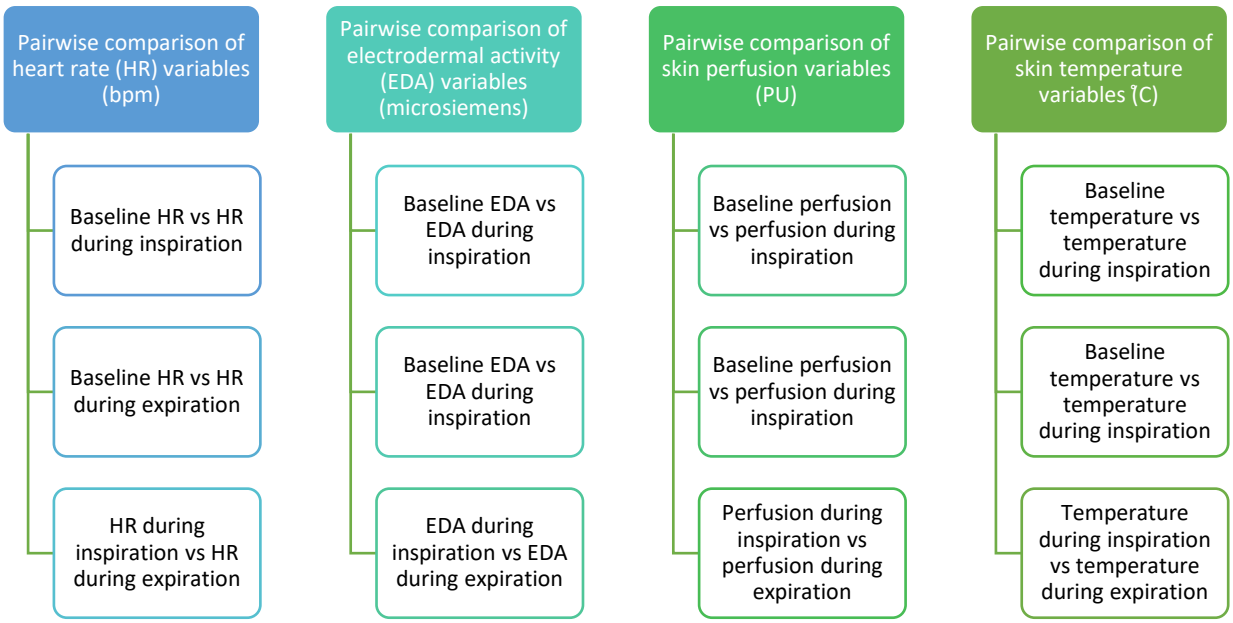



*Figure 5.4 Analysis approach for pairwise comparison of variables*

## 5.4 Results

### 5.4.1 Descriptive analysis and Pairwise comparison Wilcox Sign Rank Test:

An initial descriptive analysis was performed and normality testing using Shapiro-Wilk's test indicated the on-normal distribution of the data ($p<0.05$). Figure 5.5 shows the mean and standard deviation (SD) for the four parameters. Then, a pairwise comparison of parameters as per Figure 5.4 was performed using the Wilcox Sign Rank test and the significant relationships are shown in Figure 5.5 as a link.

#### *5.4.1.1 Heart Rate (HR)*

A respiratory sinus arrhythmia pattern was observed; that is the heart rate increased with inspiration and decreased with expiration. The HR synchronised with the breathing, which was paced at the rate of 6 breaths per minute. The mean HR during inspiration increased during inspiration and the group mean HR during inspiration was 68.97 bpm (SD 13.88; Range 49.24). Meanwhile, the mean HR during expiration was significantly ($p<0.05$) decreased during expiration and the group mean HR during expiration was 65.03 bpm (SD 14.76; Range 53.36) as shown in Figure 5.4. Also, the figure illustrates the findings that the HR measured during inspiration was significantly higher than that which was measured during baseline ($p = 0.047$; $z = -1.988$; and HR measured during inspiration was significantly higher than that measured during expiration was confirmed using Wilcoxon signed rank test pairwise comparison of related samples ($z = -2.395$; $p = 0.017$) as seen in Table 5.1. Additionally, the HR variation

which is determined by taking the difference between the average HR during inspiration from the average HR during expiration showed a group mean of 3.93 bpm (SD 3.25; range 8.55).

#### *5.4.1.2 Electrodermal Activity (EDA)*

In the right foot, the group mean EDA during baseline was inspiration was 5 micro-Siemens (SD 4.72; Range 15.82). and the group mean EDA during expiration was 5.09 micro-Siemens (SD 4.57; Range 15.58). In the left foot, the group mean EDA during inspiration was 4.16 micro-Siemens (SD 3.62; Range 12.61). Meanwhile, the mean EDA during expiration decreased during expiration and the group mean EDA during expiration was 4.22 micro-Siemens (SD 3.50; Range 12.47). Although on both occasions the EDA was decreased during expiration the findings showed no significant difference when using the Wilcoxon signed rank test pairwise comparison of related samples ($p > 0.05$). However, the Wilcoxon signed rank test results showed that there was a significant difference between the EDA measured during inspiration vs baseline (right foot $z = -2.666$; $p = 0.008$; left foot $z = -2.803$; $p = 0.005$) and baseline vs expiration in both feet (right foot $z = -2.666$; $p = 0.008$; left foot $z = -2.803$; $p = 0.005$). These results are summarised in Figure 5.5 and Table 5.1.

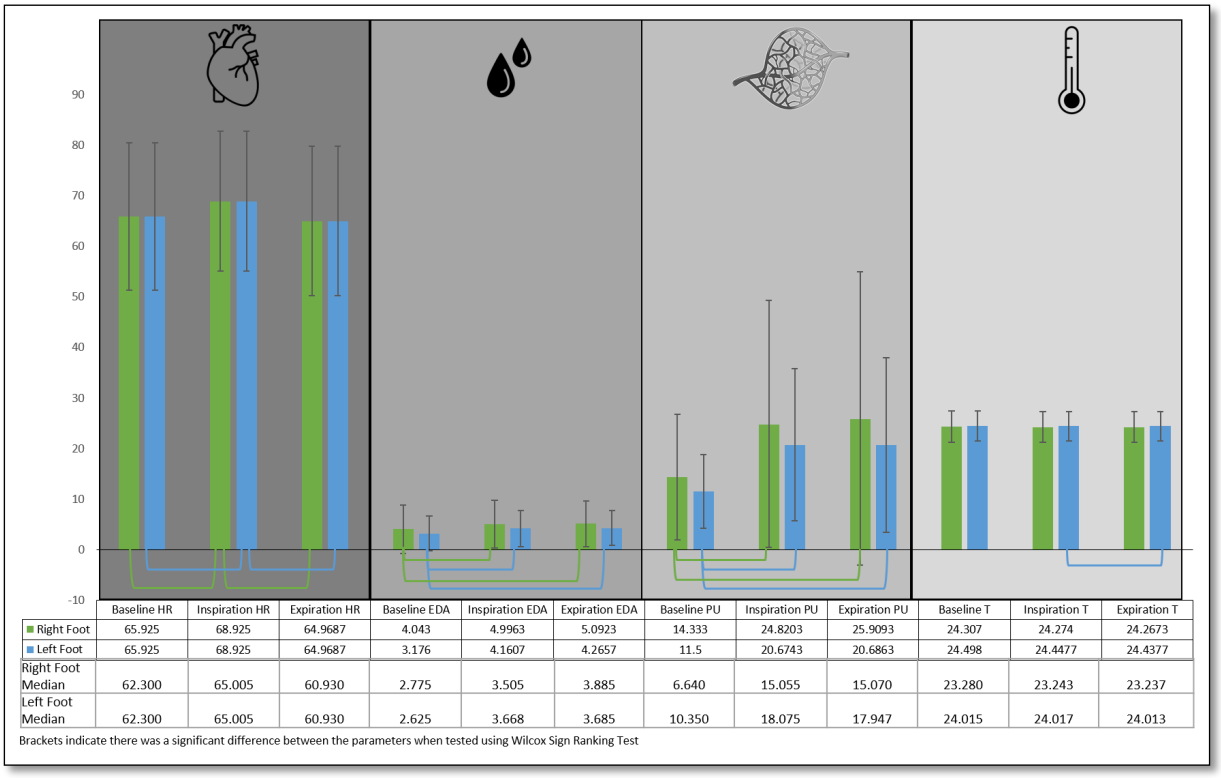
#### *5.4.1.3 Skin perfusion (PU):*

In the right foot, the group mean perfusion during inspiration was 24.82 PU (SD 24.41). Meanwhile, the mean perfusion during expiration was 25.91 PU (SD 29.07). Although the perfusion decreased with inspiration and increased during expiration, this was not statistically significant ($p >0.05$), the findings showed no significant difference when using Wilcoxon signed rank test pairwise comparison of related samples ($p > 0.05$). The findings were similar on the left foot as well. In the left foot, the group mean perfusion during inspiration and expiration was 20.67 PU (SD 14.99) and 20.69 PU (SD 17.26), respectively. The findings showed no significant difference between the perfusion measured in the left foot during inspiration and expiration, which was confirmed using Wilcoxon signed rank test pairwise comparison of related samples ($p > 0.05$). Nevertheless, Wilcoxon signed rank test results showed that there was a significant difference between the PU measured during inspiration vs baseline (right foot $z = --2.497$; $p = 0.013$; left foot $z = -2.803$; $p = 0.005$) and baseline vs expiration in both feet (right foot $z = -2.803$; $p = 0.005$; left foot $z = -2.803$; $p = 0.005$). These results are summarised in Figure 5.5 and Table 5.1.

#### *5.4.1.4 Skin temperature (T) at probe site:*

In the right foot, the group mean T during inspiration was 24.27°C (SD 3.06) and during expiration was 24.27°C (SD 3.05). In the left foot, the group mean T during inspiration was 24.45°C (SD 2.92) and during expiration was 24.44°C (SD 2.90). Wilcox Sign Ranking test no significant differences ($p > 0.05$) between

the measures during the three phases except for the T during expiration vs inspiration in the left foot (z = -2.033; p = 0.042) as shown in Figure 5.5 and Table 5.1.




| | Baseline HR | Inspiration HR | Expiration HR | Baseline EDA | Inspiration EDA | Expiration EDA | Baseline PU | Inspiration PU | Expiration PU | Baseline T | Inspiration T | Expiration T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Right Foot | 65.925 | 68.925 | 64.9687 | 4.043 | 4.9963 | 5.0923 | 14.333 | 24.8203 | 25.9093 | 24.307 | 24.274 | 24.2673 |
| Left Foot | 65.925 | 68.925 | 64.9687 | 3.176 | 4.1607 | 4.2657 | 11.5 | 20.6743 | 20.6863 | 24.498 | 24.4477 | 24.4377 |
| Right Foot Median | 62.300 | 65.005 | 60.930 | 2.775 | 3.505 | 3.885 | 6.640 | 15.055 | 15.070 | 23.280 | 23.243 | 23.237 |
| Left Foot Median | 62.300 | 65.005 | 60.930 | 2.625 | 3.668 | 3.685 | 10.350 | 18.075 | 17.947 | 24.015 | 24.017 | 24.013 |

Brackets indicate there was a significant difference between the parameters when tested using Wilcox Sign Ranking Test

*Figure 5.5 Mean of HR, EDA, PU and T with SD and Results from Pairwise Comparison using Wilcox Sign Ranking Test*

*Table 5.1 Wilcox Sign Ranking Test Comparing Parameters across Phases*

| | Right foot | | | Left foot | | |
|---|---|---|---|---|---|---|
| **Tests** | **Z** | **Effect size** | **Asymp. Sig. (2-tailed)** | **Z** | **Effect size** | **Asymp. Sig. (2-tailed)** |
| Mean HR during Inspiratory vs Baseline Phase | -1.988[b] | -0.445 | 0.047 | -1.988[b] | -0.445 | 0.047 |
| Mean HR during the Expiratory vs Inspiratory Phase | -2.395[c] | -0.536 | 0.017 | -2.395[c] | -0.536 | 0.017 |
| Mean HR during Baseline vs Expiratory Phase | -.357[b] | -0.080 | 0.721 | -.357[b] | -0.080 | 0.721 |
| Mean EDA during Inspiratory vs Baseline Phase | -2.666[b] | -0.596 | 0.008 | -2.803[b] | -0.627 | 0.005 |
| Mean EDA during the Expiratory vs Inspiratory Phase | -.059[b] | -0.013 | 0.953 | -.415[b] | -0.093 | 0.678 |
| Mean EDA during Baseline vs Expiratory Phase | -2.666[c] | -0.596 | 0.008 | -2.803[c] | -0.627 | 0.005 |
| Mean PU during Inspiratory vs Baseline Phase | -2.497[b] | -0.558 | 0.013 | -2.803[b] | -0.627 | 0.005 |
| Mean PU during the Expiratory vs Inspiratory Phase | -.561[c] | -0.125 | 0.575 | -.459[c] | -0.103 | 0.646 |
| Mean PU during Baseline vs Expiratory Phase | -2.803[c] | -0.627 | 0.005 | -2.803[c] | -0.627 | 0.005 |
| Mean T during Inspiratory vs Baseline Phase | -1.125[c] | -0.252 | 0.26 | -1.276[c] | -0.285 | 0.202 |
| Mean T during Expiratory vs Inspiratory Phase | -1.439[c] | -0.322 | 0.15 | -2.033[c] | -0.455 | 0.042 |
| Mean T during Baseline vs Expiratory Phase | -1.125[b] | -0.252 | 0.26 | -1.428[b] | -0.319 | 0.153 |

[a] Wilcoxon Signed Ranks Test

| | | | | | | |
|---|---|---|---|---|---|---|
| [b] Based on negative ranks. | | | | | | |
| [c] Based on positive ranks. | | | | | | |
| According to Cohen's classification of effect sizes which is 0.1 (small effect), 0.3 (moderate effect) and 0.5 and above (large effect) (Sullivan and Feinn, 2012). | | Small effect | | Moderate effect | | Large effect |

### 5.4.2 Correlation tests

The Spearman's correlation testing was used owing to the non-normal distribution of data. The test results showed that the PU and T were strongly correlated and were statistically significant ($p < 0.05$) in all phases (except for the Baseline phase in the right foot). On the right foot, the PU and T showed a very strong correlation during inspiratory phase (correlation coefficient = 0.855; $p = 0.002$) and expiratory phase (correlation coefficient = 0.818; $p = 0.004$). Furthermore, PU and T showed strong correlation during all three phases of baseline (correlation coefficient = 0.758; $p = 0.011$), inspiration (correlation coefficient = 0.782; $p = 0.008$) and expiration (correlation coefficient = 0.758; $p = 0.011$) in the left foot. The results of this correlation testing are tabulated in Table 5.2 and 5.3 below.

*Table 5.2 Right foot Spearman's correlation results*

| Variables | Tests | B PU | B T | B HR | B EDA RF` | Mean PU IN | Mean T IN | Mean HR IN | Mean EDA IN | Mean PU EX | Mean T EX | Mean HR EX | MEAN EDA EX |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| B PU | Correlation Coefficient | 1 | 0.6 | 0.164 | -0.2 | .818** | .636* | -0.467 | -0.261 | .867** | .636* | -0.03 | -0.152 |
| | Sig. (2-tailed) | . | 0.067 | 0.651 | 0.58 | 0.004 | 0.048 | 0.174 | 0.467 | 0.001 | 0.048 | 0.934 | 0.676 |
| B T | Correlation Coefficient | 0.6 | 1 | -0.103 | -0.333 | .806** | .988** | -0.455 | -0.442 | .770** | .988** | -0.467 | -0.382 |
| | Sig. (2-tailed) | 0.067 | . | 0.777 | 0.347 | 0.005 | <.001 | 0.187 | 0.2 | 0.009 | <.001 | 0.174 | 0.276 |
| B HR | Correlation Coefficient | 0.164 | -0.103 | 1 | 0.261 | -0.164 | -0.042 | .648* | 0.345 | -0.091 | -0.042 | .806** | 0.503 |
| | Sig. (2-tailed) | 0.651 | 0.777 | . | 0.467 | 0.651 | 0.907 | 0.043 | 0.328 | 0.803 | 0.907 | 0.005 | 0.138 |
| B EDA RF` | Correlation Coefficient | -0.2 | -0.333 | 0.261 | 1 | -0.503 | -0.358 | 0.176 | .939** | -0.406 | -0.358 | 0.358 | .855** |
| | Sig. (2-tailed) | 0.58 | 0.347 | 0.467 | . | 0.138 | 0.31 | 0.627 | <.001 | 0.244 | 0.31 | 0.31 | 0.002 |
| Mean PU IN | Correlation Coefficient | .818** | .806** | -0.164 | -0.503 | 1 | .855** | -0.6 | -0.612 | .976** | .855** | -0.406 | -0.527 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sig. (2-tailed) | 0.004 | 0.005 | 0.651 | 0.138 | . | 0.002 | 0.067 | 0.06 | <.001 | 0.002 | 0.244 | 0.117 |
| **Mean T IN** | Correlation Coefficient | .636* | .988** | -0.042 | -0.358 | .855** | 1 | -0.442 | -0.479 | .818** | 1.000** | -0.43 | -0.406 |
| | Sig. (2-tailed) | 0.048 | <.001 | 0.907 | 0.31 | 0.002 | . | 0.2 | 0.162 | 0.004 | . | 0.214 | 0.244 |
| **Mean HR IN** | Correlation Coefficient | -0.467 | -0.455 | .648* | 0.176 | -0.6 | -0.442 | 1 | 0.358 | -0.515 | -0.442 | .818** | 0.418 |
| | Sig. (2-tailed) | 0.174 | 0.187 | 0.043 | 0.627 | 0.067 | 0.2 | . | 0.31 | 0.128 | 0.2 | 0.004 | 0.229 |
| **Mean EDA IN** | Correlation Coefficient | -0.261 | -0.442 | 0.345 | .939** | -0.612 | -0.479 | 0.358 | 1 | -0.491 | -0.479 | 0.539 | .964** |
| | Sig. (2-tailed) | 0.467 | 0.2 | 0.328 | <.001 | 0.06 | 0.162 | 0.31 | . | 0.15 | 0.162 | 0.108 | <.001 |
| **Mean PU EX** | Correlation Coefficient | .867** | .770** | -0.091 | -0.406 | .976** | .818** | -0.515 | -0.491 | 1 | .818** | -0.261 | -0.406 |
| | Sig. (2-tailed) | 0.001 | 0.009 | 0.803 | 0.244 | <.001 | 0.004 | 0.128 | 0.15 | . | 0.004 | 0.467 | 0.244 |
| **Mean T EX** | Correlation Coefficient | .636* | .988** | -0.042 | -0.358 | .855** | 1.000** | -0.442 | -0.479 | .818** | 1 | -0.43 | -0.406 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sig. (2-tailed) | 0.048 | <.001 | 0.907 | 0.31 | 0.002 | . | 0.2 | 0.162 | 0.004 | . | 0.214 | 0.244 |
| **Mean HR EX** | Correlation Coefficient | -0.03 | -0.467 | .806** | 0.358 | -0.406 | -0.43 | .818** | 0.539 | -0.261 | -0.43 | 1 | .648* |
| | Sig. (2-tailed) | 0.934 | 0.174 | 0.005 | 0.31 | 0.244 | 0.214 | 0.004 | 0.108 | 0.467 | 0.214 | . | 0.043 |
| **MEAN EDA EX** | Correlation Coefficient | -0.152 | -0.382 | 0.503 | .855** | -0.527 | -0.406 | 0.418 | .964** | -0.406 | -0.406 | .648* | 1 |
| | Sig. (2-tailed) | 0.676 | 0.276 | 0.138 | 0.002 | 0.117 | 0.244 | 0.229 | <.001 | 0.244 | 0.244 | 0.043 | . |

B - Baseline; IN – Inspiratory phase; EX – Expiratory phase; PU – Perfusion; T – Temperature; EDA – Electrodermal activity; HR – Heart Rate

*Table 5.3 Left foot Spearman's correlation results*

| Variables | Tests | B PU | B T | B HR | B EDA LF | Mean PU IN | Mean T IN | Mean HR IN | Mean EDA IN | Mean PU EX | Mean T EX | Mean HR EX | Mean EDA EX |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B PU** | Correlation Coefficient | 1 | .758* | -0.212 | -0.333 | .855** | .758* | -0.6 | -0.358 | .903** | .758* | -0.358 | -0.43 |
| | Sig. (2-tailed) | . | 0.011 | 0.556 | 0.347 | 0.002 | 0.011 | 0.067 | 0.31 | <.001 | 0.011 | 0.31 | 0.214 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B T** | Correlation Coefficient | .758* | 1 | -0.358 | -0.333 | .782** | 1.000** | -0.527 | -0.418 | .758* | 1.000** | -.648* | -0.503 |
| | Sig. (2-tailed) | 0.011 | . | 0.31 | 0.347 | 0.008 | . | 0.117 | 0.229 | 0.011 | . | 0.043 | 0.138 |
| **B HR** | Correlation Coefficient | -0.212 | -0.358 | 1 | 0.248 | -0.491 | -0.358 | .648* | 0.273 | -0.455 | -0.358 | .806** | 0.297 |
| | Sig. (2-tailed) | 0.556 | 0.31 | . | 0.489 | 0.15 | 0.31 | 0.043 | 0.446 | 0.187 | 0.31 | 0.005 | 0.405 |
| **B EDA LF** | Correlation Coefficient | -0.333 | -0.333 | 0.248 | 1 | -0.564 | -0.333 | 0.236 | .988** | -0.455 | -0.333 | 0.297 | .976** |
| | Sig. (2-tailed) | 0.347 | 0.347 | 0.489 | . | 0.09 | 0.347 | 0.511 | <.001 | 0.187 | 0.347 | 0.405 | <.001 |
| **Mean PU IN** | Correlation Coefficient | .855** | .782** | -0.491 | -0.564 | 1 | .782** | -0.576 | -0.612 | .976** | .782** | -0.515 | -.661* |
| | Sig. (2-tailed) | 0.002 | 0.008 | 0.15 | 0.09 | . | 0.008 | 0.082 | 0.06 | <.001 | 0.008 | 0.128 | 0.038 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Mean TIN** | Correlation Coefficient | .758* | 1.000** | -0.358 | -0.333 | .782** | 1 | -0.527 | -0.418 | .758* | 1.000** | -.648* | -0.503 |
| | Sig. (2-tailed) | 0.011 | . | 0.31 | 0.347 | 0.008 | . | 0.117 | 0.229 | 0.011 | . | 0.043 | 0.138 |
| **Mean HR IN** | Correlation Coefficient | -0.6 | -0.527 | .648* | 0.236 | -0.576 | -0.527 | 1 | 0.2 | -0.576 | -0.527 | .818** | 0.273 |
| | Sig. (2-tailed) | 0.067 | 0.117 | 0.043 | 0.511 | 0.082 | 0.117 | . | 0.58 | 0.082 | 0.117 | 0.004 | 0.446 |
| **Mean EDA IN** | Correlation Coefficient | -0.358 | -0.418 | 0.273 | .988** | -0.612 | -0.418 | 0.2 | 1 | -0.503 | -0.418 | 0.321 | .988** |
| | Sig. (2-tailed) | 0.31 | 0.229 | 0.446 | <.001 | 0.06 | 0.229 | 0.58 | . | 0.138 | 0.229 | 0.365 | <.001 |
| **Mean PU EX** | Correlation Coefficient | .903** | .758* | -0.455 | -0.455 | .976** | .758* | -0.576 | -0.503 | 1 | .758* | -0.455 | -0.552 |
| | Sig. (2-tailed) | <.001 | 0.011 | 0.187 | 0.187 | <.001 | 0.011 | 0.082 | 0.138 | . | 0.011 | 0.187 | 0.098 |
| **Mean T EX** | Correlation Coefficient | .758* | 1.000** | -0.358 | -0.333 | .782** | 1.000** | -0.527 | -0.418 | .758* | 1 | -.648* | -0.503 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sig. (2-tailed) | 0.011 | . | 0.31 | 0.347 | 0.008 | . | 0.117 | 0.229 | 0.011 | . | 0.043 | 0.138 |
| **Mean HR EX** | Correlation Coefficient | -0.358 | -.648* | .806** | 0.297 | -0.515 | -.648* | .818** | 0.321 | -0.455 | -.648* | 1 | 0.394 |
| | Sig. (2-tailed) | 0.31 | 0.043 | 0.005 | 0.405 | 0.128 | 0.043 | 0.004 | 0.365 | 0.187 | 0.043 | . | 0.26 |
| **Mean EDA EX** | Correlation Coefficient | -0.43 | -0.503 | 0.297 | .976** | -.661* | -0.503 | 0.273 | .988** | -0.552 | -0.503 | 0.394 | 1 |
| | Sig. (2-tailed) | 0.214 | 0.138 | 0.405 | <.001 | 0.038 | 0.138 | 0.446 | <.001 | 0.098 | 0.138 | 0.26 | . |

B - Baseline; IN – Inspiratory phase; EX – Expiratory phase; PU – Perfusion; T – Temperature; EDA – Electrodermal activity; HR – Heart Rate

### 5.4.3 Partial correlation

As a next step, in order to check if the correlation between these two variables were influenced by the changes in HR and EDA, partial correlation tests were conducted. Preliminary assessments were performed to ensure no violation of the assumptions of normality and linearity. Also, as recommended in the literature the regression analysis was carried out (next step) to identify the predictor variable for PU, which is discussed later. The Table 5.4 and 5.5 summarises the results from partial correlation analysis.

#### *5.4.3.1 Right foot:*

- **In the baseline phase,** firstly, there was a strong, positive partial correlation between PU and T when controlling for HR, (r = 0.8, n = 10, p 0.010). Secondly, there was a strong, positive partial correlation between PU and T when controlling for EDA, (r = 0.843, n = 10, p 0.004). Finally, there was a strong, positive partial correlation between PU and T when controlling for both HR and EDA (r = 0.841, n = 10, p 0.009). An inspection of the zero-order correlation coefficient (r = 0.810, n = 10, p 0.005) suggested that controlling for HR and EDA, both individually and together had very little effect on the strength of the relationship between these two variables. Additionally, there was a strong, positive partial correlation between HR and EDA when controlling for PU (r = 0.766, n = 10, p 0.016), T (r = 0.722, n = 10, p 0.028) and PU and T (r = 0.750, n = 10, p 0.032). An inspection of the zero-order correlation coefficient (r = 0.750, n = 10, p 0.012) suggested that controlling for PU and T, both individually and together had very little effect on the strength of the relationship between these two variables.
- **In the inspiratory phase,** firstly, there was a strong, positive partial correlation between PU and T when controlling for HR, (r = 0.741, n = 10, p 0.022). Secondly, there was a strong, positive partial correlation between PU and T when controlling for EDA (r = 0.754, n = 10, p 0.019). Finally, it was observed that there was a strong, positive partial correlation between PU and T when controlling for HR and EDA (r = 0.841, n = 10, p 0.009). An inspection of the zero-order correlation coefficient (r = 0.790, n = 10, p 0.007) suggested that controlling for HR and EDA, both separately and together had very little effect on the strength of the relationship between these two variables. Additionally, there was a positive partial correlation between HR and EDA when controlling for PU (r = 0.696, n = 10, p 0.037) and when controlling for T (r = 0.709, n = 10, p 0.032). An inspection of the zero-order correlation coefficient (r = 0.752, n = 10, p 0.012) suggested that controlling for PU had very little effect on the strength of the relationship between these two variables.
- **During the expiratory phase,** there was a strong, positive partial correlation between PU and T when controlling for EDA (r = 0.676, n = 10, p 0.045). An inspection of the zero-order

correlation coefficient (r = 0.720, n = 10, p 0.019) suggested that controlling for EDA had very little effect on the strength of the relationship between these two variables. Additionally, there was a strong, positive partial correlation between HR and EDA when controlling for PU (r = 0.854, n = 10, p 0.003), T (r = 0.855, n = 10, p 0.003) and PU and T (r = 0.850, n = 10, p 0.007). An inspection of the zero-order correlation coefficient (r = 0.875, n = 10, p <0.001) suggested that controlling for PU and T, both individually and together had very little effect on the strength of the relationship between these two variables.

#### *5.4.3.2 Left foot:*

- **In the for the baseline phase,** there was a strong, positive partial correlation between PU and T when controlling for HR, (r = 0.932, n = 10, p <0.001), EDA (r = 0.930, n = 10, p <0.001), and HR and EDA, (r = 0.931, n = 10, p <0.001). An inspection of the zero-order correlation coefficient (r = 0.938, n = 10, p <0.001) suggested that controlling for HR and EDA, both separately and collectively had very little effect on the strength of the relationship between these two variables. Besides, there was a strong, positive partial correlation between HR and EDA when controlling for PU (r = 0.697, n = 10, p 0.037) and T (r = 0.680, n = 10, p 0.044). An inspection of the zero-order correlation coefficient (r = 0.731, n = 10, p 0.016) suggested that controlling for PU and T separately had very little effect on the strength of the relationship between these two variables.
- **In the inspiratory phase,** there was a strong, positive partial correlation between PU and T when controlling for HR (r = 0.833, n = 10, p 0.005), EDA (r = 0.829, n = 10, p 0.006), and HR and EDA (r = 0.825, n = 10, p 0.012). An inspection of the zero-order correlation coefficient (r = 0.868, n = 10, p 0.001) suggested that controlling for HR and EDA, both individually and together had very little effect on the strength of the relationship between these two variables. Furthermore, there was a strong, positive partial correlation between HR and EDA when controlling for T (r = 0.676, n = 10, p 0.046) and an inspection of the zero-order correlation coefficient (r = 0.742, n = 10, p 0.014) suggested that controlling for T had very little effect on the strength of the relationship between these two variables.
- **In the expiratory phase,** there was a strong, positive partial correlation between PU and T when controlling for HR (r = 0.749, n = 10, p 0.020), EDA (r = 0.745, n = 10, p 0.021), and HR and EDA (r = 0.748, n = 10, p 0.033). An inspection of the zero-order correlation coefficient (r = 0.804 n = 10, p 0.005) suggested that when HR and EDA were used as controls separately or together, they had no effect on the strength of the relationship between these two variables. Moreover, there was a strong, positive partial correlation between HR and EDA when controlling for PU (r = 0.810, n = 10, p 0.008), T (r = 0.816, n = 10, p 0.007), and PU and T (r =

0.812, n = 10, p 0.014). An inspection of the zero-order correlation coefficient (r = 0.855, n = 10, p = 0.002) suggested that controlling for PU and T had very little effect on the strength of the relationship between these two variables.

*Table 5.4 Partial correlation table for right foot*

| **Baseline Phase** | | | | |
|---|---|---|---|---|
| **Test variable 1** | Test variable 2 | Control variable(s) | Partial Correlation Coefficient r | p value |
| **PU** | Temp | HR | 0.800 | p 0.010 |
| | | EDA | 0.843 | p 0.004 |
| | | HR + EDA | 0.841 | p 0.009 |
| | | None | 0.810 | p 0.005 |
| **HR** | EDA | PU | 0.766 | p 0.016 |
| | | Temp | 0.722 | p 0.028 |
| | | PU + Temp | 0.750 | p 0.032 |
| | | None | 0.750 | p 0.012 |
| **Inspiratory Phase** | | | | |
| **Test variable 1** | Test variable 2 | Control variable(s) | Partial Correlation Coefficient r | p value |
| **PU** | Temp | HR | 0.741 | p 0.022 |
| | | EDA | 0.754 | p 0.019 |
| | | HR + EDA | 0.841 | p 0.009 |
| | | None | 0.790 | p 0.007 |
| **HR** | EDA | PU | 0.696 | p 0.037 |
| | | Temp | 0.709 | p 0.032 |
| | | PU + Temp | 0.695 | p 0.056 |
| | | None | 0.752 | p 0.012 |
| **Expiratory Phase** | | | | |
| **Test variable 1** | Test variable 2 | Control variable(s) | Partial Correlation Coefficient r | p value |
| **PU** | Temp | HR | 0.661 | p 0.053 |
| | | EDA | 0.676 | p 0.045 |

| | | | | |
|---|---|---|---|---|
| | | HR + EDA | 0.661 | p 0.075 |
| | | None | 0.720 | p 0.019 |
| **HR** | EDA | PU | 0.854 | p 0.003 |
| | | Temp | 0.855 | p 0.003 |
| | | PU + Temp | 0.850 | p 0.007 |
| | | None | 0.875 | p <0.001 |
| **Key** | | | | |
| **0.0 - 0.20** | Negligible correlation | | p > 0.05 | |
| **0.20 - 0.39** | Weak correlation | | | |
| **0.40 - 0.59** | Moderate correlation | | | |
| **0.60 - 0.79** | Strong correlation | | | |
| **0.80 - 1** | Very strong correlation | | | |

*Table 5.5 Partial correlation table for left foot*

| **Baseline Phase** | | | | |
|---|---|---|---|---|
| **Test variable 1** | Test variable 2 | Control variable(s) | Partial Correlation Coefficient r | p value |
| **PU** | Temp | HR | 0.932 | p <0.001 |
| | | EDA | 0.930 | p <0.001 |
| | | HR + EDA | 0.931 | p <0.001 |
| | | None | 0.938 | p <0.001 |
| **HR** | EDA | PU | 0.697 | p 0.037 |
| | | Temp | 0.680 | p 0.044 |
| | | PU + Temp | 0.680 | p 0.064 |
| | | None | 0.731 | p 0.016 |
| **Inspiratory Phase** | | | | |
| **Test variable 1** | Test variable 2 | Control variable(s) | Partial Correlation Coefficient r | p value |
| **PU** | Temp | HR | 0.833 | p 0.005 |
| | | EDA | 0.829 | p 0.006 |
| | | HR + EDA | 0.825 | p 0.012 |

| | | | | |
|---|---|---|---|---|
| | | None | 0.868 | p <0.001 |
| **HR** | EDA | PU | 0.646 | p 0.060 |
| | | Temp | 0.676 | p 0.046 |
| | | PU + Temp | 0.648 | p 0.082 |
| | | None | 0.742 | p 0.014 |
| **Expiratory Phase** | | | | |
| **Test variable 1** | Test variable 2 | Control variable(s) | Partial Correlation Coefficient r | p value |
| **PU** | Temp | HR | 0.749 | p 0.020 |
| | | EDA | 0.745 | p 0.021 |
| | | HR + EDA | 0.748 | p 0.033 |
| | | None | 0.804 | p 0.005 |
| **HR** | EDA | PU | 0.81 | p 0.008 |
| | | Temp | 0.816 | p 0.007 |
| | | PU + Temp | 0.812 | p 0.014 |
| | | None | 0.855 | p 0.002 |
| **Key** | | | | |
| **0.0 - 0.10** | Negligible correlation | | p > 0.05 | |
| **0.10 - 0.39** | Weak correlation | | | |
| **0.40 - 0.69** | Moderate correlation | | | |
| **0.70 - 0.89** | Strong correlation | | | |
| **0.90 - 1** | Very strong correlation | | | |

### 5.4.4 Regression analysis

As a final step, in order to identify the best predictor of PU, a multiple regression analysis was conducted separately for both feet. A standard multiple regression was run to predict PU from T, HR and EDA. Only T statistically significantly predicted PU in both feet. Of the three independent parameters only T contributed to the model significantly. In the right foot, the baseline phase: [F (3, 6) = 5.649, p < .0035, $R^2$ = 0.739, Coefficient table p = 0.009]. Although the ANOVA results in the inspiratory phase showed no statistical significance the individual coefficient table showed significant results for T as a predictor [F (3, 6) = 3.662, p < .083, $R^2$ = .647, Coefficient table p = 0.036]. In the expiratory phase, the ANOVA and the individual coefficient did not show any statistical significance [F

(3, 6) = 2.302, p < .177, $R^2$ = .535, Coefficient table p = 0.075]. Furthermore, in the left foot, T significantly contributed to the prediction of PU for all three phases and T was the only independent predictor of PU: baseline [F (3, 6) = 15.302, p < .0032, R2 = 0.884, coefficient table p = <0.001], inspiratory [F (3, 6) = 7.215, p < .020, $R^2$ = 0.783, coefficient table p = 0.012] and expiratory [F (3, 6) = 4.274, p < .062, R2 = 0.681, coefficient table p = 0.033].

**Coefficients[a]**

| Model | | Unstandardized Coefficients | | Standardized Coefficients | | | 95.0% Confidence Interval for B | | Correlations | | | Collinearity Statistics | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | t | Sig. | Lower Bound | Upper Bound | Zero-order | Partial | Part | Tolerance | VIF |
| 1 | (Constant) | -58.676 | 29.822 | | -1.968 | .097 | -131.648 | 14.296 | | | | | |
| | B_TEMP | 3.407 | .893 | .850 | 3.815 | .009 | 1.221 | 5.592 | .810 | .841 | .796 | .877 | 1.140 |
| | B_HR | -.217 | .275 | -.254 | -.788 | .461 | -.889 | .456 | -.226 | -.306 | -.165 | .421 | 2.375 |
| | B_EDA_RF` | 1.109 | .821 | .428 | 1.351 | .225 | -.899 | 3.117 | -.017 | .483 | .282 | .435 | 2.298 |

a. Dependent Variable: B_PU

*Figure 5.6 Right Foot Baseline Prediction Model*

By using the model and substituting the data in Figure 5.5 for Baseline values in the regression equation using the B constant value -58.676 in Figure 5.6 the PU can be predicted as below.

- Baseline predicted PU calculated using in the equation -58.676 + (3.4 * T) – (0.217 * HR) + (1.109 * EDA) = -58.676 + (3.4 * 24.31) – (0.217 * 65.92) + (1.109 * 4.04) = 14.15
- This calculated (Predicted) PU as per the model and by using regression equation is approximately the Baseline PU value of 14.33 in Figure 5.5.
- Thus, the prediction model can be used to calculate predicted PU values based on T.

## 5.5 Discussion

The purpose of this study was to understand the relationship between skin microcirculation and autonomic functions of the small fibre nerves such as thermoregulation, heart rate and electrodermal activity in the foot. The results of the study indicate that there is a robust association between skin microcirculation and temperature. The initial inspection of the scatterplot showed a monotonic relationship between the two. A monotonic relationship is a relationship such that as the value of one parameter increases, the value of the other parameter also increases, or as the value of one parameter increases, the value of the other parameter decreases. The Spearman's rho correlation analysis determined the degree to which a relationship is monotonic, the strength and direction of a monotonic relationship. The results from the correlation analysis that showed that PU and T were very strong and positively correlated during inspiratory phase (correlation coefficient $r_s$ = 0.855; p = 0.002) and expiratory phase (correlation coefficient $r_s$ = 0.818; p = 0.004) in the right foot and strong and

positively correlated during baseline (correlation coefficient $r_s$ = 0.758; p = 0.011), inspiration (correlation coefficient $r_s$ = 0.782; p = 0.008) and expiration (correlation coefficient $r_s$ = 0.758; p = 0.011) in the left foot. These results imply that when the skin perfusion increases the skin temperature increases and vice-versa. Also, it indicates that when the skin perfusion decreases the skin temperature decreases and the other way around too. This is in line with the mechanism explained in the literature that the sympathetic vasoconstrictor system is known to be active in thermoneutral environments and is responsible for the small changes in skin blood flow that maintain normal body temperature with slight changes in activity or environmental temperature (Johnson *et al.*, 1986; Pergola *et al.*, 1994). The findings are also in line with studies which have explored the relationship of skin perfusion and temperature (Stoner *et al.*, 1991; Li, Zhang and Yi, 2002). Skin microcirculation is involved with the nutrition supply as well as thermoregulatory mechanisms (Bharara, Cobb and Claremont, 2006). The latter is responsible for the maintenance of body temperature. The use of thermal techniques to evaluate various pathological conditions such as diabetic foot, cerebral injury, Raynaud's mechanisms, dental issues, fibromyalgia and many more. The application of thermal techniques for the early detection and prevention of diabetic foot complications such as ulcers has largely remained a key area of research interest. However, the literature highlights that there is no comprehensive understanding of the importance of small local temperature variations in the lower extremities, particularly in patients with diabetic neuropathy (Bharara, Cobb and Claremont, 2006). Whilst there are studies that have explored the vasoreflex (neurogenic) aspects of microcirculation which mediates microcirculatory response during rapid-heating and the Nitric-Oxide (NO) aspects which mediates microcirculatory response during slow local heating, there is very little known about the variations that occur due to thermoregulatory mechanisms in a thermoneutral environment (Vas and Rayman, 2011; Dawson *et al.*, 2015). The latter is worth investigating in order to identify any inflammatory signs in the foot (G. Balasubramanian, Chockalingam and Naemi, 2021; Balasubramanian, Chockalingam and Naemi, 2021). Moreover, studies have highlighted that while local heating of the probes to 33°C is an additional option besides maintaining a thermoneutral space, it is less physiologically accurate, so skin temperature should be recorded instead (Cracowski *et al.*, 2006; Barwick, Lanting and Chuter, 2015). This study helped to address the gap in literature and provided insights regarding the relationship between microcirculation and temperature in a thermoneutral environment in the foot. Additionally, as autonomic functions such as heart rate and sweating may influence blood flow and temperature, the effects of these were studied (Inoue and Shibasaki, 1996; Charkoudian, 2003). The partial correlation analysis (Table 5.4 and 5.5) showed that PU and T had a strong to a very strong positive correlation which was significant ($p < 0.05$) and controlling for HR and EDA had very little effect on the relationship between PU and T in all the three-

phased in both feet (the only exception being in the right foot the results when controlling for HR and both HR and EDA during expiratory phase showed to be insignificance $p > 0.05$). The reason why the HR and EDA had very little effect on the P and T could be due to the fact that the participants were in an environment with ambient temperature with resting blood flow. The resting skin blood flow is approximately 250 mL/min resulting in heat dissipation of approximately 80 to 90 kcal/h, which is nearly the level of resting metabolic heat production (Charkoudian, 2003). When there is an increased physiological demand such as during exercise, physical stimuli like exposure to heat or psychological/emotional stress trigger cutaneous vasodilation and sweating (Johnson, 1998; Kamei et al., 1998; Charkoudian, 2003; Yano, Sone and Yamazaki, 2009; Harker, 2013). Based on the demands, larger increases require increased cardiac output and redistribution of blood flow from the splanchnic region (the abdominal gastrointestinal organs, which receives blood supply through splanchnic circulation), that demonstrate vasoconstriction (Charkoudian, 2003). These are adaptive changes that occur in the body to meet the demand for increased skin blood flow so that the oxygen supply to organs such as the heart is not compromised (Charkoudian, 2003). In this study, the environment was at ambient temperature and there were no additional stimuli used except for deep slow breathing, which was inspiration and expiration for 10 seconds each in such a way that there are 6/minute. Slow breathing relaxes an individual and RSA is present, which was observed in this study. The HR increased during inspiration (mean = 68.93; SD = 13.87) and decreased with expiration (mean = 64.97; SD = 14.77) as shown in Figure 5.4. Moreover, the Wilcox Sign Ranking test showed a significant difference ($p < 0.05$) between the two (Figure 5.4 and Table 5.1). The HR percentage increase from baseline to inspiration phase was 4.55%. The observed HR percentage decrease from inspiration to expiration phase was 5.74% and from baseline to expiration phase was 1.45%. The HR variation, calculated by taking the difference between the averages HR during inspiration from the average HR during expiration, showed a group mean of 4 bpm (SD 3.25; range 8.55). According to literature, HR variation greater than 15 beats per minute (bpm) is considered normal, less than 10 bpm is abnormal in younger patients (<40 years) and less than 5 bpm is abnormal at any age (Phillips and Donofrio, 2009). The less than 10 bpm variation in young people (<40 years) observed in this study may be due to the fact that the breathing was not deep enough to see such variation and the breathing depth could not be controlled. Some participants may have overbreathed whereas others could have under breathed at the same breathing rate. As previous literature suggests this may have potentially resulted in individual shifts in the carbon dioxide concentration that are likely to differently affect the circulation in different subjects as excessive lung inflation changes the effect of respiration on sympathetic neural activity (Phillips and Donofrio, 2009; De Burgh Daly, 2011). Furthermore, the current study did not use ant device guided breathing session such as the use of RESPeRATE device for guided slow breathing,

which could be recommended for future study in the area. Nonetheless, the derived insights on the HR have aided to accomplish the purpose of the study was to understand the autonomic function dynamics (sympathetic neural activity) and its relationship with microcirculation. Whilst the HR synchronised with breathing, there were no variations in the EDA on either of the foot in the different phases. This may be due to the fact that in a thermoneutral environment and by the application of slow breathing techniques the participants may have been in a relaxed state, which may have resulted in no or very minor fluctuations in the EDA. This can be substantiated by the existing evidence that slow breathing calms an individual (Russo, Santarelli and O'Rourke, 2017). Finally, the standard regression analysis showed that temperature was a predictor of microcirculation. Finally, the standard regression analysis showed that temperature was a predictor of microcirculation. This finding is consistent with studies have shown that when there was an increase in perfusion, an increase in the temperature was observed and the other way round too (Inoue and Shibasaki, 1996; Nakamoto et al., 2012; Balasubramanian et al., 2020; Balasubramanian, Chockalingam and Naemi, 2021). As highlighted earlier, the application of thermal techniques to evaluate diabetic foot is a constant area of research focus to determine a foot at risk. This is valuable as it may not always need expensive equipment like perfusion measurement, which may be convenient to use for mass screening and resource limited settings. Temperature measurements may be potentially used to indicate microcirculatory dysfunction or small fibre nerve dysfunctions. A study, which established the differences in thermographic patterns between the various complications in diabetic foot disease showed that the temperature measured at the feet and toes were significantly higher in the group with complications than in healthy adults and healthy groups with diabetes mellitus (Gatt *et al.*, 2018). It was also observed that higher the temperature of the foot in people with diabetes mellitus, the higher the probability that it was affected by neuropathy, neuroischaemia or peripheral arterial disease, which was confirmed using logistic regression models (Gatt *et al.*, 2018). Furthermore, outside of the area of diabetes, it was observed that when the perfusion measured above tender points in people with fibromyalgia using LDF, when the skin perfusion decreased, the temperature measured using infrared thermometer decreased as well (Jeschonneck *et al.*, 2000). The found that vasoconstriction occurs in the skin above tender points in people with fibromyalgia supporting the hypothesis that fibromyalgia is related to local hypoxia in the skin above tender points (Jeschonneck *et al.*, 2000). This finding is useful and relevant to diabetic foot as well because literature suggests that prolonged glycation causes damage to the microvasculature that supply nerves, which causes hypoxia to the nerves, thereby, damaging the nerves resulting in neuropathy (Singh et al., 2014). Therefore, the findings of the current study may have practical implications when assessing the foot at risk in

people with diabetes using temperature as the results have implied a strong relationship between temperature and microcirculation.

Elevated temperatures under the foot combined with reduced or complete loss of sensation may predispose the patient to foot ulcers (Bagavathiappan *et al.*, 2010). Previous studies have shown that the temperature in affected regions with vascular disease was either low due to occluded arteries in the extremities or 0.7 to 1 °C higher than normal due to inflammation and changes in venous flow (Bagavathiappan *et al.*, 2009). The study showed that thermal imaging results correlated well with clinical findings and had high reliability. It was found that the areas of higher temperature contrast were not discernible on clinical examination. It is suggested that the contralateral foot temperature differences of 2.2℃ can be considered as an "hot spot", which needs attention (van Netten *et al.*, 2014; Jones *et al.*, 2020). This underscores the need for further research examining temperature variations in relation to various vascular parameters to understand the underlying mechanisms, which may aid in diagnosis when not evident through clinical examination. The current study helped fill this gap by demonstrating a strong positive correlation between temperature and perfusion. Further studies in this area examining temperature variations in relation to microcirculation in people with diabetes will help better understand the predictive or prognostic nature of this assessment. A similar study showed that thermal imaging aids in the detection of diabetic neuropathy and therefore may be a useful additional tool for the assessment of high-risk diabetic feet (Bagavathiappan *et al.*, 2010). The results from the study showed that people with diabetic neuropathy (VPT values on biothesiometry greater than 20 V) had a higher foot temperature (32–35 °C) compared to those without neuropathy (27–30 °C).

The foot in people with diabetic neuropathy is considered as warm with palpable pulses and distended veins, indicating increased blood flow in the affected limb (Bagavathiappan *et al.*, 2010). Sympathetic autonomic neuropathy in the lower extremity results in reduced sweating and dry skin, and in the absence of peripheral vascular disease, increased blood flow, arteriovenous shunt, and the warm foot (Boulton, 2000). In contrast, the clinical symptoms of diabetic neuropathy in the early stages can present with positive symptoms such as pain, hypersensitivity, tingling, cramps and cold feet (Hidmark *et al.*, 2014). Cutaneous microcirculation may be reduced or there may be diminished neurovascular response in people with diabetes (Balasubramanian *et al.*, 2020). Impaired neurovascular response in people with diabetic neuropathy results in a significant reduction of blood flow under demands owing to injury or infection (Hile and Veves, 2003; Bagavathiappan et al., 2010; Balasubramanian, Chockalingam and Naemi, 2021b). All of these studies highlight that temperature measurement can be an additional tool to identify a vulnerable foot. Most of these only focused on the macrovascular or neuropathy-related aspects, but not on the microcirculation. However, microcirculation is

important for tissue health and meeting local needs in injury and inflammation. People with diabetes can be vulnerable to acute and critical limb ischemia, resulting in significant morbidity and death rates that must be promptly identified and treated to avoid amputation (Shishehbor, 2014). Assessment of skin perfusion using multiple methods is recommended to be considered for revascularisation in order to restore blood perfusion, when time is limb (Shishehbor, 2014). However, microcirculation assessment may not always be feasible due to lack of time or resources. Equipment for non-invasive microcirculation measurement such as LDF or LSCI can be expensive, although many inexpensive smartphone-based and webcam-based laser speckle imagers are in ongoing research (Heeman et al., 2019; Balasubramanian *et al.*, 2020). Frequent monitoring of temperature as an indirect measurement for microcirculation can be helpful to monitor perfusion over time to identify inflammatory signs or a foot at risk of ulceration or vascular issues. Devices for measuring temperature are relatively inexpensive than those of microcirculation. Infrared thermographic cameras are capable of capturing the temperature of multiple locations in the extremity in a short time (Sroczyński, Bresler and Cinciała, 1989; Bagavathiappan *et al.*, 2010; Gatt *et al.*, 2018). Therefore, it can be useful for mass screenings and in time- and resource-constrained environments. In addition, the measurement of the foot temperature is not complex and can be carried out easily. People with diabetes can also be trained to monitor foot temperature at home. Studies have found that measuring foot temperature at home has helped reduce the occurrence of foot ulcers, but mere monitoring would not be enough; appropriate measures such as reducing ambulatory activity can help (Lavery *et al.*, 2004; Bus *et al.*, 2021). With a better understanding of temperature variations in relation to microcirculation, leading to better diagnosis and treatment, unwanted complications such as ulcers and amputations can be reduced. Patient education and empowerment using self-management tools can promote the prevention of adverse foot-related complications. The current study has facilitated development in this direction.

According to the literature, this increase in temperature in the foot could be explained by autolysis that occurs preceding an imminent ulcer (Armstrong et al., 1997; Bus et al., 2021b). Tissues of the body often shrink to a smaller size (as in muscles during long periods of inactivity) or autolysis occurs, removing damaged cells or damaged cell parts from tissues (Hall and Hall, 2021). Lysosomes are responsible for much of this regression and autolysis (Hall and Hall, 2021). The damage to the cell can occur due to factors such as heat, cold, trauma, chemicals, or others that cause lysosomes to rupture (Hall and Hall, 2021). The released hydrolases immediately begin to digest the surrounding organic matter. In the case of minor damage, only part of the cell is removed, and the cell is then repaired (Hall and Hall, 2021). If the damage is severe, the entire cell is digested, a process called autolysis replacing the old one with a new cell of the same type, usually by mitotic reproduction of an adjacent cell (Hall and Hall, 2021). In addition, an increase in temperature accelerates autolysis (Olakanye and

Ralebitso-Senior, 2018). Therefore, temperature monitoring and timely action when hot spots appear can help prevent an ulcer as suggested by Bus et al. (2021). Although current guidelines suggest temperature monitoring, it is not widely implemented (Schaper *et al.*, 2019; Bus *et al.*, 2021). The results of this study underscore the potential use of foot temperature measurements as a surrogate measure of microcirculation and temperature rise due to underlying cellular mechanisms such as inflammation or autolysis, which may be able to prevent pathological tissue damage leading to open ulcers.

## 5.6 Conclusion

This study was conducted to assess the relationship between microcirculation and autonomic functions of the small nerve fibres. PU, T, HR and EDA were measured. The results from the study showed that there is a relationship between microcirculation (PU) and autonomic thermoregulatory functions of the foot (T). Other autonomic physiological measures such as the HR and EDA only had little effects on this relationship. First, this research has contributed to the understanding of the relationship between microcirculation and temperature. Second, the practical implications of these results could be that temperature can be an indicator of perfusion-related changes in the skin due to inflammation, injury, or tissue autolysis, thus potentially using thermal techniques to assess foot risk in people with diabetes. Third, measuring foot temperature can be a simple and less expensive means of assessing foot risk, which can be particularly helpful in settings with limited time and resources, for mass screening and self-management. Finally, although foot temperature measurement is included in international guidelines, it is not currently widely implemented, possibly because more research is awaited to support this in practice. This study has provided evidence for the use of foot temperature measurement reflecting cutaneous microcirculation. This, in turn, can prevent adverse complications such as ulcers and amputations.

# CHAPTER VI

CONCLUSION

# Chapter 6 Conclusion

## 6.1 Introduction

In this final conclusion chapter, the key insights generated through this PhD research along with its strengths and limitations are discussed. The practical applications of the finding are highlighted. Overall, this PhD research was conducted with the vision of contributing to the solution of one of the main problems related to foot complications in diabetes, the diabetic foot ulcer.

## 6.2 Summary of Key findings

The current comprehensive assessment of the foot does not include microcirculatory and small fibre nerve assessments. There are many reasons for this. Firstly, there was a lack of understanding of the relationship between the two and their role in tissue injury, inflammation and diabetic foot ulceration. Secondly, excessively long times are needed for a such comprehensive examination of the foot that is inclusive of microcirculation and small fibre nerve function assessments. For example, for decades, micromyography has been considered the gold standard method for assessing microvascular structural changes by measuring the media-lumen ratio (MLR) or wall-lumen ratio (WLR) of subcutaneous small vessels dissected from tissue biopsies. However, the use of invasive methods is not always convenient, and the literature has highlighted the need for further research to move toward the use of non-invasive bench-to-bedside assessment. Laser Doppler flowmetry of microvascular function and structure is one of such assessment, which is currently lacking robust evidence for its prognostic value, that limits its widespread use in daily clinical practice (Rizzoni *et al.*, 2022). Thirdly, depending upon the resource availability, generally equipment to measure microcirculation and small fibre nerve functions are expensive technology making it unattractive for clinical practice.

### 6.2.1 Key findings

#### *6.2.1.1 There is a relationship between microcirculation and small fibre nerve functions.*

The literature review (Chapter 1) established that microcirculation and functions of the small fibre neuropathy influence each other. Also, microcirculation plays a major role in tissue injury and inflammation, which may, in turn, contribute to ulceration. The temperature at or nearer to injury and inflammation usually increases (cardinal signs of inflammation). Furthermore, the review aided to understand the link between temperature, which is mediated by the small fibre nerves and the corresponding changes to the microcirculation. This laid a foundation to develop effective and less time-consuming protocols to measure microcirculation in the foot that can be clinically viable. Since microcirculatory measurements require expensive

equipment and can be complex to measure in resource-limited settings and for mass screening, temperature, which is relatively easier to measure, was used. This, in turn, aided to explore the prospective of using temperature as surrogate measure for microcirculation in the foot.

#### 6.2.1.2 *Microcirculation of the foot can be assessed systematically and reliably using PORH test with 30 seconds occlusion time*

Simple tests such as PORH can help assess the physiological function of microvessels systematically and reliably in the foot using a protocol with an occlusion time as short as 30 seconds. The vasodilating capacity of the microvessels, which is also influenced by the sensory small nerve fibres, can be measured and compared at the same place with and without a temperature control protocol (Chapter 4).

#### 6.2.1.3 *There is a strong relationship between cutaneous microcirculation and foot skin temperature, with temperature being an independent predictor of microcirculation.*

Finally, in Chapter 5, it was shown that the autonomic nerve fibres and microcirculation influence each other with attest using a deep breathing technique. It was established that the cutaneous microcirculation and foot temperature have a strong relationship and other parameters such as skin electrodermal activity and heart rate had very little effect on them. In particular, skin temperature was an independent predictor of cutaneous microcirculation that can be used as a surrogate measure of microcirculation, which may be useful in clinical practice, especially in an environment with limited time and resources. This also emphasises that foot temperature measurement may be significant in the prevention and early diagnosis of diabetic foot ulcer incidents. The next section summarises the key contributions of this doctoral research to the field, discussing these key findings in light of some of the key real-world challenges in managing diabetic foot-related problems and how this thesis can potentially help to overcome them.

## 6.3 Contribution of this Research to the Field

Although this current research used healthy participants as a proof-of-concept, the findings suggest that the measurements of cutaneous microcirculation and foot temperature can help understand the physiology and underlying mechanisms in the presence of stimuli. Further studies are required, especially to understand the outcomes of these measurements in the presence of pathology like diabetes. However, from the gathered information through the literature review and the insights derived from the primary research data, the below section focuses on the current problems in the area of diabetic foot and how evidence from studies like the current one can be used in the future to

address some of these problems. The intention here is to suggest potential benefits in this area of research and not to make any clinical recommendations such as the temperature range, using the data to conduct foot screening for diabetes or for self-diagnosis.

### 6.3.1 An exposition of causes of nerve damage in people with type 2 diabetes

#### *6.3.1.1 Problem*

As highlighted earlier diabetes-related complications continue to be key areas in research. The priority setting partnerships have listed identifying the causes of nerve damage in people with type 2 diabetes, who is most affected, raising awareness of the issue, and how best to prevent and treat it as one of their top ten priorities (James Lind Alliance, 2022a). Therefore, there is a need for further research to elucidate the key mechanisms behind nerve damage in people with type 2 diabetes.

#### *6.3.1.2 Solution*

This research has contributed to this area by generating knowledge and understanding of the mechanisms of nerve damage, particularly small fibre nerves. One of the main causes of nerve damage is impaired microcirculation, as nerve hypoxia occurs when nerve oxygenation is impaired (the oxygen supply to them is affected) due to damage to microvessels caused by glycation (Singh et al., 2014; Balasubramanian et al., 2020). The comprehensive literature review in Chapter 1 conducted as part of this research highlighted the neurovascular interactions that emphasise the influence of microcirculation on nerve functions and vice versa. The function of the small fibre function and its role in mediating microcirculatory responses was investigated through selected assays that are non-invasive and less time-consuming. A deeper understanding of the neurogenic and vascular components of the responses has been achieved. Therefore, the assessment methods discussed in this review such as PORH, PIV and vasodilation to local heating can help to assess the neurovascular aspects simultaneously in routine practice. In Chapter 1, the role of microcirculation played in tissue injury and inflammation was established. Microcirculation and neurovascular functions play a protective role in the human body. In particular, microcirculation plays important homeostatic and defensive roles during tissue injury and inflammation. Impaired microcirculation, which also alters nerve functions, may be the missing link in the chain of events that lead to foot ulcers in people with diabetes. This knowledge was disseminated through publication in a peer-reviewed journal (Appendix 1 and 2) to raise awareness of the topic by reviewing existing literature and bringing new perspectives, and highlighting the areas that require attention, such as: the paucity of research that focuses on the relationship between microcirculation and small fibre nerve functions in the foot. These results are small steps toward understanding the mechanisms behind neurovascular responses, better

understanding them in the presence of glycation-related complexities, and preventing and treating them, as previously mentioned as one of the top ten priorities.

### 6.3.2 Unpacking the impact of poor foot health on people's lives

#### *6.3.2.1 Problem*

Feet are the foundation of our body and play an important role in leading a happy, healthy lifestyle. Each foot is an intricate structure made up of 26 bones, 33 joints, and over 100 muscles, tendons, and ligaments (Manganaro *et al.*, 2021). Its unique architecture allows the foot to a lot of force every day. It is estimated that healthy adults typically take between 4,000 and 18,000 steps per day (Tudor-Locke *et al.*, 2011). The foot helps with locomotive and weight-bearing activities like running, walking, skipping, jumping, dancing, low-impact aerobics, elliptical training machines, stair climbing and gardening. Therefore, Leonardo da Vinci rightly said, "The human foot is a masterpiece of engineering and a work of art." Considering the weight and stress that is put on feet every day, it is easy to perceive how people experience a foot-related problem at some point in life.

Foot health issues impact daily life. Foot pain is most common and is associated with female gender, older age and obesity (Menz *et al.*, 2010; Gates *et al.*, 2019). The literature shows that 8% of musculoskeletal consultations involve foot and ankle problems (Menz *et al.*, 2010). The effects are manifold in the areas of physical health, psychological and emotional well-being, social issues, work and finance. Foot pathologies negatively and adversely affect measures of health-related quality of life (López-López *et al.*, 2018, 2021; Reinoso-Cobo *et al.*, 2020). Foot problems contribute to reduced ability to perform activities of daily living, balance and gait problems and increased risk of falls (Menz, Morris and Lord, 2005, 2006). The skeletal system of ageing individuals tends to become fragile, reducing bone mineral density and various biomechanical aspects, including the interactions between traumatic loading and bone strength, determine bone resistance to fracture (Leali *et al.*, 2011). These foot problems can be exacerbated by pathological conditions such as diabetes, which has long been recognised as a cause of accelerated ageing (Morley, 2008). Diabetic foot is a common complication of diabetes with consequent disruption in patients' daily lives, and the co-existence of depression and anxiety in these patients is a common phenomenon (Ahmad *et al.*, 2018).

The role of the classic triad: neuropathy, peripheral vascular disease, and mechanical trauma is well established in the literature. However, the sequence of events leading to an ulcer is labyrinthine rather than linear. Trauma can arise from a variety of intrinsic and extrinsic factors, as discussed in detail in Chapter 1. Factors like repetitive stress on the foot can place an increased physiological demand on microcirculation. Both mechanical and metabolic stress induce injury or inflammatory responses (Barr and Barbe, 2002). With restricted microcirculation or small nerve fibre functions, the increased

demand is not met, which leads to an ulcer. The intricate mechanism of wound healing occurs in four phases: haemostasis, inflammation, proliferation, and remodelling (Wallace, Basehore and Zito, 2022). Regardless of wound aetiology, both macrocirculation and microcirculation, are critical to healing (Li *et al.*, 2017). Insufficient perfusion impairs angiogenesis, collagen deposition and epithelialisation resulting in delayed wound healing. Relative hypoxia is essential to instigate wound healing as it plays a role in regulating all the critical processes involved in tissue repair (Tandara and Mustoe, 2004; Li *et al.*, 2017). However. prolonged hypoxia, an impaired response to hypoxia or an abnormal local oxygen gradient (severe hypoxia) disrupts cellular mechanisms such as angiogenesis and induction of multiple growth factors and cytokines such as vascular endothelial growth factor (VEGF), Transforming growth factor-beta (TGF-β), Platelet-derived growth factor (PDGF) and others necessary to stimulate the proliferation and migration of endothelial cells, fibroblasts and keratinocytes, which are inherent to wound healing (Botusan *et al.*, 2008; Hong *et al.*, 2014). Thus, impaired neurovascular responses are critical to both preventing ulceration and to promoting wound healing. In the presence of microcirculatory complications leading to foot problems, the impact is not only at the foot level. As discussed in the thesis introduction in Chapter 1, diabetic foot problems can severely impact mental and emotional health, social life, sexual life and financial matters. Therefore, in order to prevent the negative impact of diabetes foot problems, there is a need for more comprehensive assessments to identify a foot at risk and adopt preventive strategies.

#### *6.3.2.2 Solution*

As emphasised above, not only macrocirculation but also microcirculation plays a vital role in the provision of nutrition, defence, maintenance of homeostasis, protection and promotion of healing. Any impairment to the neurovascular responses to demands, especially in times of injury, inflammation and healing can result in adverse complications. Early diagnosis of neruovasculatory dysfunctions may help to delay the progression of peripheral neuropathy, promote tissue microcirculation or prevent an ulcer when hot spots have occurred through relevant interventions (Bus et al., 2021b; Li, 2021; Ren et al., 2021). This research has contributed by emphasising the role of neurovascular responses in the foot, the simple and reliable methods to assess them (PORH) and the fact that foot temperature measurements can be used as a surrogate measurement of microcirculation. This could be an addition to improve existing risk assessment tools to make them more comprehensive. In addition, a previous study pointed out that while recommendations and guidelines such as IWGDF suggest measuring foot temperature, it is not always implemented (Bus et al., 2021b). The effectiveness of at-home skin temperature monitoring in reducing the incidence of foot ulcer recurrence in people with diabetes depends on adherence, which remains as a barrier to implementation as stated in IWGDF guidelines and in the study by Bus et al., (2021b). The current

study underscores the significance of temperature measurement as it can reflect microcirculatory changes. This a stepping stone towards generating evidence for making a strong case that supports evidence-based decision-making in creating awareness to educate and empower patients to monitor foot temperature.

### 6.3.3 Preventive strategies for people who are categorised as 'high risk' for foot health problems

#### *6.3.3.1 Problem*

Two of the top ten priorities identified specifically in the foot health space are: how can people prevent foot health problems and what people at high risk can do to prevent foot health problems, particularly those with poor circulation, diabetes or other conditions (James Lind Alliance, 2022b). This draws attention to the need for simple solutions that would make it easier to monitor foot health at home and take action to prevent dreaded complications.

#### *6.3.3.2 Solution*

The existing literature indicates that the assessment of microcirculation is complex and would require expensive equipment to study. However, the current PhD thesis has shown that temperature is an independent predictor of microcirculation, and they are strongly correlated (Chapter 5). This opens up possibilities for using temperature measuring devices that are relatively cheaper and simpler than devices for assessing microcirculation. People can be trained to use hand-held devices that measure foot temperature to monitor their feet regularly and take appropriate action to prevent ulcers and amputations. There are studies to support this recommendation, as inflammation helps detect underlying pathological processes before an ulcer occurs, and heat (calor) is one of the cardinal signs of inflammation (Lavery et al., 2004; Bus et al., 2021b). As previously mentioned, the literature highlights that foot temperature measurement is not implemented despite the fact that it is included in the recommendations and guidelines for preventative diabetic foot care and management (Bus et al., 2021b). Evidence from this research emphasizes the importance of measuring temperature in the foot as it may reflect microcirculation at the tissue level. Microcirculation plays an important role in tissue injury and inflammation (Chapter 1) (Balasubramanian, Chockalingam and Naemi, 2021). It can be the missing link to ulceration. Therefore, simple at-home solutions can help to regularly monitor foot health, thereby preventing ulcers and amputations.

### 6.3.4 Exploring the impact of delayed or infrequent foot assessment

#### *6.3.4.1 Problem*

Any delay in treating a foot problem in someone with diabetes can be disastrous. When caring for people with diabetes, it is often said that ‘time is tissue’ or ‘time is limb’. Delayed detection of skin

lesions and signs of trauma or injury can result in a wound that, in turn, can become susceptible to infection. As discussed in Chapter 1, the literature shows that delayed referrals can be an issue causing a lot of adverse complications in people with diabetes (Meloni and Apelqvist, 2018; Pankhurst and Edmonds, 2018). Many medical professionals fight for time. Especially when demand increases, physicians and nurses struggle to devote sufficient clinical time to meet the needs of people with diabetes in the community. Therefore, there is a need for efficient and time-effective methods to conduct comprehensive assessments of foot that might help with limbic salvage.

#### *6.3.4.2 Solution*

While addressing all of these issues to provide expedited access to appropriate diagnosis and treatment is complex, there are specific steps that can be taken to help. One of the steps could be to help educate people with diabetes or healthcare professionals who are their first contact about foot examination using simple tools such as foot temperature measuring thermometers both handheald and mounted that are time efficient. In addition to podiatrists, other healthcare professionals like wound care specialists, primary care physicians, vascular/orthopaedic surgeons, nurses, diabetes educators and community healthcare workers could receive training and education (NHS does provide training to conduct foot screening) to increase awareness on early diagnosis, prevention and periodic assessment of the foot, which is also an area of top priority, to use these tests to accurately assess the feet and refer them to specialists as appropriate (Collings et al., 2022; James Lind Alliance, 2022b). Use of simple and non-invasive tools to measure temperature (handheld thermometers or infrared thermography equipment of different brands like FLIR and Exergen DermaTemp) can be helpful and easier to train individuals to use them (Balbinot, L. *et al.*, 2012; Mufti et al., 2015). The recommended sites for temperature assessment are the first, third, and fifth metatarsal head, the hallux, the central midfoot and the calcaneum (heel) (Lavery *et al.*, 2004). If the hallux or a toe and metatarsal had been previously amputated, the adjacent anatomic area could be measured instead (Lavery *et al.*, 2004). Even if a site had a callus, a temperature assessment at this site was performed as per the literature. However, more studies are needed to expose the impact of callus on foot skin temperature measurements.

The current research has shown that temperature is an effective and a means to observe temperature and accompanied microcirculatory changes. Whilst observing lesions and swelling is subjective, the temperature is objective. Making decisions based on observations can be challenging for people with diabetes with little to no experience in differentiating skin lesions. Furthermore, they are likely to have vision disturbances owing to cataracts because of age or other eye conditions such as diabetic retinopathy. This contention is in accordance with the existing literature, which emphasises that swelling and redness are difficult to grade objectively even for experienced clinicians and most lay

people cannot understand or accurately evaluate these subtle parameters (Lavery *et al.*, 2004). The same study highlighted that temperature measurements can provide subjective and quantitative information, which can be easily taken by patients, their spouses or carers (Lavery *et al.*, 2004). There have been previous studies that have shown the predictive nature of temperature measurement (Schubert and Fagrell, 1991; Armstrong and Lavery, 1997; Armstrong *et al.*, 1997; Gatt *et al.*, 2018). The findings from this current PhD have further demonstrated the potential of using temperature as a surrogate measure for skin microcirculation. The results suggest that the proposed approach is a promising alternative to time-consuming or expensive measurements of skin perfusion. This is in line with existing literature, which showed that when a post-occlusive reactive hyperaemia was studied in the areas prone to pressure ulcers (decubitus ulcers), there is initially a gradual rise in temperature, caused by the increase in the skin blood flow (cutaneous microcirculation) in the region (hyperaemic response). Initially, the temperature had increased, as did the skin blood flow, but the time-lapse to maximum temperature was found to be longer than time to peak skin blood flow in most cases, suggesting that temperature reactivity occurs in response to changes in skin blood flow. These findings were observed mainly in healthy participants and the elderly group, but not in those hospitalised in a geriatric ward, suggesting impairment of the physiological microcirculatory response, which in turn affects the temperature changes in those bedridden and those under constant pressure. Of course, the underlying complications in these patients cannot be negated and a variety of various mechanisms can accentuate the producing a decubitus ulcer. In the same way, several mechanisms have been suggested to explain the cause of a foot ulcer in diabetes but there are no doubts that impaired skin microcirculation is a contributor even though it may not be a standalone cause (Flynn and Tooke, 1992; Korzon-Burakowska and Edmonds, 2006a). Therefore, simple solutions such as regular foot temperature measurements can potentially be useful in averting the adverse impact of delayed or infrequent foot assessment.

### 6.3.5 Importance of specialised tests in the diagnosis of foot health problems

#### *6.3.5.1 Problem*

The need for a comprehensive foot assessment has been highlighted in the literature (Boulton *et al.*, 2008; Iseli *et al.*, 2021). However, current risk assessment guidelines, tools or strategies are not comprehensive enough to include microcirculation or small fibre dysfunctions (Shahbazian, Yazdanpanah and Latifi, 2013; National Institute for Health and Care Excellence, 2015; National Health Service (NHS), 2016; Schaper *et al.*, 2019).

#### *6.3.5.2 Solution*

This PhD research has led to findings demonstrating that both microcirculation and small fibre dysfunctions are known to play a role in the mechanisms of injury and inflammation (Chapter 1) (Balasubramanian, Chockalingam and Naemi, 2021b). In addition, it is known that certain neurovascular responses are protective in nature (Chapter 2) (Balasubramanian *et al.*, 2020). Study 1 of the current PhD research (Chapter 3) showed that microcirculatory assessments, particularly neurovascular hyperaemic response, were measured with PORH in a simple, systematic, reliable and time-efficient method with a minimal occlusion time of 10 seconds with hallux occlusion and 30 seconds with ankle occlusion in the foot (Balasubramanian, Chockalingam and Naemi, 2021a). Furthermore, study 2 (Chapter 5) has shown that foot temperature measurement can be used as an indirect method to assess microcirculation of the foot or even a combined assessment with a deep breathing technique can provide a comprehensive picture. This can useful particularly in those with autonomic neuropathy as it uses a sympathetic-vasoreflex principle. Future studies in people with diabetes, with and without neuropathy, would help to understand its value in practical applications. Time is limb, so such simple tests can help strengthen existing risk assessment guidelines, tools or strategies to save limbs. Also, comprehensive does not have to be expensive. Tools to measure foot temperature are cheaper than other invasive tests and imaging equipment.

### 6.3.6 Impact of neglected foot health problems on health and social care services

#### *6.3.6.1 Problem*

Another concern highlighted in the foot health priority list was the impact on health and social services when known foot health issues are neglected (James Lind Alliance, 2022b). As highlighted in chapter 1, the cost of healthcare for ulceration and amputation in diabetes in 2014-2015 is estimated to be 0.8% to 0.9% of the NHS budget for England imposing a significant financial burden (Kerr, 2017; Edmonds, Manu and Vas, 2021). Therefore, the need for simple, economical and less time-consuming assessment methods for the prevention and early detection of diabetic foot complications, especially for mass screening of the foot, is important.

#### *6.3.6.2 Solution*

Findings from the current doctoral thesis have demonstrated not only a simple and time-efficient microcirculation assessment by PORH, but also the fact that temperature measurement can be a surrogate method for assessing microcirculation, which may be altered by inflammation, which is a precursor to an ulcer. Many studies have shown that microcirculation measuring devices such as LDF are expensive (Ghouth *et al.*, 2018; Gazyakan *et al.*, 2019; Balasubramanian, Chockalingam and Naemi, 2021a). Although not as expensive as the kits to measure microcirculation, equipment to measure foot

temperature such as infrared thermography can be expensive. But handheld skin thermometers could be a cheaper alternative. Overall, as always said, “Prevention is better than cure”, the costs of using these methods to prevent or aid early diagnosis may outweigh the costs of treatment, wound care, surgeries and rehabilitation as depicted in Figure 6.1 below.

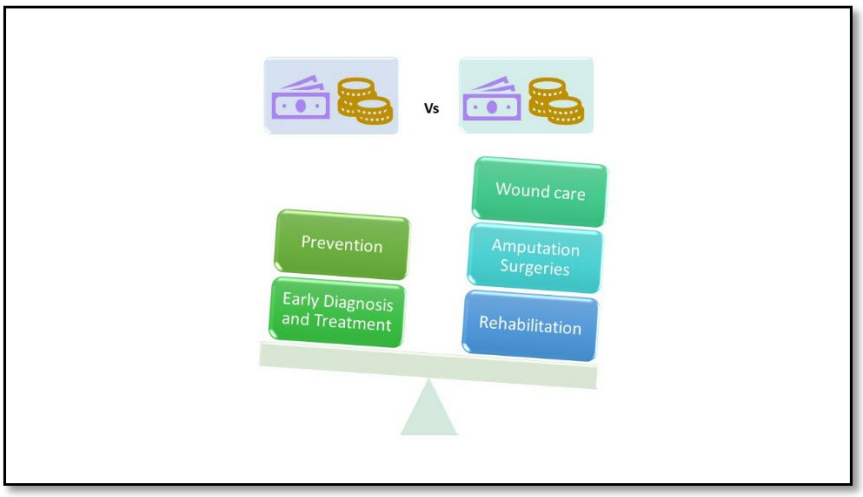



*Figure 6.1 Costs of using prevention and early diagnosis methods vs cost of care*

## 6.4 Strengths

Although microcirculation is well recognised in delayed wound healing, not enough research has been conducted to understand its role in tissue damage and ulceration to develop prognostic tools to identify a foot at risk. This doctoral thesis focused on this topic and generated evidence that microcirculation is an important factor in tissue response to physiological demands including the times of injury. Healthy microcirculation contributes to healthy functions of the small fibre nerves. They both influence each other; therefore, microcirculation and small fibre function should be one of key areas of focus when monitoring a foot at risk.

As already mentioned, the current comprehensive risk system does not include microcirculation measurements. This may be one of the shortcomings of the existing comprehensive foot evaluation system. Studies have clearly shown that diagnosing macrovascular problems and only correcting them is not enough to restore limb health. For example, one study has shown that impaired vasodilation in people with diabetic lower extremity neuropathy leads to functional ischemia, which improves significantly but is not fully resolved by successful bypass surgery. Therefore, patients with diabetes and neuropathy, despite adequate correction of macrovascular abnormalities, i.e. restoring blood flow in large vessels, will still be at high risk of developing foot ulcers or delayed healing of an existing ulcer (Arora *et al.*, 2002). One of the main strengths of this thesis is that it emphasises the importance

of assessing the microcirculation according to physiological demands. Not only the macrovascular measurements like ABI and TBI are useful, but also microcirculation measurements to understand if tissue needs are being met locally are important. The same applies to blood flow at the tissue level, microcirculation that satisfies nutritional needs, excretion, thermoregulation and more. Particularly, there is an increased demand during times of injury, inflammation, increased pressure, temperature variations, and other intrinsic or extrinsic factors. When these needs are not met due to functional impairment of the neurovascular network at the tissue level, it breaks down and can potentially lead to ulcers. Therefore, a focus on microcirculation is a necessity to improve overall foot health and quality of life for people with diabetes. It is important to think holistically (lower extremity) and act locally (tissue level).

It is often thought that assessing structural damage to the microcirculation can be complex, but the results of this thesis have shown that there are functional assessments following a well-established protocol to measure physiological responses such as PORH, temperature and microcirculatory responses to tasks such as deep breathing. In addition, there is a need for inexpensive and simple methods of identifying vulnerable feet, and this study demonstrated that temperature can be used as a surrogate measure of microcirculation. Vasodilation to local warming (LDI flare), which are easy to assess. Most commonly, people with diabetes are an ageing population who may be restricted in mobility and frail. Conducting lengthy studies and exposing your sensitive skin to extreme stimuli such as heat and cold can be risky and extremely uncomfortable. This thesis has shown that the use of simple techniques, comparable with methods used to take arm and leg blood pressure measurements, with only 30 seconds of occlusion is sufficient to study reactive hyperaemia. No change in posture or movement was required to study autonomic functions. Neither the Valsalva manoeuvre is required, which has several important contraindications, stroke, including aortic stenosis, hypertension, recent myocardial infarction, glaucoma, and retinopathy that tend to be the common comorbidities in people with diabetes (Morehead, 2008; Hayes, 2018; Srivastav, Jamil and Zeltser, 2022). Furthermore, potential complications of performing a Valsalva manoeuvre include perforation of the tympanic membrane of the ear (Baum *et al.*, 2010). In order to overcome these challenges, the doctoral thesis offers the safest approach where participants could simply lie down, relax, and use a deep breathing technique that allowed microcirculation and temperature to be studied simultaneously. The equipment used to measure blood flow and temperature is also completely non-invasive. In clinical practice, these two tests can be easily replicated.

## 6.5 Limitations

Some of the limitations of this thesis are discussed here. First, sample sizes were small in both Study 1 (25 participants) and Study 2 (10 participants). Owing to the pandemic COVID and related lockdowns

in 2020, the access to the laboratory and recruitment of participants to increase the sample the study was affected. Based on a heuristic in the literature, similar studies used a comparable type of sample size. An obvious strength was that the research question could be addressed in a relatively short space of time and with limited resources, especially when recruitment and access to the laboratory was challenging during the pandemic time owing to lockdowns. It was also better to test a new research hypothesis on a small number of participants first. This avoids wasting too many resources, time and money trying to find an association between two factors (Hackshaw, 2008). If an association is found, it can lead from a hypothesis-generating study to a larger confirmatory study. Small studies may also use surrogate markers when examining associations and may not have obvious effects for participants to identify (Hackshaw, 2008). As this current research focused on identifying the link between microcirculation and small fibre nerve functions and identifying simple tests to measure microcirculation, a small study design was helpful. However, increasing the sample size might be beneficial to improve the generalisability and validity of the research results. Second, participants in both Study 1 and Study 2 were young and healthy with no injuries, trauma, or neurovascular complications that do not compare to the demographics of people with diabetes who are at risk of developing foot problems in the real-world. Although care was taken to exclude participants with neurovascular complications, active or recent injury, and trauma, the menstrual cycle of the female participants was not considered in this research. Certain evidence from literature suggests that peripheral skin circulation and forearm muscle blood flow in healthy female volunteers exhibit significant variability during the hormonal changes in a menstrual cycle (Bartelink *et al.*, 1990; Song *et al.*, 2020). The microcirculatory skin perfusion in the superficial tissues of select Xi-cleft acupoints (SP8 and LR6 Yin meridians of the lower limb) was lower during the menstrual phase in comparison to the ovulation and luteal phases in healthy young college students (Bartelink *et al.*, 1990; Song *et al.*, 2020). In contrast, a previous study conducted on healthy ovulatory women demonstrated that microvascular responses do not have a clear cycle-dependent variation and that there is no clear influence of changing oestrogen levels throughout the menstrual cycle on these responses (Ketel *et al.*, 2009). Furthermore, a systematic review and metanalysis conducted to understand the impact of the menstrual cycle on peripheral vascular function in premenopausal women found that the menstrual cycle appears to have a small effect on macrovascular endothelial function but not on microvascular or vascular smooth muscle function (J. S. Williams et al., 2020). The contrasting evidence found in the existing literature can be attributed to methodological and demographic differences. Since microcirculatory assessment may be carried out in women to asses foot risks, future studies need to include protocols to understand and take into account such factors like hormonal influence. The understanding of the influence of hormonal changes on neurovascular changes, can

help to improve the accuracy of using microvascular or temperature measurements as a prognostic tool for foot microcirculation and associated temperature assessment. Also, the chosen sample may not be true representatives of the population of interest because of the use of a non-probability sampling method. There is a possible risk of bias with the use of convenience sampling. Therefore, the results of this study may not be directly generalisable or transferable to people with diabetes in the real-world. However, diversity of the participants is a strength to the convenience samples used in this research. There are several ways of further improving the current study. Before the current findings can be used to assess people at risk for diabetic foot complications in the real-world, the next step is to examine them in a sample representative of the population with diabetes. One strategy is that the proposed methods need to be studied in a sample who are demographically similar to those who are likely to have diabetic foot related problems such as older age group (Kurkela *et al.*, 2022). This would help to gain a better understanding of the safety and efficiency aspects before studying the use of a population with complex pathologies because of factors such as the assessment requires participants to stay in a supine position without fidgeting to avoid movement artefacts and the occlusion time (though minimal comparative to previous used duration as per literature). The use of randomised sampling methods for such a study in the future can help to improve the validity and generalisability of the research finding. Third, since a thermostatic probe was used to measure blood flow as well as temperature, microcirculation measurements and temperature were taken at one site. Studies that focus on multiple sites and compare these measurements between healthy people and those with active injury or inflammation can help in understanding the variations in microcirculation measurements related to temperature changes due to increased physiological demands due to exposure to various extrinsic or intrinsic factors will be helpful in generating evidence for evaluating the prognostic value of these proposed methods. In lieu of LDF with a thermostatic probe, devices such as Laser Speckle Contrast Imager for microcirculation measurements and thermal imaging for temperature measurements can be useful to expand research in this regard. Finally, the equipment used to measure microcirculation and temperature was the LDF, which is expensive equipment. Also, testing multiple different protocols can be time-consuming, and participants are required to remain still as even the slightest movement can cause artefacts when using the LDF system. This might be difficult to perform if participants were elderly and had questionable complications. However, the equipment used are already in clinical use. Throughout this work, these factors have always been taken into account and the results have been interpreted in their light.

## 6.6 Recommendations for Further Research

This study has established the association between microcirculation and small fibre nerve functions and their relevance in understanding the neurovascular responses in the foot. The next step is to

explore their role and prognostic value in people with diabetes to prevent ulceration. The answer likely lies in more large studies of people with diabetes, people with diabetic foot complications, and those deemed at risk of developing foot-related complications. Comparative studies with participants with diabetes and healthy adults would help to understand perfusion and temperature variations. This helps to understand a normal and abnormal range to predict a foot at risk. Studies that use methods that cover multiple sites of measurement or the whole would help to save time and screen the entire foot.

## 6.7 Conclusion

In this chapter, the main results and highlights of the conducted doctoral research were discussed. The contributions that this research has made to the diabetic foot area are also discussed in light of the James Lind Alliance's key diabetes and foot health priorities. This research has established that there is a relationship between microcirculation and small fibre nerve functions, both sensory and autonomic using non-invasive methods. This choice may suffice in many practical cases for assessing a foot at risk in people with diabetes. One of the major findings to emerge from the doctoral research is that there is an association between microcirculation and small fibre nerve functions. Secondly, microcirculation of the foot can be assessed non-invasively, systematically and reliably using PORH test with 30 seconds occlusion time. Finally, temperature can be a surrogate method to assess microcirculation, which emphasises the importance of measuring foot temperature and monitoring it regularly. These results can potentially be useful in clinical practice to prevent or aid in early diagnosis of ulcers to prevent adverse complications such as amputations. Perhaps in the future mHealth and mHealth technology could be leveraged to develop tools to report observed changes such as foot temperature fluctuations or the presence of lesions and enable a better way of communicating between patients and healthcare professionals to avoid delays in foot care. In conclusion, a thorough understanding of microcirculatory function and its impaired response mechanisms is essential and will comprehensively contribute to the understanding of soft tissue biomechanics and help to develop strategies for a comprehensive assessment of the diabetic foot. This, in turn, will aid in the prevention and early diagnosis of ulcers, thereby, reducing amputations.

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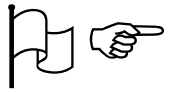

# List of Appendices

## Appendix 1: Review Article 1

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**A Synoptic Overview of Neurovascular Interactions in the Foot**

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Diabetes is a worldwide public health concern as it is associated with various complications. One of the major complications of diabetes is diabetic foot syndrome that results in catastrophic events such as ulceration and amputation. Therefore, the main four strategies of diabetic foot care involve risk prediction, prevention, and early diagnosis and prompt intervention. The drivers of ulceration are multifactorial, and importantly, include microcirculatory changes in the diabetic skin. Cutaneous microcirculation on the foot is greatly influenced by the small fibers which mediate thermal sensation and pain perception in addition to sympathetic activities such as thermoregulation and vasodilation. The interdependence between the neurovascular elements means with the loss of small fiber functions, the corresponding microcirculatory responses may be compromised. Thus, it can be hypothesized that the impairment of the microcirculation may follow the order of the corresponding small fiber nerve dysfunction or vice versa. In this review, select neurovascular investigations that inform the cutaneous microcirculatory and small fiber nerve function in response to pain, cold, and heat and pressure stimuli are reviewed and discussed in this order of sensory loss: the loss of pain, cold, warmth, touch and deep pressure sensation. We also discuss the neurological and vascular characteristics of each of these neurovascular responses. This review highlights the influence of small fibers on cutaneous microcirculation and the need for prospective studies that can determine the course of microcirculatory impairment over time. This, in turn, may help clarify the exact role of microcirculatory changes in the pathway of ulceration. The insights from this review can be pertinent to understand key microcirculatory disturbances and given that the microcirculatory impairment develops at an early stage, relevant interventions can be implemented to possibly reverse or regress

the course of the disease. Therefore, knowledge of the neurovascular interactions aids to map the disease progression for early diagnosis and prevention of adverse complications.

**Diabetes and its Complications: A Growing Concern**

Diabetes is a growing public health concern worldwide. Diabetes imposes huge health and economic burden across the nations. Since it is a chronic condition, commonly associated with various complications, the direct and indirect costs of care are high. According to the International Diabetes Federation (IDF), the total health-care spending on diabetes has more than tripled over the period 2003 to 2013 worldwide. Hence, diabetes and its related complications are a persisting problem that demand attention.

There is a spectrum of complications associated with diabetes, with the most common ones being retinopathy, neuropathy, nephropathy, peripheral vascular disease, and foot disease. These complications are the consequences of various glycation related changes that occur after the onset of diabetes over time. These complications significantly decrease the quality of life and may progress to become fatal. Hence, prevention and early diagnosis, treatment and constant care remain the cornerstone for diabetes care.

Diabetic foot ulceration (DFU) is one of the most devastating complications of diabetes. Development of DFU is associated with significant impairment of quality of life, decreased mobility, decreased independence, increased morbidity, and mortality and with impact on health care resources. Moreover, it is estimated that around 85% of non-traumatic amputations are preceded by DFU (1). The literature highlights that the annual population-based incidence of DFUs is around 1.9 to 2.2% (2). There are significant costs associated with DFU—a recent estimate has suggested that the National Health Service in England spends more than £1 billion treating the condition, higher than treatment costs of the lung, prostate and breast cancer combined (3). Development of infection can complicate over 50% of all DFUs which further increases the risk of non-healing. The common sites for ulceration are dorsal or plantar aspects of the toes, plantar metatarsal heads, and heel. In some patients, the ulcers heal with re-epithelialization, which is the restoration of the epithelium in the denuded wound area (2). However, in the absence of healing, there can be infections and adverse outcomes such as amputation. Therefore, early diagnosis and prevention are keys for better management. Effective evidence-based prevention programme with strategies for early detection and control are known to reduce the amputation rate by 50% (4). Thus, knowledge synthesis and understanding the risks associated with a foot ulcer, their interactions and role in the development of an ulcer incident is essential. This can potentially throw light on some of the predictive factors to develop strategies for early interventions.

The key drivers for the development of a DFU are complex and multifactorial (5–7). Rarely does a foot ulcerates due to a single underlying cause, and often, there are several extrinsic and intrinsic risk factors that trigger the diabetic foot to ulcerate (5). Whilst the extrinsic factors include trauma, ill-fitting shoes, walking barefoot, key intrinsic factors include peripheral neuropathy and peripheral arterial disease (5). A combination of two or more of these factors increases the risk of ulceration (5). From previous research, it is well-established that the triad of macrovascular disease, neuropathy and mechanical stress are involved in the pathogenesis of diabetic foot ulceration. In the absence of macrovascular issues and occlusive arterial diseases, a neuropathic foot with palpable pulses may imply microcirculation as a causative factor in the development of an ulcer (8–10). The role of microcirculation in foot complications is evident and well-realized in ulceration and delayed wound healing (2, 6, 8, 11). However, there has been no detailed investigation of its specific role or causal relationship in ulceration. Although the microvascular disease cannot be a standalone cause for a DFU incident, the interaction of microcirculation with the triad and its involvement cannot be denied. Moreover, microcirculatory complications commence at a much earlier stage and microvascular functional changes are predicted to occur even in the prediabetes state and progress with time (12). Hence, identifying the impairment and timely targeted interventions may help reverse some of the changes or delay progression of the disease. Therefore, studies that comprehend neurovascular interactions and underpins the core mechanisms are required. Understanding the underlying pathophysiology associated with the multitude of factors that trigger diabetic foot syndrome is a continuing concern within diabetic foot research. More research focused on the fundamental concepts of neuropathy and vascular diseases can be instrumental in understanding the risks that lead to a DFU incident.

**Overview of Interaction between Neurological and Vascular Aspects in the Foot**

There is evidence that patients with diabetic foot syndrome present with both microcirculatory and neurological disturbances. This neurovascular dysfunction affects the microcirculatory response at the tissue level under conditions of physical stress such as injury or infection, and chemical stress such as contact with heat, cold, chemicals. This may be present even in the absence of major macrocirculatory disturbances and the presence of satisfactory blood flow under normal conditions (5, 6, 10). Whilst both small and large fiber neuropathy is known to play a vital role and is predictive of incident ulceration, the role of microcirculation needs further investigation (6). Therefore, it is easier to explore the unknown through what is known in terms of neurovascular interactions.

This review aims at exploring the role of the microcirculation and the neurovascular interactions by appraising the microcirculatory responses mediated by the small fiber nerves and its significance. This

review intends to understand some of the neurovascular interactions, especially in relation to small fibers and microcirculation in the diabetic foot. As mentioned earlier, microvascular functional changes are detectable even in the prediabetes state and progress over time with diabetes (12). With the presence of peripheral diabetic neuropathy, a higher degree of dysfunction is observed (12). Probably, understanding microvascular disease progression and tailored investigation, may aid in the early diagnosis of microcirculatory and accompanied small fiber dysfunctions. Plausibly, this knowledge can be translated to effectively predict diabetic foot complications. Consequently, the practical implications from this review can be valuable for screening, early diagnosis, treatment, and enhancing prognosis by devising management and adapting prevention strategies that can change the paradigm of diabetic foot care in future.

The scope of this review is to identify the microcirculatory responses through functional assessment of small fiber nerves and to discuss cutaneous neurovascular interactions in the foot. Hence, this synoptic overview focuses on key investigations and responses of small fiber functions with relation to microcirculation in the diabetic foot. This is to gain some understanding of the thermoregulatory and biomechanical aspects corresponding to the actions of the thermoreceptors and mechanoreceptors that mediate microcirculation of the foot. Select neurovascular investigations that use stimuli such as pain, cold, heat, and pressure are identified and discussed. Furthermore, the investigation of neurovascular responses must aid to isolate the neurological and vascular components to identify the course of the disease. However, the review boundaries are that conventional tests such as Quantitative Sensory Testing (QST), Quantitative Sudomotor Axon Reflex Testing (QSART), electrochemical skin conductance (SUDOSCAN), iontophoresis, and skin biopsies used to test small fiber nerve functions and microcirculation are not discussed. Therefore, only studies that described small fiber nerve function in relation to microcirculatory responses or vice versa using combined neurovascular testing through brief methods were reviewed. Specific insights generated from the literature are presented and discussed below. Besides, an attempt is made to discuss the neurovascular responses in the order of sensory loss associated with small fiber dysfunction based on their response to local anesthesia, where the fibers of less diameter respond first (13, 14). This is to draw attention to the fact that the corresponding microcirculatory responses may follow the same trend. This may be useful in practice for early diagnosis and monitor disease progression concerning diabetic foot.

**Investigating Neurovascular Aspects**

Microvascular dysfunction in diabetes plays a crucial role in the development of diabetic complications. In recent years, functional changes of the microcirculation have gained much attention

for their potential role in the development of diabetic complications, especially diabetic foot syndrome. The skin is generally preferred to study microcirculatory functions as it is one of the most accessible organs. The skin microcirculatory bed is rich in capillaries whose functional assessment facilitates understanding of pathophysiological mechanisms that lead to microvascular and small fiber dysfunction. The skin has an intrinsic ability to auto-regulate its blood flow that depends on some of the external or internal factors. Such functions are facilitated by a complex regulatory system that includes local regulation of cutaneous microcirculation involving sensory and autonomic fibers (12). Glabrous skin has highly innervated arteriovenous shunts and plays a major role in thermoregulation (12). In contrast, non-glabrous hairy skin has fewer arteriovenous shunts and is primarily involved in defense and nutrition (12). Additionally, there are various nociceptors in the skin.

Nociceptors are peripherally localized sensory receptor neurons which are sensitive to a noxious stimulus or a prolonged stimulus that eventually becomes noxious (15). Nociceptors detect signals from tissues vulnerable to injuries or from damaged tissue (15). The speed of transmission is directly correlated to the diameter of axons of sensory neurons and whether they are myelinated. Most nociceptors have small diameter unmyelinated axons (C fibers) which support conduction velocities of 0.4–1.4 m/s or A fibers whose axons are myelinated and support conduction velocities of approximately 5–30 m/s (Aδ range) (15, 16). The nociceptors that can be found in the skin, joints and viscera exchange messages respond to a wide range of noxious stimuli (15, 17). Following an incident of injury and inflammation, the nociceptors are sensitized by pro-nociceptive mediators, such as prostaglandins, glutamate, kinins, cytokines, extracellular ATP, protons and other tropic factors (18–20). Also, there are various subcategories of nociceptors that respond based on the site of stimuli application and the type of the stimuli such as chemical, thermal and mechanical (15, 21). Stimulation and activation of the terminal branches of the sympathetic and nociceptor fibers result in axon reflex mediated neurogenic inflammatory reaction, sweating and vasodilation (22).

The skin nociceptors mediate pain, which can be protective in nature. They differ based on their responses to various types of stimuli. The skin nociceptors are categorized by their function in response to the noxious stimuli as illustrated in Figure 1 (15, 23, 24). Additionally, the skin has polymodal nociceptors respond to high-intensity stimuli such as mechanical, thermal and to chemical substances (23, 24). The skin nociceptors associated with small fibers mediate pain, which can be protective in nature and the skin microcirculation responds to these stimuli. Furthermore, several humoral, neural and external factors are involved in the regulation. In general, cutaneous microcirculatory disturbances in diabetic neuropathy is of interest to understand diabetic foot syndrome and adverse complications such as ulceration and delayed wound healing.

**FIGURE 1**

**Figure 1**. Skin nociceptors and their functions.

The investigation of the microcirculation in patients with diabetes is an increasing field of interest, fuelled by the availability of novel integrated research techniques used specifically to test microvascular function. Also, these investigations identify the role of small fiber nerves in relation to the microcirculatory responses. This paves the way to understand the neurovascular interaction and contribution to diabetic foot complications. Several non-invasive imaging techniques, mostly laser-Doppler-based methods have been developed in recent times to assess microvascular function in the skin. There are various devices and methods available to evaluate the microvascular changes such as Laser Doppler Flowmetry, Laser Speckle Contrast Image Analysis (LASCA), flow-video microscopy, cannulation measurements of capillary pressure, and transcutaneous oxygen tension measurements. Various provocation tests such as heating, cold, pressure, postural changes and iontophoresis are used to assess the impairment of microcirculation. These provocation tests dependent on the stimulation of the small nerve fibers and are mediated by them to invoke the respective microcirculatory responses. For instance: Endothelium-dependant and independent vasodilation have been studied using the laser Flowmetry through the method of iontophoresis. The indirect effect of the vasoactive substance on skin microcirculation results from the stimulation of C fibers (small fibers), typically through a nerve-axon-related hyperaemic response (25, 26). Thus, the microcirculatory function and the involvement of peripheral nociceptive C fiber function has been assessed simultaneously by measurement of the axon flare-reflex in research. Furthermore, laser-Doppler measurements of the skin microcirculation at the dorsum of the foot following postural change help to understand vascular disturbances in the form of reduced capillary blood flow, observed as an enhanced reduction in skin blood flux, and impaired fluid filtration after sitting up (25). The evidence generated from such studies shows that sympathetic innervation plays a major role in the regulation of skin microcirculation by opening and closing arteriovenous anastomoses and pre-capillary arterioles during postural changes (10, 12, 25). Impairment of endothelium-dependent microvascular regulation is known to correlate closely with the presence of sudomotor dysfunction (12). This microcirculatory impairment is of importance as autonomic neuropathy caused by sympathetic denervation can play a pathogenic role in the development of a diabetic foot; as skin dryness that eventually cracks paves way for infections

and ulceration (25). Therefore, neurovascular investigations are useful in understanding and evaluating the association between somatic/autonomic neuropathy and microcirculatory changes.

**Neurovascular Interactions**

In polyneuropathy, the small nerve dysfunction is characterized by symptoms such as pain, burning, numbness, and autonomic dysfunction characterized by lack of sweating show a stocking-glove distribution (27). The literature shows that the order of sensation loss is as follows: The loss of pain sensation, cold, warmth, touch and deep pressure upon application of local anesthetics as the smallest fibers respond first (13, 28–30). Research shows an association between microvascular impairment and small fiber neuropathy; microvascular dysfunction contributes to small fiber neuropathy and vice versa as micro-vessels supply the small fibers and small fibers innervate blood vessels (31). Glycation related changes to the microvasculature such as thickening of the basement membrane and altered permeability cause hypoxia of the nerve fibers resulting in the functional loss (32, 33). Increased glucose levels in the cells and tissues stimulate glycolytic and polyol pathways in the peripheral nerve. Furthermore, the modification of proteins with Advanced Glycation End-products (AGEs) and the accumulation of AGEs results in both structural (fiber loss or demyelination of nerve fibers and thickening of the basement membrane of the endothelium in microvessels) and functional damage in the small fiber nerves and microvessels (26, 33). Although glycation related changes in the microvasculature and small fibers are known to occur at a very early stage, it is not clear which precedes the other and how the cycle continues. Based on the severity of the neuropathy and the extent of the small fiber functions lost, the related microcirculatory response may be compromised. If the neurovascular elements are related, with the impairment of small fiber nerve functions, the corresponding microcirculatory responses may be compromised. Thus, the impairment of the microcirculation may follow a similar trend corresponding to small fiber nerve dysfunction (34). This order of sensory loss is upon application of local anesthetics is usually pain, temperature, touch and deep pressure based on the sequential sensory block and for the purpose of this review the neurovascular tests are discussed in this sequence (35). However, the sequence of loss of sensation in case of pathological conditions like diabetes may be different. This depends on the distribution and the number of receptors and of their sensory nerve fibers which are impacted by both aging and diabetes (36). Although the evidence strongly suggests that small fiber neuropathy precedes large fiber neuropathy, there is too little evidence to say which sensory loss mediated by the small fibers is the first one to be lost. The structure and function of small fiber nerves and microvessels are investigated using QST, QSART, electrochemical skin conductance (SUDOSCAN), iontophoresis, and even biopsies (22, 37). Many studies have shown the role of small fiber nerves in microcirculatory responses. Their role is well-established in certain responses more than the other. Previous research

has shown the specific role of nerves on certain microcirculatory responses such as following heat provocation tests, topical application of agents such as histamine or capsaicin and iontophoresis (31, 38–42). Whereas, their role in other mechanisms such as Post-Occlusive Reactive Hyperaemia (PORH) and Pressure-induced vasodilation (PIV) needs further exposition. This can be challenging as more than one factors contribute to most of these neurovascular responses. This review focused on providing an account of some of these responses that are non-invasive, less time-consuming and allows for an objective quick assessment of both neuro and vascular function.

**Pain Sensation Mediated By Small Fiber Nerves**

Loss of pain or painful neuropathy can be a major cause of foot complications as it can lead to misdiagnosis or late diagnosis of neuropathic complications (43). The pain receptors can be stimulated using certain substances. At the same time, these nociceptors can be sensitized by which the threshold to stimulation is decreased through the use of such substances, which helps to treat the symptoms of pain (44–46).

Sensitization happens following nerve/tissue injury or inflammation through the repetitive exposure to noxious stimuli which triggers an action potential to be propagated to the central terminal via the sensory neurons and the peripheral terminal via the collateral axon branches (17, 47). This instigates the membrane depolarization along with $Ca^{2+}$ influx via the VOCC, inducing the transmitters to be released at the site of the injury and activates the surrounding nociceptors. There are nociception-specific receptors that are present at the afferent terminals: the capsaicin receptor, transient receptor potential cation channel, subfamily V (TRPV1) or vanilloid receptor for capsaicin (VR1) (22, 47). The signaling mechanism pathways involved in the afferent terminal sensitization have included elevation of the $Ca^{2+}$ and activation of G-protein coupled receptors (GPCRs) that results in the elevation of adenylyl cyclase (AC)/cAMP/PKA, phospholipase C (PLC)/inositol triphosphate (IP3)/$Ca^{2+}$ or PLC/DAG/PKC activities. Additionally, neurogenic inflammation can occur through the antidromic release of the transmitters from the collateral branches of the afferent nerves when inflammatory mediators like Substance P, CGRP and neurokinin A are released locally by the afferent neurons (47, 48).

Substances such as histamine, capsaicin, and menthol induce axon reflex or the neurogenic flare response. The most commonly used substance is capsaicin. Capsaicin is a powerful vasodilator and is known to significantly increase skin perfusion (49, 50). Pain-related small fiber functions, symptomology and microcirculatory response are studied through histamine- or capsaicin-evoked axon flare responses are currently visualized by LASCA or photoplethysmography (22, 41, 49). The study by Unal-Cevik (41) characteristics of the flare which depended on the amount of activated small

nerve fibers and the function mediated by the C fibers (41). Two components can be isolated from the flare response. The size of the flare and the maximum perfusion represented the neurogenic and vascular components, respectively. The reduction in the size of the flare or its intensity at 5 min following provocation indicated reduced small fiber functions (influenced by the integrity and overlap of C fibers) (41). Besides, the spatial measure, the temporal measures such as prolongation or absence of the latency to reach 3-fold of baseline skin microcirculation denoted diminished small fiber functions (41). On the other hand, the maximum perfusion indicated the microcirculatory function and the spatial distribution of blood pulsation amplitude (BPA) and redness can be attributed to the high perfusion due to vasodilation induced by capsaicin (41, 49). In addition to the monitoring of flare, BPA dynamics measured by imaging photoplethysmography enables to visualize areas highly sensitive to capsaicin, which is indicated to be a novel sensitive non-invasive biomarker of migraine-associated changes in microcirculation (46, 49). However, its role in diabetes is yet to be explored.

It is worth mentioning the influence of certain topical anesthetics on the neurovascular reactions. The use of EMLA cream was found to decrease the responses and pain symptoms, however, it did not completely blocked the small fibre functions (41). Possibly the lower skin innervation layers were only partially inhibited by EMLA cream or certain features of the flare response were not completely influenced by the small fibres (41). Use of lidocaine can induce vasomotor effects similar to capsaicin and also mask its effect, however, perfusion changes do not seem to be influenced by low concentrations of lidocaine (46). These observations raise intriguing questions regarding the nature and extent of small fiber nerve functions on cutaneous microcirculation, especially in the case of neuropathy.

In people with diabetes, peripheral neuropathy causes the loss of these protective responses. The loss of protective sensation and dry skin predisposes the skin to cracks, infection and ulceration. Impaired neurogenic inflammation following capsaicin-induced desensitization can be demonstrated through impaired sudomotor axon reflex and nociceptor axon reflex responses (22). People with diabetes-related small fiber neuropathy present with a range of pain symptoms such as burning sensation, shooting pain, allodynia, and hyperesthesia. In such population, the possible absence of the flare response in people with diabetes may indicate either a severe small fiber dysfunction because of non-receptive superficial C fibers or dysfunction of fibers even in deeper layers of skin. Therefore, further exposition may aid to understand disease progression, small fiber dysfunction and related microcirculatory impairment. This would help to proceed with relevant intervention. For instance, capsaicin is being recommended to treat painful neuropathy and there is ongoing research on whether it will provide additional benefits in terms of improving microcirculation (45, 51). Similar interventions

that are tailored and timely may help to reverse impairments and potentially prevent further damage to the neurovascular structures of the skin.

**Heat Perception Mediated By Small Fiber Nerves**

Heat stimulus is one of the common provocative tests to study the small fiber functions. During a QST, a range of thermal challenges that are non-nociceptive and nociceptive are used. Heat-induced pain and the threshold is one of the parameters measured which help to assess C fiber functions (37). A most commonly used provocation test used to assess cutaneous microcirculation is local heating as well. This induces nociceptive stimuli-mediated vasodilation and a neurogenic flare by an axon reflex response involving the C fibers. These flare tests are specific to C fibers that can be activated by a stimuli and produces a neurogenic vasodilation (flare response) surrounding the injured site. Apart from thermal stimulus, even electrical stimulus can induce a flare response but such tests are minimally invasive (39, 52). The most commonly used equipment for measuring this effect is the laser Doppler imager (LDI) and the resultant axon reflex, which is a flare response is known as the LDI flare. The dorsum of the feet is preferred for the test as the skin is less influenced by the thermoregulatory blood flow due to the absence of arteriovenous anastomoses (53). The method either involves heating the local area of the skin to 44°C for 20 min or 6 min in a stepwise fashion (44°C for 2 min, 46°C for 1 min and finally 47°C for 3 min) in a temperature-controlled room to evoke the flare followed by scanning the site using an LDI to measure the area (38, 39, 54). The latter protocol is known to produce a significantly larger and consistent response (54, 55). A heating probe that allows direct heating of the skin or probe filled with deionized water is used to assess heat-induced vasodilation and the axon flare response. Similar to the study by Unal-Cevik (41), the LDI flare area or the size of the area with a hyperaemic response is known to be reflective of the small fiber function. Thus, the size of the LDI flare is known to be influenced by the C fiber function, the cutaneous small fiber neural network underneath the probe and its extent (38, 42, 54). The changes in perfusion of the skin immediately beneath the heating probe are a direct response to heating and are reflective of non-neurogenic components involved and may represent the endothelial function (38, 42, 54). Therefore, the intensity of the hyperaemic response depended on the microvascular ability to vasodilate, but on the other hand, the size of the flare was dependent on the small fiber function. This assessment showed reduced neurogenic flare along with microcirculatory dysfunction in people with either type 1 or 2 diabetes (54, 56). Overall, the LDI flare test helps to assess both small fiber nerve dysfunction as well as the associated impairment in cutaneous microcirculation. Further research may aid to isolate the impairment's origin, vascular or neurological, thereby, facilitating to have a better grasp of disease progression.

**Cold Perception Mediated By Small Fiber Nerves**

Small fibers and microcirculation are integral for thermal homeostasis in the skin. Although foot temperature is not monitored in routine practice, research suggests that it facilitates risk prediction and early diagnosis of complications (57–60). The common devices used to measure/monitor foot temperature are infrared thermographic cameras, infrared handheld thermometers, Laser Doppler Flowmetry systems and more recently the in-shoe temperature-based sensors designed to fit in prescribed footwear or offloading devices (58).

Plantar thermography is used as a complementary diagnostic method for various foot-related complications (61). Assessing plantar skin temperature can aid to detect the presence of either inflammation or neuropathy (58, 61). To facilitate foot temperature evaluation in people with diabetes, certain provocative tests may be useful (61, 62). One such is the cold stress test, in which the plantar aspect of the feet is covered with thin plastic and immersed in cold water for 1 min or longer (61). Data collection and analysis process involves recording infrared images for baseline and 10 min post-cooling immersion, and calculation of a rewarming index (61).

The cold stress test is also used to study the microcirculation in relation to thermal changes. Through the cold stress test, the afferent nerves that mediate pain and thermal perception in the skin and sympathetic efferent vasoconstrictor aspect are evaluated. The response following the cold stress test might be reflective of a sympathetic vasoconstrictor and the protective vasodilatory activities (63, 64). The initial exposure to cold temperature leads to cutaneous vasoconstriction witnessed by low perfusion (65, 66). However, prolonged exposure to cold increases skin perfusion, a protective hyperaemic vasodilatory mechanism (64, 67). This could be due to the sensitization of the thermal nociceptors to cold. But, the peripheral microcirculatory adaptations to cold exposure-response is known to be unpredictable (67). The cold provocation test is commonly used to study Raynaud's phenomenon, systematic sclerosis, and other conditions (68–71). However, few studies have been conducted to explore the neurovascular responses in people with diabetes (61, 72, 73).

The microcirculatory response to cold stress test is impaired in people with diabetes (74, 75). Increased vascular activity in the digits of people with diabetes (with and without neuropathy), following cold stress, seems to be corresponding with the arteriovenous shunting and the abnormal vascular regulation (74). In accordance with this finding, foot thermography through cold stress test is proven to be beneficial in diagnosing neuropathic complications and mere observation of temperature changes are known to indicate foot at risk (59, 61, 76). Therefore, plantar thermography, which relies on the small fibers function and related vascular responses can be useful in the early diagnosis of diabetic foot complications. There are few imaging techniques such as thermal imaging

and Laser Doppler methods used to visualize the skin temperature changes following cold stress tests in people with diabetes to assess the relationship with neurovascular complications in the diabetic foot (49, 61, 74, 76). Studies have demonstrated the changes in skin temperature, perfusion and BPA dynamics in response to thermoregulation following a cold challenge and these changes can be observed in the images using corresponding testing (49, 61, 74, 76). However, there are no studies that identify and isolate the neurogenic and vascular components in the image such as LASCA (49, 61, 76). Such studies may help to further the knowledge of the neurovascular relationship.

**Pressure Sensation Mediated By Small Fiber Nerves**

The polymodal mechanothermal receptors in the foot respond to mechanical stimuli such as application of pressure in addition to thermal stimuli. The microcirculatory responses that correspond to the pressure changes are reflected as a change in skin perfusion. The influence of external pressure application on the neurovascular aspects helps to build a foundation that can further be expanded to understand the basics of neurovascular interaction in foot under pressure. The autoregulation of blood flow upon application of extrinsic pressure is known as reactive hyperaemia and the most commonly used provocation tests (Post-occlusive reactive hyperaemia and pressure-induced vasodilation) are discussed in this review.

**Post Occlusive Reactive Hyperaemia (PORH)**

Post-occlusive reactive hyperaemia (PORH) is a measure of the reactive hyperaemia to arterial occlusion with pneumatic cuffs. During a PORH test, at occlusion, the blood flow goes to a biological zero followed by a PORH response when the pressure is released. PORH is a transient increase in blood flow because of the induced vasodilation in the organ or tissue following that brief period of the arterial occlusion. During the hyperaemia, the tissue becomes re-oxygenated and reperfusion occurs. PORH is considered to be both endothelial dependant and independent (11, 40). The response is known to be of myogenic, metabolic, neuronal and endothelial (31, 40). Research from as early as in the'90s has demonstrated the involvement of sensory nerves in PORH (13, 77). PORH is mediated by a local reflex involving sensory nerves and an endothelium-derived hyperpolarizing factor, which is known to play a vital role in vasomotor tone for microvessels (40, 78, 79). Although the involvement of a cyclooxygenase product (possibly a vasodilator prostaglandin) was suggested earlier, more recent research shows contradictory results and there is no strong evidence to substantiate the participation of prostaglandins in PORH (13, 40, 78). Larkin and Williams (13) showed that the hyperaemic response could be significantly decreased by the use of topical anesthetic creams through a mechanism that did not alter the vasodilation induced by exogenous calcitonin gene-related peptide (CGRP) or capsaicin (13). The rest flow and blood flow during the recovery seemed to be the same with or without

anesthetics. This finding implies that there is a neuronal component involved in the hyperaemic process of the provocation test. The study showed that the use of capsaicin to provoke a microcirculatory response through the stimulation of sensory nerves did increase the blood flow. Similarly, CGRP increased cutaneous blood flow. The increase of blood flow induced by both of these mechanisms were unhindered by the use of topical anesthetics (13). But, the flare produced was abolished (13). This showed that the use of the local anesthetics altered the neurogenic component, which is the axon reflex flare and not the release of endogenic vasodilators (characterized by increased blood flow). The reduced maximum peak flow or magnitude of the PORH response could be attributed to the slower conduction speed of the sensory nerves (80). Sensory nerve function seems to influence the peak perfusion and decrease in time to peak (31, 40). Therefore, in a PORH output, the magnitude and duration of hyperaemia can be considered as the neurogenic component and the increase in blood flow (maximum perfusion/hyperaemia) as the vascular component.

The understanding PORH mechanisms and measures can be valuable as its impairment is found to be associated with both early complications like the presence of peripheral sensory neuropathy in diabetes and late complications such as ulcer (11, 31). PORH is generally measured in the arms but in recent times studies have explored the association of PORH measures at foot in diabetes (11, 13, 40, 81–83). Findings show that for each second increase in time to peak, the likelihood of a participant having a history of foot complication is increased by 2% (11). Therefore, further understanding of the temporal and spatial measures of a PORH response, whether it is indicative of small fiber or microcirculatory dysfunction or both, can help with risk prediction. The neurovascular involvement on the PORH flare was discussed above, however, the isolation of the neurogenic and vascular components for various other PORH measures such as changes in hyperaemic area, hyperaemic repayment, time to recovery, time to latency and such can be of added value. Studies have also highlighted the need to understand this component in order to understand the role of microcirculation and neuropathy which are major contributors to DFU (11, 31). Since microcirculatory and small fiber neuropathy related complications begin at a much earlier stage, such knowledge can help implement appropriate interventions that regress the course of the disease and prevent adverse complications.

**Pressure-Induced Vasodilation (PIV)**

Research suggests that Pressure-Induced Vasodilation (PIV) is one of the cutaneous microcirculatory reactive mechanisms to low pressure (84). PIV works through a vascular and neuronal mechanism (84, 85). It results from the interaction of primary afferent nerves and vascular endothelium of skin vessels. The local application of pressure over a particular threshold at a specific location over time

may act as a stimulus and the sensations are mediated by the afferent nociceptive C fibers. This response is observed during local application of progressive pressure over time. It is known to be a transient increase in cutaneous blood flow initially before it decreases in response to the stimuli induced by the pressure strain (84–87). This assessment once again shows the relationship between small fiber nerve function and microcirculation. PIV is considered to be more than a transient phenomenon rather an important physiological response allowing the skin to respond adequately to a mechanical stimulus (88). Cutaneous receptors in the skin respond to local mechanical stresses such as local pressure strain (86). These receptors are found to be of mechanothermal nature as the PIV response required certain cutaneous thermal condition (86). The acid-sensing ion channel 3 (Asic3), a neuronal sensor is known to play a pivotal role in causing the vasodilatory response to direct pressure and also for protecting against pressure ulcer (89). The cutaneous Asic3 channels in the skin act as a mechanosensor triggering the microvascular responses through CGRP and produces PIV at low pressures (89). This highlights the fact that small fiber dysfunction as noticed in people with diabetes may result in the absence of certain protective microcirculatory response to temperature or mechanical stimuli or to both. Absence of PIV in people with diabetes showing impaired vasodilation to acetylcholine (endothelium dependent) suggests that PIV is endothelial dependent as well (87). The cutaneous blood flow in response to applied pressure at 5.0 mmHg/min indicated PIV to be absent in the foot of people with type 1 diabetes whereas it existed in healthy subjects (87). This was despite the fact that the study was conducted in a temperature (29.5 +/−0.2°C) controlled environment as low skin temperature in people with diabetes is known to interfere with microcirculation (87). However, a similar study did not observe PIV at 28.7 +/– 0.4°C skin temperature even in healthy subjects and the study suggested the influence of temperature on the mechanism due to the involvement of mechanothermal receptors (86). Further research is required to understand the nature of the mechanoreceptors and their corresponding neurovascular response. Koïtka et al. (87) revealed that in the participants, the non-endothelial-mediated response to sodium nitroprusside was preserved, whereas the endothelial-mediated response to acetylcholine was impaired. This behavior is suggestive of the association between endothelial dysfunction and PIV. On the other hand, the role of small fibers in the response has been implied through the findings of a study that found that vasodilatory axon reflex response to local pressure strain was absent when the capsaicin-sensitive nerve terminals were pre-treated with local anesthetic or chronically applied capsaicin (90).

As mentioned earlier, PIV is a short-lived response, which is believed to be a protective response to non-noxious pressure application. Effect of aging or existing pathology is characterized by the absence of PIV, decreased response or following the PIV the cutaneous blood flow is observed to progressively decrease with the application of increasing local pressure for a prolonged period (86, 91). Upon

application of local pressure over time, in comparison to the healthy controls (48.8 mmHg), the cutaneous blood flow was found to decline significantly from baseline at much lower applied pressure (7.5 mmHg) in people with diabetes without neuropathy and with subclinical or clinical neuropathy (6.3 mmHg) (86, 92). The findings suggested that the easily compressible arterial wall and surrounding tissue coupled with impaired response mediated by mechanoreceptors caused an early decrease in cutaneous blood flow (86). Whilst it is apparent that PIV is mediated by C fibers, studies need to isolate the role of the small nerve fibers and the endothelial component possibly through the use of imaging methods.

Largely in people with diabetes, the neurovascular responses to local pressure are compromised and the tissues are very compressible (86, 93). Previous studies have suggested that this microcirculatory response appears to be a protective cutaneous response that relies on the excitation of unmyelinated afferent C nerve fibers (86–88, 94–96). The phenomenon of PIV has been observed even in the pediatric population (96). PIV impairment could contribute to the development of lesions such as pressure ulcers and DFUs (86–88, 94, 95). A recent study demonstrated that the cutaneous vasodilation in response to pressure is decreased in people with DFU in comparison to people without (97). Moreover, these responses are known to be impaired in the cohort of the aging population (84). Thus, the physiological process of aging and progression of pathological conditions of diabetes may worsen the neurovascular functions making certain groups of people (elderly population with diabetes) more vulnerable to complications such as ulcers. Collectively, these findings reveal the significance of protective microcirculatory responses mediated by small fibers that can be important in assessing the risk for ulcers. Therefore, in-depth research analyzing the neurogenic and vascular components and its relationship with local pressure is necessary to understand the tissue vulnerability to ulceration. This can aid with early diagnosis and risk prediction of DFU.

**Conclusion**

On the whole, in this review, we have provided an overview of small fiber function and its role in mediating microcirculatory responses. Select tests that are non-invasive and less time-consuming that allows the simultaneous assessment of small fiber functions and the respective microcirculatory response were summarized. The commonly used evaluation methods discussed in this review helps to explore the neurovascular aspects simultaneously in routine practice. Besides, most of these tests facilitate isolating the neurogenic and vascular components of the responses.

In people with neuropathy small fiber dysfunction is known to precede large fiber dysfunction. The small fibers play their role in pain and temperature perception and cutaneous superficial touch/pressure sensations. The impairment of the microcirculation may correspond to the

dysfunction of the small fiber nerves. However, prospective studies are required to substantiate it. More research is required to expand the knowledge on these certain responses and their value to understand disease progression. As microcirculatory impairment and small fiber neuropathy are known to precede many other diabetic foot-related complications, outcomes of such research can aid in early diagnosis and better prognosis. Furthermore, whilst the role of microcirculation is well-realized in wound healing, its role played in ulceration remains speculative. Future research in the progression of microcirculatory impairment in the diabetic foot may fetch interesting evidence for resolution.

**Author Contributions**

GB and RN made substantial contributions to the conception of the study, data synthesis, and interpretation along with drafting the work. NC and PV contributed to the interpretation of the work and revising it critically for important intellectual content. All authors provided final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**Conflict of Interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Appendix 2: Review Article 2

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The Role of Cutaneous Microcirculatory Responses in Tissue Injury, Inflammation and Repair at the Foot in Diabetes

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Diabetic foot syndrome is one of the most costly complications of diabetes. Damage to the soft tissue structure is one of the primary causes of diabetic foot ulcers and most of the current literature focuses on factors such as neuropathy and excessive load. Although the role of blood supply has been reported in the context of macro-circulation, soft tissue damage and its healing in the context of skin microcirculation have not been adequately investigated. Previous research suggested that certain microcirculatory responses protect the skin and their impairment may contribute to increased risk for occlusive and ischemic injuries to the foot. The purpose of this narrative review was to explore and establish the possible link between impairment in skin perfusion and the chain of events that leads to ulceration, considering the interaction with other more established ulceration factors. This review

highlights some of the key skin microcirculatory functions in response to various stimuli. The microcirculatory responses observed in the form of altered skin blood flow are divided into three categories based on the type of stimuli including occlusion, pressure and temperature. Studies on the three categories were reviewed including: the microcirculatory response to occlusive ischemia or Post-Occlusive Reactive Hyperaemia (PORH); the microcirculatory response to locally applied pressure such as Pressure-Induced Vasodilation (PIV); and the interplay between microcirculation and skin temperature and the microcirculatory responses to thermal stimuli such as reduced/increased blood flow due to cooling/heating. This review highlights how microcirculatory responses protect the skin and the plantar soft tissues and their plausible dysfunction in people with diabetes. Whilst discussing the link between impairment in skin perfusion as a result of altered microcirculatory response, the review describes the chain of events that leads to ulceration. A thorough understanding of the microcirculatory function and its impaired reactive mechanisms is provided, which allows an understanding of the interaction between functional disturbances of microcirculation and other more established factors for foot ulceration.

Chapter 2 Diabetes Is a Global Health Issue

Diabetes is a common condition which has a considerable impact on the health and economy of nations around the world. There is an annual upsurge in the number of patients being diagnosed with diabetes. The International Diabetes Federation estimates that total global health-care spending on diabetes more than tripled over the period 2003 to 2013 (World Health Organization, 2016). The estimated direct annual cost of diabetes to the world is more than US$ 827 billion and the projected losses in gross domestic product (GDP) for the period 2011 to 2030 is a total of US$ 1.7 trillion worldwide incurred by both the direct and indirect costs (World Health Organization, 2016). This indicates that diabetes imposes a large economic burden on the global health-care system and the wider global economy. As diabetes is a chronic condition, many complications arise as the disease progresses.

Chapter 3 Diabetes Complications and the Role of Microcirculation

Microcirculation is vital for the efficient exchange of gases and nutrients and the removal of the waste products of metabolism. In addition, the cutaneous microcirculation plays an important role in thermoregulation (Flynn and Tooke, 1992). Some of the common complications of diabetes are retinopathy, neuropathy, nephropathy, peripheral vascular diseases, and diabetic foot syndrome. One of the important aspects that resonate with all these complications is microcirculation. Endothelial damage and dysfunction of the microvasculature have been observed in various parts of the body such as the eyes, kidneys and the foot in people with diabetes (Goldenberg et al., 1959; Flynn and

Tooke, 1992; Hile and Veves, 2003; Williams et al., 2004; Boulton et al., 2006; Schramm et al., 2006; Körei et al., 2016). Both structural and functional microvascular disturbances (known as microangiopathy or disease to small blood vessels) are commonly observed in people with diabetes as a result of glycation related changes that occur due to the prolonged hyperglycaemic state (Boulton et al., 2006; Singh et al., 2014; Stirban et al., 2014). Besides, glycation related direct changes in the microvessels, both sensory and autonomic neuropathies contribute to the functional changes of the microvasculature (Schramm et al., 2006). As early as in 1983 Parving et al. introduced the "haemodynamic theory" to explain microangiopathy in diabetes (Flynn and Tooke, 1992; Veves et al., 2006; Chao and Cheing, 2009). The theory proposes that the increased microvascular blood flow triggers endothelial injury response, followed by microvascular sclerosis (Flynn and Tooke, 1992; Veves et al., 2006). This, in turn, may lead to functional abnormalities such as impaired maximum hyperaemic response, reduced tissue response to injury or trauma, autoregulation of blood flow and changes to vascular tone (Flynn and Tooke, 1992; Boulton et al., 2006; Veves et al., 2006).

Chapter 4 Diabetic Foot Disease as a Significant Complication and the Role of Microcirculation

In the foot, the adverse complications of diabetes are ulceration and amputation. The annual population-based incidence of diabetic foot ulcers is estimated to be 1.9–2.2% (Levin et al., 2008). Once the skin on the foot is ulcerated, it is susceptible to infections leading to an urgent medical problem (Bakker et al., 2016). It is estimated that only two-thirds of diabetic foot ulcers will eventually heal, but approximately 28% may result in some form of lower extremity amputation (Bakker et al., 2016). Hence, understanding the risks associated with foot ulcer development and its course is crucial. While the role of peripheral vascular disease and neuropathy resulting in diabetic foot ulcers is well-established, more research is needed to understand the contribution of microcirculation (Schaper et al., 2016).

The role of microcirculation in diabetic foot ulcers is a continuing area of research, where there are many theories put forth by several studies on microcirculation and the concept of "small vessel disease" was proposed (Goldenberg et al., 1959). Although this theory of an exclusive microvascular disease is widely debated, historical evidence for structural and functional microcirculation and related disturbances exist (Boulton et al., 2006). Also, studies have shown that capillary pressure is increased in the foot of people with diabetes due to arteriovenous shunting caused by sympathetic denervation (Deanfield et al., 1980; Flynn and Tooke, 1992; Boulton, 2000; Korzon-Burakowska & Edmonds, 2006). Collectively, these studies outline a critical role for microcirculation in ulceration.

With respect to diabetic foot ulcers, it is proposed that the impaired microcirculatory response may induce microcirculatory failure, resulting in tissue necrosis and ulceration (Flynn and Tooke,

1992; Korzon-Burakowska & Edmonds, 2006). Although microvascular disease may not be the single cause of pathogenesis of diabetic foot ulcers, the co-existence of abnormal microcirculatory function with both peripheral arterial disease and neuropathy may be associated with tissue damage (Flynn and Tooke, 1990; Boulton et al., 2006). This is supported by the evidence from studies that demonstrate the role of microcirculation in the development of ulceration, gangrene, necrosis and wound healing (Flynn and Tooke, 1992; Boulton et al., 2006; Levin et al., 2008; Lanting et al., 2017). Therefore, understanding functional abnormalities is of importance when studying diabetic foot ulcers.

Injury, Inflammation and Soft-Tissues

To gain a better understanding of microcirculatory function and recognise appropriate methods to evaluate it, it is important to look at the bigger picture of the body's defence, injury, inflammation and repair mechanisms. In the host defence mechanism, both lymphatic and blood vessels play an important role in an inflammatory response (Granger and Rodrigues, 2016; Parnham, 2016). Changes in the inflammatory mediators are known to correlate with the risk of developing a diabetic foot ulcer and inflammation is one of the earliest signs of ulcer (Lanys et al., 2021). Inflammation is a microcirculation-dependent tissue response to extrinsic and intrinsic stimuli (Granger and Rodrigues, 2016). During such an inflammatory response, the cardinal signs of inflammation that can be observed are heat (calor), pain (dolor), redness (rubor), and swelling (tumor), which may eventually lead to the loss of tissue function. In general, microcirculation is highly reactive to inflammatory response and plays a pivotal role in it as all components of the microvasculature such as the arterioles, capillaries, and venules respond and work towards the delivery of inflammatory cells to the injured or infected tissue/site (Granger and Senchenkova, 2010). The microvasculature isolates the infected or injured region from the healthy tissue and the systemic circulation, to facilitate tissue repair and regeneration (Johnson, 1973; Granger and Senchenkova, 2010; Bentov and Reed, 2014). The inflammatory responses of microcirculation include impaired vasomotor function, reduced capillary perfusion, leukocytes and platelets adhesion, activation of the coagulation cascade, enhanced thrombosis, increased vascular permeability, and an increased proliferation rate of blood and lymphatic vessels (Granger and Senchenkova, 2010). Other common microcirculatory changes result in shunting and hypoxia (reduced oxygen capacity of the tissues) caused by endothelial cell injury induced by a severe form of infection like sepsis, stasis of red blood cells due to vascular resistance, increased distances in oxygen diffusion in case of oedema owing to capillary leak syndrome (Guven et al., 2020).

In the foot, defence mechanisms (stimulation–response) plays a vital role. The role of microcirculation in wound repair and healing is well-realised (Shapiro and Nouvong, 2011; Ambrózy et al., 2013).

Evidence suggests that despite the reasons behind an ulcer incident, the microcirculatory role in the process of healing remains the same and that the subpapillary perfusion plays a major role in the formation of granulation tissue, which was studied through the use of Laser Doppler Flowmetry system in patients with venous ulcers (Ambrózy et al., 2013). Microvasculature aids with tissue perfusion, fluid homeostasis, cutaneous oxygen delivery and recruiting collateral vessels to facilitate healing process (Bentov and Reed, 2014). Transcutaneous Oxygen Pressure (TcPo2) technique allows the measurement of cutaneous oxygen supply, which is found to be reduced in type 2 diabetic patients with the foot at risk of ulceration (Zimny et al., 2001). This was related to an impaired neurogenic blood flow regulation, which may contribute to capillary hypertension, endothelial dysfunction leading to oedema and skin damage (Zimny et al., 2001). Other non-invasive methods such as the measurement of skin perfusion pressure allow to assess healing (wound is likely to heal if pressure is above 30 mmHg) and to determine amputation levels (Sarin et al., 1991; Shapiro and Nouvong, 2011). Newer technology such as Laser Speckle Perfusion Imaging allows visualising the blood in the microvaculatore in and around the ulcer area, which may indicate the ability to heal (Shapiro and Nouvong, 2011). However, this device images cutaneous circulation to a depth no greater than 1 mm (Shapiro and Nouvong, 2011). While recent research focuses on assessing microcirculation to predict ulcer outcomes, further studies are needed to gain a deeper understanding of the microcirculatory changes in the ulcers with respect to the stages of healing for better prediction of wound healing.

Although the responses of the inflammatory system are regarded as defence mechanisms (stimulation–response) it may also be considered as a homeostatic system that operates continually to maintain organ and organism function (Tracy, 2006). Based on the dual nature of inflammation, stimulation–response and homeostatic, research suggest the use of biomarkers such as C-reactive protein or interleukin-6 to assess the activity level of the inflammatory process (Tracy, 2006). These biomarkers may represent normal homeostatic function, a response to a pathological condition or to both, which can take place to varying degrees depending on the differences in the person, time and condition (Tracy, 2006). Whilst in younger, healthier people, the biomarkers may likely represent the ongoing homeostatic activity, with increasing age and in the presence of underlying pathology such as chronic inflammatory changes due to diabetes or triggered atherosclerotic changes in cardiovascular conditions, these biomarkers may indicate a stimulation–response type inflammation (Payne, 2006; Tracy, 2006; Pahwa et al., 2020). Overall, there is consensus that inflammation biomarkers are independent predictors of the future occurrence of chronic disease outcomes and events (Tracy, 2006). Similarly, physiological markers such as skin temperature, galvanic skin response and perfusion measurements that indicate homeostatic and stimulation-response in relation to microcirculation

may be pertinent to predict the future occurrences of chronic disease outcomes or events such as ulcers.

Assessment of Microcirculation

Diabetic foot ulcers are multifactorial and there are new and emerging technologies that enable the assessment of these factors to aid prevention and management. Some of the methods are various nerve function tests (quantitative sensory testing, vibration perception, galvanic skin response and sudomotor activity testing), temperature measurement (infrared thermography), biomechanical properties measurements (plantar pressure and ultrasound indentation tests/elastography), macrovascular assessments (ankle-brachial index and toe brachial index) and microvascular assessments (TCPO2, laser doppler flowmetry, hyperspectral imaging and laser speckle contrast imager) and such (Pham et al., 2000; Naemi et al., 2017; Balasubramanian et al., 2020; Lung et al., 2020). However, in this review the main focus would be to discuss the assessment of microcirculation in tissue injury and inflammation to better understand its role in ulceration.

In the past, the key signs of inflammation were predominantly detected through mere observation. However, nowadays contactless and pain-free non-invasive techniques have facilitated objective assessment of inflammatory signs, tissue injury responses, repairs and healing. Laser Doppler flowmetry (LDF) technique is one such non-invasive technique, which allows assessment of microvascular blood flow when reflection and scattering of the laser light occurs due to the movement of the red blood cells (Nakamoto et al., 2012; Balasubramanian et al., 2020). Although the depth the laser penetrates is relatively low (~1 mm), it is a useful device for the evaluation of cutaneous microcirculation. This device is gaining popularity in the field of research in diabetes, cerebrovascular conditions, Raynaud's phenomenon and others. The use of LDF is being explored in dentistry as well, especially for perioperative procedures to gain a better understanding of soft tissue diagnosis. Apart from LDF, other non-invasive methods used to evaluate microcirculation are Laser Speckle Contrast Imager (LSCI) and photo-plethysmography. At times, since small fibre nerve functions and thermal changes influence microcirculation, methods such as quantitative sensory testing, skin electrodermal activity assessment and thermography are also used in conjunction with microvascular testing.

Scope of This Review

This narrative review of literature focuses on key microcirculatory responses in relation to diabetic foot in order to understand some of the functional aspects of microcirculation. Firstly, search terms such as "Post-Occlusive Reactive Hyperaemia", "PORH" "pressure-induced vasodilation", "PIV" and "skin blood flow" "local application of pressure", "LDI flare" and "axon-mediated flare" were listed

and used to identify articles (the search was not limited to these terms only). PubMed and Medline databases were searched to identify relevant publications in journals. Secondly, the reference lists of the selected articles were scrutinised to find additional studies. However, the data sources were not limited to articles published in journals, but also included grey literature. The sources for grey literature included: 1) Reports from International Diabetes Federation and Diabete UK 2) Websites of equipment manufacturers (Perimed AB, Moor Instruments, FLIR and Impeto Medical Solutions) 3) OpenGrey, and 4) Google. The articles of interest from MEDLINE, PubMed, and PubMed Central (PMC) included in the review were identified through the initial phase of title and abstract sifting. Subsequently, after the title and abstract sifting, relevant articles that adequately described cutaneous microcirculatory responses were retrieved for further study. Later, the data were extracted from relevant articles. Specific insights generated from the literature are presented and discussed below.

Various Microcirculatory Responses and Their Association in Diabetes Foot-Related Complications

The foot is continuously under mechanical stress due to weight-bearing activities of daily living such as walking, exercise, and standing. It is exposed to various trauma, physical injury due to sudden or violent action, exposure to dangerous toxins or repetitive mechanical stress. Some of the extrinsic factors for trauma are thermal (Example: hot surfaces), mechanical (Example: repetitive damage from ill-fitted shoes), and chemical (Example: corn treatments) (Boulton, 2000; Armstrong and Lavery, 2005; Boulton et al., 2006; Vanderah, 2007; Hawke and Burns, 2009). On the other hand, some of the intrinsic factors that contribute to the risk of trauma are foot deformity and glycation related changes in case of diabetes.

Both neuro and vascular aspects are essential for healthy foot function. The nerves of the feet can respond to the thermal, mechanical and chemical stimuli, provoking a reflex withdrawal from the respective harmful stimulus (Hawke and Burns, 2009). For instance, jerking the foot away from a sharp object. This protective mechanism may be absent due to neuropathy in people with diabetes (Boulton et al., 2006; Hawke and Burns, 2009). On the other hand, microcirculation is important for tissue injury response to stimuli such as local heat or pressure (Abraham et al., 2001; Korzon-Burakowska & Edmonds, 2006). Such neurovascular mechanisms of the foot appear to play a vital role to prevent tissue injuries.

Previous research shows that there are certain protective microcirculatory responses to stimuli, which are controlled by neural mechanisms, metabolic aspects, hormones and chemicals (Guyton, 1991). A microcirculatory hyperaemic response is induced on the application of a stimulus. This transient hyperaemic response to various stimuli, witnessed by an increase in blood perfusion is one of the measures to assess microcirculatory function known as reactive hyperaemia. Reactive hyperaemia is

an indicator of the intrinsic ability of an organ or tissue to locally autoregulate its blood supply, which is found to be impaired in people with diabetes (Flynn and Tooke, 1992; Korzon-Burakowska & Edmonds, 2006; Merrill, 2008; Klabunde, 2012). For the purpose of this review, based on the select stimuli, the microcirculatory responses observed are stratified into:

1) Vasodilation in response to occlusive ischemia or Post-Occlusive Reactive Hyperaemia (PORH)

2) Microcirculatory response to locally applied pressure;

(a) Pressure-induced vasodilation (PIV);

(b) Reduced skin blood flow;

3) Interplay between microcirculation and temperature -vasodilation in response to local heating

The reviewed studies demonstrate the inability of cutaneous microcirculation to respond normally to non-painful stimulation, such as the application of pneumatic pressure, local pressure and local heating in people with diabetes (Fromy et al., 2002). This may be significant in understanding tissue response to injuries. During incidents of prolonged pressure, injury or infection, more demands are made upon the capillary circulation (Flynn and Tooke, 1992; Abraham et al., 2001). Owing to the microcirculatory dysfunction, the hyperaemic response may be impaired and tissue demands are not met (Flynn and Tooke, 1992). Vascular insufficiency to the tissues that leads to breakdown may contribute to adverse complications and increase the risk of ulceration (Flynn and Tooke, 1992). Nevertheless, there are very limited studies that evaluate the vasodilatory responses to stimuli in subjects with diabetes. Furthermore, only a handful number of research articles address these vasodilatory responses in diabetic foot syndrome, including ulcerated and non-ulcerated cohorts. Key articles on this subject were appraised and discussed in this review.

Vasodilation in Response to Occlusion or Post Occlusive Reactive Hyperaemia (PORH)

Reactive hyperaemia to occlusion is the transient increase in blood flow in the organ or tissue that occurs following a brief period of arterial occlusion. During the process of occlusion, the blood flow goes to a biological zero that is defined as the “no flow” Laser Doppler signal during a PORH test. Following the release of the occlusion, blood flow rapidly increases, which is reactive hyperaemia (Klabunde, 2012). This process is known as post-occlusive reactive hyperaemia (PORH). During the hyperaemia, the tissue becomes re-oxygenated and reperfusion occurs. Simultaneously, the vasodilator metabolites are removed from the tissue, which restores the vascular tone of the resistant vessels causing the blood flow to return to normal (Klabunde, 2012). The longer the period of occlusion, the greater the metabolic stimulus for vasodilation leading to an increase in peak reactive

hyperaemia and duration of hyperaemia (Guyton, 1991; Larkin and Williams, 1993; Klabunde, 2012). Based on the time taken to occlude the blood supply to the tissue from few seconds to several hours, the blood flow post-occlusion increases four to seven times in the tissue than normal and lasts from few seconds to hours in relation to the initial occlusion time (Guyton, 1991). Additionally, depending upon the organ or tissue, maximal vasodilation as indicated by peak flow varies (Klabunde, 2012).

PORH is predominantly an endothelial-dependent process, however, it also aids combined assessment of both endothelial-dependent and independent function (Maniewski et al., 2014; Lanting et al., 2017). Hyperaemia occurs because of the shear stress, the tangential frictional force-acting at the endothelial cell surface caused by arterial occlusion (Maniewski et al., 2014). A mechanical stimulation occurs when the shear stress vector is directed perpendicular to the long axis of the arterial vessel (Maniewski et al., 2014). The endothelium responds to this mechanical stimuli, thereby, releasing vasodilatory substances (Maniewski et al., 2014). The factors that are known to contribute to vasodilation are myogenic, neurogenic, and other local factors, such as potassium ions, hydrogen ions, carbon dioxide, catecholamines, prostaglandins, and adenosine (Maniewski et al., 2014; Lanting et al., 2017). Few studies mention that endothelial nitric oxide and other endothelium-derived agents, such as prostaglandins and endothelium-derived hyperpolarizing factors are known to be involved in the mechanism of PORH (Maniewski et al., 2014; Carasca et al., 2017; Marche et al., 2017). However, some researchers contend that nitric oxide and prostaglandins may not be contributing to the mechanism (Cracowski et al., 2011; Maniewski et al., 2014). It is argued that whilst nitric oxide is known to play a major role in the vasodilation of macrovessels, endothelium-derived hyperpolarizing factors are found to play a substantial role in the dilation of microvessels (Quyyumi and Ozkor, 2006; Cracowski et al., 2011). Apart from these substances, the sensory nerves make a vital contribution to the PORH mechanism (Larkin and Williams, 1993; Lorenzo and Minson, 2007; Cracowski et al., 2011; Lanting et al., 2017; Marche et al., 2017). To summarise, various studies have shown that PORH response is elicited with temporary tissue hypoxia upon occlusion through the accumulation of vasodilators (substances that cause the blood vessels to dilate or expand) and other complex factors that are myogenic, endothelial, neurogenic and metabolic (Guyton, 1991; Klabunde, 2012; Lanting et al., 2017).

The PORH test has a wide range of applications. Previously, PORH has been used to assess microcirculatory function in people with arterial diseases, certain ophthalmologic conditions and cardiovascular disorders (Morales et al., 2005; Maniewski et al., 2014; Carasca et al., 2017). It is impaired in people with peripheral arterial disease and has been associated with increased cardiovascular risk (Morales et al., 2005). The test was observed to be useful as an early marker of cardiovascular damage (Busila et al., 2015). PORH test is also used to assess the altered microvascular

reactivity in patients with advanced renal dysfunction (Busila et al., 2015). Besides, the use of the PORH test has also been explored in the area of diabetes.

A limited amount of research has been conducted in people with diabetes using PORH measures, both in type 1 and 2. PORH vasodilation is significantly decreased in patients with type 1 diabetes (Marche et al., 2017). In 1986, Rayman et al. demonstrated the impaired hyperaemic response to injury in people with diabetes for the first time (Rayman et al., 1986). Prolongation of the hyperaemic reaction and decrease in response was observed in patients with insulin-dependent diabetes and peripheral occlusive arterial disease (Maniewski et al., 2014). PORH is known to be impaired not only in adults but also in children with type 1 diabetes (Schlager et al., 2012). The results from children in terms of diabetic foot complications is as important as the studies conducted in adults because of two main reasons. Firstly, although this segment of the population is less likely to be vulnerable to foot complications at a younger age, but they are likely to develop complications as they advance in age. Therefore, understanding the microvascular reactivity from an earlier period may prove to be useful. Secondly, this particular study explored other less commonly assessed variables such as biological zero and reperfusion time, which can shed more light on understanding PORH. It was identified that peak perfusion was higher and biological zero was lower in children with type 1 diabetes in comparison to the controls. A key implication from this study was that higher peak perfusion might reflect a decline in the vasoconstrictive ability of arteriolar smooth muscle cells upstream of capillary beds in children with type 1 diabetes (Schlager et al., 2012).

Few studies have explored PORH more specifically in diabetic foot complications (Cheng et al., 2004; Barwick et al., 2016; Lanting et al., 2017). The presence of peripheral sensory neuropathy in people with type 2 diabetes is found to be associated with altered PORH in the foot (Barwick et al., 2016). A study on the relationship between active or previous foot complication and PORH measured by LDF in people with type 2 diabetes revealed that the increase in time to Peak, which is a variable that shows the time taken for a maximum flux post occlusion, increased the likelihood of a participant having a history of foot complication by 2% (Lanting et al., 2017). This association was not reflected in people with an active foot ulcer (Lanting et al., 2017). These findings in a cohort with type 2 diabetes with a previous history or existing foot-related complications support the need for further investigation into the relationship between measures of microvascular function and development of diabetic foot complications, prospectively (Lanting et al., 2017). Considering this evidence, it seems that PORH is an interesting microcirculatory mechanism that may be useful to assess a foot at risk. In future, their application may be a useful indicator for determining the future risk of diabetic foot complications, especially with ulcer prediction and prevention of amputation.

In the foot, the areas prone to high pressure such as the heel, the great toe and areas under the metatarsal heads are at risk of ulceration (Veves et al., 1992; Ledoux et al., 2013). Based on this, many weight-bearing activities were considered to be a contraindication to people with neuropathy (Kluding et al., 2017). However, this has recently changed as there is emerging evidence of positive adaptations of the musculoskeletal and integumentry system to overload stress (Kluding et al., 2017). Literature suggests that peripheral neuropathy may no longer be a hindrance to promoting weight-bearing activity as it did not lead to significant increases in foot ulcers (LeMaster et al., 2008). However, in people with diabetes various other factors may interplay with pressure such as increased stiffness of tissues, aging related changes, presence of other comorbidities, mobility and vascular issues. Studies show that the accumulated mechanical stimulus affected blood perfusion in the foot and should be considered when assessing the risk of developing ulcers (Ledoux et al., 2013; Pu et al., 2018). However, more understanding on the relationship between pressure stimulus and microvascular responses could shed more light on the effect of different levels of accumulated mechanical stimulus on microvascular response and their significance in an ulcer incident.

Responses to local mechanical stresses are mediated through a considerable number and variety of cutaneous receptors and some of these receptors are connected to the small fibres (Abraham et al., 2001). The vasodilation to pressure strains not only occur for noxious stimuli but also non-noxious stimuli applied over a period (Abraham et al., 2001). Local pressure strain to the skin is recognised to play a vital role in cutaneous microcirculatory impairment (Fromy et al., 2000; Abraham et al., 2001). It is presumed that this may be linked to the development of cutaneous lesions such as pressure sores and diabetic foot ulcers (Abraham et al., 2001; Fromy et al., 2002). Two important microcirculatory responses to locally applied pressure identified through the literature review are discussed below.

**Pressure-induced Vasodilation**

The transient increase in cutaneous blood flow initially before it decreases in response to a progressive locally applied pressure strain is known as pressure-induced vasodilation (PIV). This microcirculatory response appears to be a protective cutaneous response that relies on the excitation of unmyelinated afferent nerve fibres (Fromy et al., 2002; Koïtka et al., 2004; Körei et al., 2016). PIV is considered to be more than a transient phenomenon and an important physiological response allowing the skin to respond adequately to mechanical stimuli (Abraham et al., 2001). Cutaneous receptors in the skin respond to local mechanical stresses such as local pressure strain and these receptors are found to be of mechanothermal nature (Fromy et al., 2002). This response is noted to be compromised in the aging population (Fromy et al., 2010; Fouchard et al., 2019). Furthermore, the impairment of PIV is

postulated to contribute to the development of lesions such as pressure ulcers and diabetic foot ulcers (Abraham et al., 2001; Saumet, 2005; Vouillarmet et al., 2019).

The interplay between biomechanical factors and physiological responses is well-realised in the development of pressure ulcers, including in people with diabetes. Current studies highlight PIV in relation to the development of pressure ulcers or decubitus ulcers in the sacral region. As discussed above, one of the key implications from the studies on PIV is that it is a protective mechanism without which certain pressure-associated lesions may develop and plausibly this could explain the high risk of decubitus and plantar ulcers in people with diabetes (Abraham et al., 2001; Fromy et al., 2002; Bergstrand, 2014). Although pressure ulcers and plantar ulcers may differ in many ways, one of the key causal pathways to foot ulceration is somatic motor neuropathy that leads to small muscle wasting, foot deformities, loss of sensation, increased plantar pressure and repetitive trauma resulting in neuropathic foot ulcer (Armstrong and Lavery, 2005). This suggests that local pressure strain increases the vulnerability of the foot to ulcerate. Similar to pressure ulcer development, reduced physiological responses may induce local ischaemia and reperfusion injury in the foot (Flynn and Tooke, 1992; Coleman et al., 2014). A similar role of reduced microcirculatory responses in foot ulcer development is widely discussed in the literature (Flynn and Tooke, 1992; Boulton et al., 2006; Korzon-Burakowska & Edmonds, 2006). This knowledge can potentially be translated to diabetic foot ulcer prediction to see if the microcirculatory response to local pressure and plantar pressure have any association. This also accords with other observations, which showed that people with impaired or absent PIV are known to be at a higher risk to develop pressure ulcers (Fromy et al., 2002; Braden and Blanchard, 2007; Bergstrand, 2014). Evidence shows that decreased hyperaemic response and absence of PIV is known to increase the risk of pressure ulcers (Bergstrand et al., 2014). However, very limited research is available on PIV in human hand and feet in relation to diabetes (Abraham et al., 2001; Koïtka et al., 2004).

A particular study by Koitka et al. (2004) observed PIV at the foot level in people type 1 diabetes (Koïtka et al., 2004). Since low skin temperature in people with diabetes is known to interfere with microcirculation, this research was performed in warm conditions of 29.5± 0.2°C (Koïtka et al., 2004). The cutaneous blood flow was studied at warm conditions using laser Doppler flowmetry on the first metatarsal head in response to applied pressure at 5.0 mmHg/min and PIV was found to be absent at foot level in people with type 1 diabetes whereas it existed in healthy subjects at 29.5±0.2°C (Koïtka et al., 2004). These findings were attributed to an interaction between functional changes in C-fibres and the endothelium in people with diabetes (Koïtka et al., 2004). A similar study found PIV to be absent at low skin temperature even in healthy subjects (28.7±0.4°C) (Fromy et al., 2002). It was explained that a skin temperature close to 34°C was optimal for the evaluation of skin vasomotor

reflexes in the lower limb and the nervous receptors involved in the PIV development are mechanothermal, and not only mechanical (Fromy et al., 2002). The results from Koitka et al. (2004) revealed that in the same subjects the non-endothelial-mediated response to sodium nitroprusside was preserved, whereas the endothelial-mediated response to acetylcholine was impaired (Koïtka et al., 2004). Therefore, suggesting the relevance of endothelial dysfunction to PIV. Also, a previous study on PIV found that the absence of vasodilatory axon reflex response to local pressure strain when the capsaicin-sensitive nerve terminals were pre-treated with local anaesthetic or chronically applied capsaicin (Fromy et al., 1998). The capsaicin-sensitive nerve fibres are the small nerve fibres and their role in neuropathic pain and related complications, especially in people with diabetes is well-established (Boulton et al., 2006). Thus, the researchers speculated that the PIV, which is associated with the stimulation of small fibre nerves, could be a missing link between neuropathy and foot ulcers in diabetes (Koïtka et al., 2004). In support of this, several studies have demonstrated that damage to C-fibres have a great impact on skin, with disrupted blood flow predisposing to foot ulcers (Vinik et al., 2001; Caselli et al., 2003; Boulton et al., 2006; Themistocleous et al., 2014). As previously discussed, impaired microcirculatory response to local pressure strain may potentially make people with diabetes more vulnerable to pressure strains and explain the high prevalence of foot ulcer that occurs in diabetic patients (Koïtka et al., 2004).

The insights from the above-discussed studies suggest that PIV is absent at the foot level in people with diabetes. Identifying the point or stage of the disappearance of PIV in the foot, during the disease progression through prospective studies, may help in understanding the progression of neurovascular dysfunction in the foot. On the other hand, since PIV may be absent from an earlier stage, its capability to indicate risk for ulceration is disputable and needs further research. Also, the current study has observed PIV only at two sites, which was the head of the first metatarsus and the area over the internal ankle bone in a small sample size. More research is required to explore various regions of the plantar aspect of the foot, especially in areas subject to increased plantar pressure. The findings from such research can aid in comprehending the association between PIV and plantar ulcers and help identify foot at risk. Furthermore, it may aid to bridge the research gap to understand the role of microcirculation in the development of diabetic foot ulcers.

**Reduced Skin Blood Flow to Locally Applied Pressure**

As discussed earlier, PIV allows the skin blood flow to increase in response to locally applied pressure. In the absence of the transient PIV response, the cutaneous blood flow is observed to progressively decrease with the application of increasing local pressure (Fromy et al., 2002). The observed cutaneous blood flow in response to locally applied pressure is found to be impaired in people with

diabetes owing to the combined effects of low cutaneous temperature and alterations in microcirculatory function (Fromy et al., 2002). Additionally, the presence of neuropathy may aggravate the condition (Fromy et al., 2002). This study used a laser Doppler flowmetry system and applied local pressure using a specially designed apparatus at the internal anklebone allowing for a 5.0 mmHg/min rate of pressure increase (Fromy et al., 2000; Fromy et al., 2002). The skin blood flow decreased significantly from baseline at much lower applied pressure of 7.5 mmHg in people with diabetes in groups without neuropathy and with subclinical or clinical neuropathy at 6.3 mmHg in comparison to the healthy controls at 48.8 mmHg (Fromy et al., 2002). The large difference between these pressures reported within this study indicate a plausible association between decreased skin blood flow to local pressure and the development of decubitus and plantar ulcers (Fromy et al., 2002). This hypothesis is consistent with the one proposed by Koitka et al. (2004) who suggested an association between microcirculatory dysfunction and the high prevalence of foot ulcer (Koïtka et al., 2004). They also postulate that the arterial wall and surrounding tissues are very compressible in people with diabetes making them vulnerable to the development of pressure ulcers (Fromy et al., 2002; Coleman et al., 2014). The application of this knowledge to understand the role of microcirculation in foot ulceration may potentially be useful.

Although the collated findings reveal the possibility of decreased skin blood flow and PIV to be associated with pressure ulcer development, more research is needed to understand the mechanism in relation to diabetic foot complications. The aetiology for decubitus ulcer and plantar ulcers may vary, nevertheless, pressure remains as a common contributing factor in both the incidents. Studies suggest pressure-induced local ischaemia and reperfusion injuries in relation to both pressure ulcers and diabetic foot ulcers (Flynn and Tooke, 1992; Korzon-Burakowska & Edmonds, 2006; Coleman et al., 2014; Shahwan, 2015). Understanding PIV, reduced skin flow and other microcirculatory responses in various regions prone to diabetic foot ulcers and in relation to plantar pressure during standing or walking are important. The need for such a study is further supported by the evidence from a study that identified subjects who lacked PIV and reactive hyperaemia in response to locally applied pressure, to be particularly vulnerable to pressure exposure (Bergstrand, 2014; Bergstrand et al., 2014). These subjects were stratified to be at a higher risk for pressure ulcer development (Bergstrand, 2014; Bergstrand et al., 2014). Thereby, translating the knowledge generated from the studies on microcirculatory responses in the development of pressure ulcers to diabetic foot ulcers can prove to be useful.

Interplay Between Microcirculation and Temperature - Vasodilation in Response to Local Heating

While specific literature on the microcirculatory responses and temperature changes in response to plantar skin tissue injuries and healing are limited, previous studies reviewed microcirculatory assessments in various organs in people with diabetes. The knowledge of microcirculatory responses to temperature changes in other organs, can reveal that external stimuli causes an increased microvascular demand. This showcases the role of cutaneous microcirculatory response in tissue injuries and healing.

When injuries and repair occur, monitoring the conditions between the skin, soft tissues or even after skin grafts can aid better prognosis. A study explored the proposed theory that conducive interface conditions between soft tissue and prostheses are necessary for a better outcome with prosthodontic treatment. This study by Nakamoto et al. (2012) focused on the gingiva and mucosa surrounding anterior implants and both LDF and thermographs were concurrently used to elucidate the relationship between temperature and blood flow as peri-implant soft tissues are often portrayed to have decreased blood flow because of the lack of blood supply from the periodontal ligament. The study also analysed the morphological changes of the cutaneous microvasculature and temperature changes between participants with and without bone grafting associated with implant placement. The findings suggested that soft tissue around implants showed decreased blood flow compared with periodontal tissue in adjacent natural teeth, despite the absence of clinical signs such as chronic inflammation. The study also highlighted the significance of bone quality to maintain blood flow in the soft as the area around implants with bone grafting showed significantly reduced blood flow. Many research studies suggest that microcirculatory blood flow is influenced by thermal changes and reportedly increases in proportion to temperature to an extent, which is not limited to dentistry but also in studies on other cutaneous microcirculation (Molnár et al., 2015). However, the observed results by Nakamoto et al. (2012) were contrary to this popular idea. The suggested explanation for this was the involvement of deeper structures that modified the thermal properties and the usually observed increase in temperature was often associated with inflammation due to infection such as periodontitis but not in case of tissue surrounding implants (Baab et al., 1990).

Although the skin and the oral mucosa have certain similarities and differences anatomically, they have some comparable physiological properties. For instance, they play a crucial role in the prevention of infections and act as a barrier against exogenous or endogenous substances, pathogens, and mechanical stresses (Liu et al., 2010). The dysfunction of these barriers can compromise the integrity of the underlying tissue as well. The combination of findings from the study provides some support for the conceptual premise that the simultaneous measurements of blood flow and temperature are useful to evaluate the microcirculation of soft tissue behaviour in injury and healing, and its significane even in the absence of noticeable signs chronic inflammation. A similar study compared the peripheral

blood flow in the lower limbs during the local heating tests with different temperature protocols in people with diabetes mellitus and healthy participants (Filina et al., 2017). The LDF was used to evaluate the adaptive changes of the microvascular bed during thermal tests and the detection of the preclinical stage of trophic disorders owing to disruption in nutritional or nerve supply (Filina et al., 2017). Research suggest that in the feet of patients with diabetic neuropathy, total skin blood flow is increased due to an increased shunt flow due to denervation (Harpuder et al., 1940; Schaper et al., 2008). Further study in the area has shown that the increased anastomotic shunt flow lead to either under- or over perfused nutritive capillaries (Netten et al., 1996). Skin temperature measurements and LDF were performed to record mainly shunt flow and capillaroscopy to study nailfold capillary blood flow (Netten et al., 1996). The study showed that in insulin-dependent diabetic patients with neuropathy, the baseline skin temperature and capillary blood-cell velocity was higher in comparison to those without neuropathy and healthy control subjects (Netten et al., 1996). The findings from the study highlighted the presence of hyperperfused nutritive capillary circulation in the feet of patients with diabetic neuropathy favouring the previously discussed hyperdynamic hypothesis and in contradiction to the capillary steal phenomenon to explain the decreased healing potential in diabetic neuropathic foot ulceration.

As suggested by previous research, microcirculatory and temperature measurements might become useful techniques to evaluate healthy, infected, injured, inflamed and treated skin and soft tissues of the foot (Netten et al., 1996; Gatt et al., 2018; Gatt et al., 2020). But, there is abundant room for further progress in determining if these two measurements may be useful for the diagnosis or prognosis of foot ulcers. Such research may aid to draw a margin between the compromised tissue and the surrounding healthy tissue when determining the course of treatment, surgery or even amputation. Furthermore, comparative studies conducted on healthy vs inflamed/injured tissue in the foot can help to identify early signs of dysfunction, inflammation and injury in a foot in order to effectively manage the condition. For instance, Ren et al. (2021) explored the stimulation of microcirculation using simple thermal stimuli such as infrared and warm bath in healthy adults to explore the options in hope to design interventions to promote better circulation in the lower extremities of the body in the geriatric population and those suffering from diabetes who are likely to have impaired microcirculation (Ren et al., 2021).

The vasodilation in response to local heating and the neurogenic flare response to nociceptive stimuli is mediated by an axon reflex involving C-fibres. This is studied using the laser Doppler imager (LDI) and the induced flare response is known as the LDI flare. The LDI flare area which is the area with the hyperaemic response is known to be reflective of the small fibre function. Therefore, the size of the LDI flare is known to be dependent on the C-fibre function and the underlying skin small fibre neural

network and its extent (Green et al., 2010; Vas et al., 2012). Whereas, the LDI max (perfusion) in the skin immediately beneath the heating probe is shown to be mediated by non-neurogenic means and to reflect the endothelial function (Green et al., 2010; Vas et al., 2012). Therefore, the intensity of the hyperaemic response depends on the microvascular ability to vasodilate. The site commonly studied is the dorsum of the feet because the underlying skin is less influenced by the thermoregulatory blood flow due to the absence of arteriovenous anastomoses (Braverman, 2000). The method used to assess this reflex involves local skin heating to 44°C for 20 min or 6 min in a stepwise fashion: 44°C for 2 min, 46°C for 1 min and finally 47°C for 3 min in a temperature-controlled room to evoke the flare followed by scanning the site using an LDI to measure the area (Krishnan and Rayman, 2004; Green et al., 2010; Vas et al., 2012). Another technique is also known to be used to observe the hyperaemic response to local heating. This involves the use of a skin-heating probe filled with deionized water and heating to 44 °C to assess heat-induced vasodilation. In summary, the LDI flare test in subjects shows reduced microcirculatory response as well as a neurogenic flare in people with either type 1 or two diabetes (Krishnan and Rayman, 2004; Vas et al., 2012). It facilitates early diagnosis of C-fibre dysfunction even before its detection by other available methods such as the quantitative sensory testing, which focuses on the testing of sensory abnormalities in the areas of temperature change sensation, vibration, and pain threshold testing (Example: Using equipment named Computer Aided Sensory Evaluator–IV - case IV) (Krishnan and Rayman, 2004). Therefore, the heat provocation or LDI flare test is commonly used with a focus on LDI flare for the assessment of C-fibre function than with a concentration on the LDI max for evaluating the microcirculatory function. However, the test can be used to assess not only C-fibre function but also microcirculation, and additionally investigate their association in neuropathy (Vas et al., 2012; Marche et al., 2017). This can further clarify the link between microcirculation impairment and tissue damage in light of impaired sensation.

## Conclusion

Microcirculation plays a vital role in homeostatic and defence states during tissue injury and inflammation. Firstly, the most obvious finding to emerge from this review is the protective role of microcirculation. Secondly, the impairment of microcirculation and the possibility of it being the missing link in the chain of events that leads to foot ulceration in people with diabetes is clearly supported by the current findings. Thirdly, assessment of microcirculatory structural damages might be complex, however, the insights emerged from this review has shown that there are responses such as post-occlusive reactive hyperaemia, pressure-induced vasodilation and vasodilation to local heating (LDI flare) that are simple to assess. In conclusion, a thorough understanding of the microcirculatory function and its impaired reactive mechanisms is imperative and will contribute extensively to understanding the soft tissue biomechanics and aid to devise strategies for comprehensive

assessment of the diabetic foot. This, in turn, will aid in prevention and early diagnosis of ulcers, thereby, reducing amputations.

## Author Contributions

GB: Conducted the literature review and wrote the first draft of the manuscript. NC: Reviewed the manuscript draft and provided comments. RN: Developed the concept and contributed to revising the draft and shaping the manuscript.

## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Abbreviations

PORH, Post-Occlusive reactive hyperaemia; PIV, Pressure-Induced Vasodilation; LDI, Laser Doppler Imager; GDP, Gross Domestic Product.

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## Appendix 3: Primary Research Article Published with the Journal of Microcirculation

**Title:** Systematic Evaluation of Cutaneous Microcirculation in the Foot Using Post-Occlusive Reactive Hyperemia

Running title: Evaluation of microcirculation in the foot

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Abstract:

Objectives: Cutaneous microcirculatory impairments are associated with skin injury to the foot. Post Occlusive Reactive Hyperemia (PORH) is one of the quick and easy methods to assess microcirculatory function. However, there are variations in the protocols currently used. Hence, this study aimed to systematically investigate the reproducibility of PORH protocols with minimal occlusion time in the foot.

Methods: PORH was measured using 12 different protocols (3 occlusion times, 2 occlusion sites and with or without temperature control) in 25 healthy adults. Each of the 12 different protocols was tested 3 times and the Intraclass correlation coefficient (ICC) was calculated.

Results: ICC showed that that ankle level occlusion produced moderate to excellent reproducibility for most PORH measures. In the right foot, 30- and 60- seconds ankle level occlusion without temperature

control showed ICC of >0.40 for all parameters except the area of hyperemia (ICC = -0.36) and biological zero to peak flow percent change (ICC = -0.46). In the left foot, 30 seconds ankle level occlusion without temperature control showed ICC of >0.40 for all parameters except time to latency (ICC = 0.29), after hyperemia (ICC = 0.37), and max (ICC = -0.01), and area of hyperemia (ICC =-0.36). But the 60- seconds protocol showed ICC >0.40 for all except time to max (ICC = 0.38). In the hallux protocols, all three 10-, 30- and 60-seconds protocols without temperature control showed moderate to excellent reproducibility (ICC >0.40). In most cases, the temporal and area under the perfusion-time curve parameters showed poor reproducibility.

Conclusion: PORH can be tested efficiently with a minimal occlusion time of 10-seconds with hallux occlusion and 30-seconds with ankle occlusion in the foot. This can suggest that microcirculatory assessment is feasible in routine practice and can be included for routine assessment of foot in people with diabetes.

Keywords: Microcirculation, PORH, Reactive Hyperemia, diabetic foot

**List of abbreviations:**

Post-Occlusive Reactive Hyperemia – PORH

Intraclass Correlation Coefficient - ICC

Ankle Brachial Index - ABI

Toe Brachial Index - TBI

Endothelium-Derived Hyperpolarizing Factors - EDHFs

Laser Contrast Speckle Imager - LSCI

Peripheral Arterial Obstructive Disease - PAOD

Laser Doppler Perfusion Monitoring – LDPM

Perfusion Units - PU

Rest Flow - RF

Biological Zero - BZ

Peak Flow – PF

Time to Latency - TL

Time to Recovery – TR

Time to Half Before Hyperemia - TH1

Time to Max – TM

Time to Half After Hyperemia - TH2

Area of Occlusion - AO

Area of Hyperemia - AH

Area of Hyperemia/Area of Occlusion; Hyperemia repayment ratio - AH/AO

**Background:**

For long the peripheral vascular function assessments have relied on macrocirculatory measures such as Ankle Brachial Index (ABI) and Toe Brachial Index (TBI). Even assessment of peripheral vascular function in at-risk individuals for complications such as foot ulcers, clinical decisions, and risk stratification are based on guidelines that have been traditionally limited to measures such as ABI and TBI 1–5. However, in recent times the measurement of microcirculation has gained importance, especially in the field of diabetes. Previous research shows that microcirculation plays a major role in diabetic foot-related complications 6–8. The recent development in medical technologies has facilitated the non-invasive assessment of the microcirculation. Various provocation tests are used to assess the cutaneous microcirculatory responses such as heat provocation, cold provocation, postural changes and application of pressure stimuli 9–11. One such test is Post Occlusive Reactive Hyperemia (PORH) which is the transient increase in blood flow in the organ or tissue that occurs following a brief period of arterial occlusion 12,13. The current study's literature review focused on identifying the knowledge gaps in measuring PORH at the foot and the aims and objectives of this research were to address some of the key gaps to strengthen the existing evidence base in order to suggest its future application in diabetic foot syndrome.

PORH is known to be primarily an endothelial-dependent process, however, both endothelial-dependent and independent mechanisms are involved 14,15. The hyperemic response is a result of the shear stress, the tangential frictional force acting at the endothelial cell surface caused by arterial occlusion. The endothelium releases vasodilatory substances in response to the mechanical stimulus 14. Various factors are known to contribute to the vasodilation which are myogenic, neurogenic, humoral and other local factors, such as potassium ions, hydrogen ions, carbon dioxide, catecholamines, prostaglandins, and adenosine 14,15. Endothelial nitric oxide and other endothelium-derived agents, such as prostaglandins and endothelium-derived hyperpolarizing factors are particularly known to play a role in the mechanism of PORH. Apart from these substances, the sensory

nerves contribute to the PORH mechanism 15–20. PORH is a quick, easy and useful method to assess microcirculation in the foot. However, the methods and equipment used to measure PORH widely. Also, there were three distinctive variations identified in the protocols occlusion time, use of temperature control at the probe site and occlusion site.

The review of existing literature identified a range of equipment that has been used to measure PORH. Recently, there are types of equipment that measure the change in blood perfusion proficiently such as commonly used Laser Doppler flowmetry or fluxmetry system with a pressure unit 15,21 or Laser Speckle Contrast Imager (LSCI) which helps to visualize the reactive hyperemia in real-time 22. The Laser Doppler flowmetry or fluxmetry system have exclusive software programs that facilitate to run automated protocols and monitor various measures of interest continually. So far, the evidence on the reliability of measuring PORH is scarce 23. The study by Barwick et al. (2015) compared the use of various techniques but not protocols. Therefore, there is a need for more studies to enable the measurement of PORH reliable and practical its application in a clinical setting 23.

As highlighted earlier, the protocols used to measure PORH varies widely and there is no standard protocol. Firstly, in terms of occlusion time, studies have used occlusion times ranging from 30- to 180-seconds in people with peripheral arterial disease, ulcers and history of ulcers 15,20–22. Whilst these studies pave a way to understand the importance of measuring PORH and its relevance to various complications, there remains a need for more evidence to support the selection of protocols in order to systematically investigate PORH in minimal time. Morales et al. (2005) recommended a protocol to measure PORH in people with peripheral arterial obstructive disease 21. According to this study conducted in people without diabetes, the PORH measurements were obtained with an occlusion that lasted for a maximum of 3 minutes or 1 minute in case of strong leg pain and the entire (both acclimatisation and measurement) session lasted for 33 minutes without stops 21. But, these long provocation tests in people with diabetes might trigger tissue damage, discomfort and pain, especially when they present with various complications. In such conditions, to decrease the risk of tissue damage, discomfort and pain, the tests need to be conducted in minimal time.

Secondly, in terms of using temperature control at the probe site, the recommendations vary. Some studies recommend temperature control at the probe site and in contrast others do not recommend the use of it 15,21,23. But, the study population varied in these studies, especially in terms of including people with diabetes 15,21,23. The influence of temperature on vascularity is one of the reasons for the use of an optimal temperature of 32-33C is recommended on order to standardize the methods for cutaneous vascular evaluation, however, this may not facilitate to identify physiological differences 23–25. A previous study has demonstrated the relationship between PORH measure (time to peak)

and sensory neuropathy, which is the involvement of small sensory nerve fibers 20. This suggests the role of sensory nerves in mediating the cutaneous microcirculation. The foot temperature in people with diabetes varies incredibly. People with diabetes may either present with warm or cold feet depending on the presence of neuropathy and its type, based on which the protocols for PORH needs to be customized 26. Therefore, the reproducibility of protocols with and without temperature control needs to be evaluated.

Thirdly, different studies have used different occlusion sites 15,21,23. Thigh occlusion may be better whilst assessing PORH in people with complications such as arteriosclerosis, diabetes, renal insufficiency and such as it may be more reliable than ankle 21,27. However, people with diabetes tend to be overweight/obese and often present with various complications (predominantly vascular and neuropathic). Hence, it may not be convenient and always possible to perform thigh occlusion. ABI is a common macrovascular measure in people with diabetes. In people where ABI could be unreliable due to arterial calcification, TBI is measured 28,29. Considering these measures of macrocirculation, similar strategies can be applied whilst measuring PORH by occluding the blood flow at either the ankle or hallux site. Furthermore, in people with digital amputations hallux occlusion is impossible. This necessitates the evaluation of reproducibility of PORH protocols measured with occlusion at the ankle and hallux sites.

In summary, upon identifying the three key gaps in the literature, this study aimed at investigating reproducibility of PORH protocols in the foot of healthy young adults. The objective was to investigate a combination of occlusion time, occlusion site and the skin temperature control protocols that can be reproducible.

**Methodology:**

Participants

There were 25 healthy participants (15 females and 10 males) who participated in this study, which was conducted upon obtaining University Ethics Committee's approval. The mean (standard deviation) age of the participants was 26.9 (9.2). The average height, weight and BMI were 168.1cm (14.2) and 72.9 kg (16), and 26.3.kg/m2 (5.6), respectively. The participants were recruited through convenience sampling. Any adult over the 18 years with no severe neurological or vascular issues and no major vascular trauma or injury (bleeding, bruising, hematoma and fractured bones) that affects circulatory measurements could participate in the study. The participants were requested not to consume any caffeinated or alcoholic beverages 2 hours before the study as this is known to affect vascular measures 30–32. Besides, they were requested not to engage in any strenuous exercise of

any form 2 hours prior to the study 33. The participants upon arrival to the Biomechanics laboratory were familiarized to the settings and the protocol. The test was performed by a single observer who has a medical and clinical research background. .

**Protocol**

The participant was requested to lie supine on the couch. The nature of the tests required the participants to be as still as possible during the recording as even minor movements cause artefacts. The study commenced after a minimum of 15 minutes of acclimatisation (Barwick, Lanting and Chuter, 2015). The laser Doppler probes (contact area 1 cm2 each) were secured using a double-sided adhesive tape from on the distal/plantar aspect of the hallux for ankle and toe pressure and PORH measurements or the pulp of the index finger for the arm pressure measurements. The ABI and TBI were measured as they are common macrocirculatory measures to confirm healthy peripheral vascular status of participants. The PORH occlusion was at the ankle and hallux levels (one followed by other), while the cuffs were inflated to a supra systolic pressure (~200 mmHg). Firstly, the PORH protocols tested were three different occlusion times 10, 30 and 60 seconds. The recording for 60 seconds protocol consisted of two minutes baseline, occlusion for 60 seconds and two minutes of post-occlusion. The recording for 30 seconds protocol consisted of one-minute baseline, occlusion for 30 seconds and one minute of post-occlusion. The recording for 10 seconds protocol consisted of 30 seconds baseline, occlusion for 10 seconds and 30 seconds of post-occlusion. Secondly, the same protocols were tested under two different conditions without and with temperature control of 33̊C at the probe site. Finally, the same set of protocols were tested with occlusion at two different sites, ankle and hallux. Both right and left feet were evaluated simultaneously. On the whole, there were 12 protocols (Figure 1) tested over 2.5 to 3 hours in a sequential manner and the rest between each testing was 60 seconds.

**Equipment**

The laser Doppler flowmetry (Perimed Periflux system 5000) system was used for data collection. There can be several main systems, each equipped with four functional units to have the desired number of channels that facilitates simultaneous measurements of various vascular parameters. The system that was used for this study was a single main unit with 4 different functional units: including: 2 perfusion units, 1 temperature unit and 1 pressure unit. The pressure unit helps to simplify and standardize tests such as peripheral vascular pressure measurements and PORH. The thermostatic laser Doppler probes were used to allow temperature monitoring for protocols without temperature control and to precisely maintain a temperature of 33̊C for protocols with temperature control at the measurement site. The kit with two Laser Doppler Perfusion Monitoring (LDPM) units allowed

simultaneous blood pressure measurements at both arms followed by both ankles and halluces. The system was attached to a laptop with exclusive software, PeriSoft that helps to run the protocols in a sequential manner and record data. The steps for the protocols and the sequence of events were defined in order to facilitate effective, precise and replicable data collection across participants. The inflation of the pressure cuffs was manual; however, the deflation was automatic. The data for ABI, TBI and the PORH measures were collected using the same equipment.

**Data analysis**

For every participant, each of the 12 protocols, three trials were performed. Upon completion of each test, the PORH area, which included a baseline, occlusion trough and a hyperemic area was marked (Figure 2) for the system to autogenerate the results with values for PORH measure. This was done for every trial. Then, the reproducibility for the 14 parameters in Table 1: Rest Flow (RF), Biological Zero (BZ), Peak Flow (PF), Time to Latency (TL), Time to Recovery (TR), Time to Half Before Hyperemia (TH1), Time to Max (TM), Time to Half After Hyperemia (TH2), Area of Occlusion (AO), Area of Hyperemia (AH), Area of Hyperemia/Area of Occlusion and Hyperemia repayment ratio (AH/AO) was analyzed through SPSS and Microsoft EXCEL was used for collating information. For every protocol, for each of these 14 measures, ICC between the three trials was statistically analyzed. Regarding ICC values, >0.75, 0.40-0.75, <0.40 were considered to be excellent, moderate (fair-to-good) and poor reproducibility 34. All these statistical tests were conducted for right and left foot data separately.

**Results:**

There were 14 different PORH measures recorded by the equipment (Figure 2). The mean with error bars (95% Confidence intervals) for 3 different measures of PORH across 12 protocols is provided in Figures 3 and 4. For the right and left foot, the mean and the trend across protocols for temporal parameters TM and TR are presented in Figures 5 and 6 and Figures 7 and 8, respectively. The terms without temperature control and no heating as well as with temperature control and heating protocols has been used interchangeably. The ICC values, along with mean and standard deviation for key parameters of interest are presented in Table 2.

Overall, ICC showed moderate to excellent reproducibility for most PORH measures with ankle level occlusion. The perfusion parameters (RF, BZ and PF) showed excellent reproducibility with all protocols. Closer inspection of the results showed that the 30- and 60-seconds without temperature control protocols showed moderate to excellent reproducibility for most PORH measures. In the right foot, 30-seconds ankle level occlusion without temperature control showed ICC of >0.40 for all parameters except AH (ICC = -0.36). More specifically, the ICC Values were > 0.75 for RF, BZ, PF, RF-

BZ, BZ-PF, TL, TH, TH2 and AO parameters (p value <0.05). For RF-PF, TR, TM, AH/AO the ICC ranged between 0.40-0.75 (p value< 0.05) showing moderate reproducibility. The 60-seconds ankle level occlusion without temperature control in the right foot showed ICC of >0.40 for all parameters except BZ-PF percent change (ICC = -0.46). The ICC values were > 0.75 for RF, BZ, PF, RF-BZ, RF-PF, TL and TM (p value< 0.05). The ICC values ranged from 0.40-0.75 for TR, TH, TH2, AO, AH and AH/AO (p value< 0.05).

Similarly, in the left foot, 30 seconds ankle level occlusion without temperature control showed ICC of >0.40 for all parameters except three temporal parameters (ICC values TL= 0.29, TH2 = 0.37, TM = -0.01) and AH (ICC =-0.36). With the 60- seconds protocol showed ICC >0.40 for all except TM (ICC = 0.38).

In the hallux protocols, all three 10-, 30- and 60-seconds protocols without temperature control fared well as they had a minimum of 12 out of 14 PORH measures that showed moderate to excellent reproducibility (ICC >0.40) (Table 2).

When temperature control was used at the probe site, be it with the ankle or hallux level occlusion, mostly the temporal and area under the perfusion-time curve parameters showed poor reproducibility with ICC values <0.40. Additionally, the mean (standard deviation) for ABI was 1 (0.13) and TBI was 0.66 (SD 0.14).

**Discussion:**

General remarks

The use of software aided protocols helps to pre-define the steps and abled to run tests systematically and time-effectively across participants. The nature of the tests required the participants to be as still as possible during the recording as even minor movements cause artefacts. Each of the 12 protocols was performed at ease in minimal time. For instance, the 60 seconds protocol took only a maximum of 5 minutes and 10 seconds (10 seconds preparation, 2 minutes baseline, 1-minute occlusion and 2 minutes hyperemia recording).

This study was conducted in healthy subjects to assess the feasibility of conducting the test and reproducibility of various protocols. This knowledge may further be translated to research and practice while investigating PORH of the foot in people with various pathological conditions. This study showed that the output for 30- and 60-seconds protocols produced more consistent results in the ankle as the output missed at least one parameter for a minimum of one of three trials in 24 participants for the 10 seconds protocol. A possible explanation for this might be that the 10 seconds occlusion time was insufficient to produce a hyperemic response. This is in line with the existing

literature which suggests that the hyperemic response corresponds to the occlusion time 19. The greater the occlusion time greater the hyperemic response; this is because longer the period of occlusion, the greater the metabolic stimulus for vasodilation resulting in an increased in peak flow and prolonged duration of hyperemia 12,19. Depending on the time taken to occlude the tissue blood supply, the reactive hyperemia increases four to seven times the baseline in the tissue and lasts from few seconds to hours in relation to the initial occlusion time. In this instance, the use of 10 seconds occlusion time at the ankle did not sufficiently provoke the vasodilation for a decent hyperemic response. However, this was not the case with hallux level occlusion. This may be due to the smaller surface area and the type of vessels in the hallux as compared to the ankle region.

Another key observation was that there was increased perfusion when protocols with temperature control were used, which suggests the role played by the thermoreceptors on the microcirculation and the effect of foot temperature on the cutaneous microcirculation. This can be observed in figure 3 and figure 4, where the perfusion measures RF, BZ and PF are higher in protocols with temperature control of 33℃. This is consistent with the suggestions from a previous study on the influence of temperature on cutaneous microcirculatory responses such as pressure-induced vasodilation 24,35. Certain microcirculatory responses such as pressure-induced vasodilation are known to be absent even in healthy subjects due to low foot temperature and the role of mechanothermal receptors in such instances are highlighted 13,24,35. These observations suggest an association between the small fiber nerve function and skin microcirculation. These findings generate interesting questions regarding the nature and extent of microcirculatory changes influenced by temperature variation. Further comparative analysis would be useful to understand the differences. While this was not within the scope of this study, it would be an area for further work.

Key remarks

The key measures of interest were the PF, RF to PF percent change, TM and TR as these measures are similar to the common parameters discussed in previous literature 15,21,34. The PF parameter showed excellent reproducibility (ICC > 0.75; p-value < 0.05) across all 12 protocols. Similarly, RF to PF percent change showed either moderate or excellent reproducibility (ICC > 0.40; p-value < 0.05) across all 12 protocols, except in one instance with 10-seconds occlusion at the left ankle without temperature control at probe site where it showed poor reproducibility (ICC = 0.15; p-value = 0.3). But, it is important to note that as highlighted earlier, 10-seconds protocols at the ankle never generated consistent reports for any parameters.

The ICC values for TM and TR showed variations across protocols. Also, it can be observed from Figure 5 and Figure 6 that the TM was higher with ankle occlusion protocols than in the hallux occlusion ones

in both feet. Furthermore, as seen in Figure 7 and Figure 8, the TR shows a similar trend. This implies an existing relationship between the occlusion site and the time taken for a hyperemic reaction. The reperfusion of the entire foot may have taken longer compared to the hallux. These findings help to realize the importance of understanding the use of different protocols and their influence on the PORH measures. Further comparative analysis may aid to understand these differences.

In the current study, the ICC showed excellent reproducibility (p-value <0.5) for all perfusion parameters (RF, BZ and PF) across all 12 protocols. The ICC for percent change measures showed moderate to excellent reproducibility for most protocols, except three occasions. However, the ICC parameters for temporal parameters varied across protocols (Table 2 shows the results for temporal parameters of interest TM and TR).

Previous research has indicated mixed results on the reliability of PORH in the upper limb 34,36,37. As indicated by Barwick et al. (2015) there is a wide variation within the literature on the reliability of PORH. A possible reason being Laser Doppler Flowmetry system is extremely sensitive and the measurement varies depending on the probe location such as being directly over an arteriovenous anastomosis. Furthermore, the PORH measures are sensitive to various factors such as temperature 23. But, Barwick et al. (2015), found that PORH can be measured reliably, especially with the use of temperature control. Similarly, the current study found that PORH can be measured reliably. However, the current study found moderate to excellent reproducibility when using without temperature control in contrast to the findings from the previous study with hallux level occlusion 23. A potential reason for this could be the fact that the current study was conducted in healthy subjects whereas the other study was conducted in people at risk of peripheral arterial disease. When the temperature control at the probe site was not used, wider variations in the PORH measures leading to poor reproducibility was observed, however, with the temperature control variations were minimised 23. Since the current study used healthy participants, even the protocols with temperature control showed good reproducibility. As suggested in the literature, temperature control at the probe site may aid to minimize the variations in the perfusion measures as mere control for room temperature may not be adequate 23. However, measurements obtained using temperature control at the probe site is a less physiologically relevant 21,23,25. Microcirculatory measurements are influenced by the underlying pathological conditions, therefore, deeper understanding of variations with protocols may aid to obtain PORH measures that are methodologically sound and also physiologically relevant.

**Future implications**

The data collected using 12 different protocols helped to assess the feasibility and reproducibility of results whilst systematically investigating PORH in the foot. The study is a stepping-stone to suggest

incorporation of less-time consuming microvascular assessments in routine practice. According to these data, we can infer that the 30-and 60-seconds protocols produced consistent results. Thus, the present study raises the possibility that PORH can be measured using minimal time as little as 30 seconds in the foot. These protocols can be easily replicated in a clinical or research setting. However, a better understanding of the variations between the measures based on the choice of occlusion time, occlusion site and temperature control is required. This may aid to interpret the PORH measures to understand the physiological significance for diagnostic purposes. The results also highlighted the influence of temperature on microcirculation. This can be further studied to pinpoint the temperature changes and the associated microcirculatory responses which may help to throw light in assessing foot complications based on thermal parameters. Having a defined protocol that can be executed in a semi-automated manner using the relevant software help to produce consistent results. This suggests that microcirculatory assessment can be performed feasibly within minimal time. In future, this knowledge can be translated in routine practice for routine assessment of foot in people with diabetes. This will be beneficial as the role of microcirculation in ulceration and wound healing is well-realized, which compels the need for a more comprehensive assessment of the foot at-risk.

**Future application in diabetic foot syndrome**

In people with type 2 diabetes, it was found that certain PORH measures such as Time to peak and percent change from baseline were associated with the presence of peripheral sensory neuropathy, cardiac autonomic deficits, critical-ischaemia in the feet and previous history of ulcers/amputations 15,20,22. These findings from previous research support the need for incorporating microcirculatory investigation in people at risk for diabetic foot complications. People with diabetes can present with a plethora of symptoms based on several foot complications. Based on pain due to underlying pathologies, surgical complications, deformities and previous or current history of ulcers/amputations, performing microvascular assessments may be challenging. In order to overcome these challenges, it is essential to customize testing protocols for microcirculatory assessment. ABI is less reliable in people with arterial calcification and in such population, TBI may be useful 28,29. Upon extrapolation of this observation, it can be implied that PORH performed at a toe level occlusion may be reliable in people with arterial calcifications. This study showed that PORH testing was reproducible in both ankle and hallux occlusion, which may be useful when a decision must be made to select an occlusion level. This study has supported the idea and shown that the testing can be performed with minimal time. There is abundant room for further progress in determining if these findings may have practical implications whilst assessing people with a diabetic foot. In future, it may have implications for determining the risk of diabetic foot complications, especially with ulcer prediction, wound healing and prevention of amputation.

**Strengths and Limitations**

This study tested the same session reproducibility. An advantage of this same session testing (similar to a real-world setting) is that the factors that influence variations in physiological measures are restricted. In contrast, the limitation is that the day to day variations in reproducibility and the potential factors influencing the measures were not studied. The test was performed by a single observer. The study examined reproducibility in healthy subjects. Although the findings from the study hold a value from a methodological standpoint, its application to detect dysfunction in people with pathological conditions needs to be determined. As mentioned earlier, PORH assessment using Laser Doppler Flowmetry system is relatively quick and easy to perform in comparison to thermal provocation tests. However, the equipment is expensive. For this reason, it is used more for research purposes rather than clinical use at present. If the assessment aids with early diagnosis and prevention of adverse complications such as ulcers and amputations, it may prove to be beneficial in a long-term. This needs further investigation. Additionally, there is scope to investigate the cost-effective options for microcirculatory assessments for clinical use.

**Conclusion:**

This study assessed the reproducibility PORH parameters measured using 12 different combinations of PORH protocols in young healthy adults in the foot. The key highlights are that PORH can be reliably tested and in a very little time. The 10-seconds occlusion time was sufficient to induce a hyperemic response with occlusion at the hallux but not the ankle, which did affect the reproducibility. The protocol using 30- and 60- seconds occlusion time fared well in comparison to 10-seconds protocol and can be considered the most minimal time for ankle occlusion.

**Data availability:**

All original anonymized data for the study is available.

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Table 6.1: Various PORH Measures

The table shows various measures of PORH in the report generated by Laser Doppler Flowmetry/Fluxmetry system

| **Perfusion measures (PU)** | **Percentage change measures (%)** | **Time measures (milliseconds)** | **The area under the curve** |
|---|---|---|---|
| **RF - Rest flow**<br>**Baseline blood perfusion** | RF - BZ: Percent change | TL - Time to latency<br>Time taken to reach baseline flow | AO - Occlusion area (Unit*sec.)<br>The area under the occlusion curve |
| **BZ - Biological zero**<br>**Temporary cessation of the blood flow during occlusion** | BZ - PF: Percent change | TR - Time to recovery<br>Time taken to recover baseline level after the occlusion is released | AH - Hyperemia Area (Unit*sec.)<br>The area under the hyperemic curve |
| **PF - Peak flow**<br>**Maximum perfusion after the release of occlusion** | RF - PF: Percent change | TH1 - Time to half before hyperemia<br>Time taken after the release of the occlusion for perfusion to reach the midpoint between no-flow and peak flow | AH/AO - Hyperemia repayment (ratio) |
| | | TM - Time to max<br>Time taken after the release of the occlusion for perfusion to reach peak flow | |
| | | TH2 - Time to half after hyperemia<br>Time taken after the occlusion release for perfusion to reach the midpoint between peak flow and baseline | |

Table 2: ICC for PORH parameters in the Foot

| Right Foot | | | | Left Foot | | | |
|---|---|---|---|---|---|---|---|
| PF | | | | PF | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.96 | 0.00 | ANKLE | 30-seconds OT without TC | 0.96 | 0.00 |
| | 30-seconds OT with TC | 0.98 | 0.00 | | 30-seconds OT with TC | 0.96 | 0.00 |
| | 60-seconds OT without TC | 0.98 | 0.00 | | 60-seconds OT without TC | 0.98 | 0.00 |
| | 60-seconds OT with TC | 0.96 | 0.00 | | 60-seconds OT with TC | 0.92 | 0.00 |
| HALLUX | 10-seconds OT without TC | 0.99 | 0.00 | HALLUX | 10-seconds OT without TC | 0.97 | 0.00 |
| | 10-seconds OT with TC | 0.97 | 0.00 | | 10-seconds OT with TC | 0.93 | 0.00 |
| | 30-seconds OT without TC | 0.97 | 0.00 | | 30-seconds OT without TC | 0.99 | 0.00 |
| | 30-seconds OT with TC | 0.97 | 0.00 | | 30-seconds OT with TC | 0.97 | 0.00 |
| | 60-seconds OT without TC | 0.98 | 0.00 | | 60-seconds OT without TC | 0.98 | 0.00 |
| | 60-seconds OT with TC | 0.95 | 0.00 | | 60-seconds OT with TC | 0.95 | 0.00 |
| RF-PF Percent Change | | | | RF-PF Percent Change | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.65 | 0.00 | ANKLE | 30-seconds OT without TC | 0.85 | 0.00 |
| | 30-seconds OT with TC | 0.73 | 0.00 | | 30-seconds OT with TC | 0.54 | 0.01 |
| | 60-seconds OT without TC | 0.92 | 0.00 | | 60-seconds OT without TC | 0.88 | 0.00 |
| | 60-seconds OT with TC | 0.61 | 0.00 | | 60-seconds OT with TC | 0.52 | 0.01 |
| HALLUX | 10-seconds OT without TC | 0.90 | 0.00 | HALLUX | 10-seconds OT without TC | 0.94 | 0.00 |
| | 10-seconds OT with TC | 0.95 | 0.00 | | 10-seconds OT with TC | 0.89 | 0.00 |
| | 30-seconds OT without TC | 0.94 | 0.00 | | 30-seconds OT without TC | 0.94 | 0.00 |
| | 30-seconds OT with TC | 0.87 | 0.00 | | 30-seconds OT with TC | 0.86 | 0.00 |
| | 60-seconds OT without TC | 0.89 | 0.00 | | 60-seconds OT without TC | 0.93 | 0.00 |
| | 60-seconds OT with TC | 0.63 | 0.00 | | 60-seconds OT with TC | 0.64 | 0.00 |
| TM | | | | TM | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.58 | 0.01 | ANKLE | 30-seconds OT without TC | -0.01 | 0.49 |
| | 30-seconds OT with TC | -0.28 | 0.73 | | 30-seconds OT with TC | 0.33 | 0.12 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 60-seconds OT without TC | 0.76 | 0.00 | | 60-seconds OT without TC | 0.38 | 0.08 |
| | 60-seconds OT with TC | 0.45 | 0.04 | | 60-seconds OT with TC | 0.31 | 0.15 |
| HALLUX | 10-seconds OT without TC | 0.83 | 0.00 | HALLUX | 10-seconds OT without TC | 0.72 | 0.00 |
| | 10-seconds OT with TC | 0.21 | 0.24 | | 10-seconds OT with TC | 0.47 | 0.03 |
| | 30-seconds OT without TC | 0.71 | 0.00 | | 30-seconds OT without TC | 0.75 | 0.00 |
| | 30-seconds OT with TC | 0.60 | 0.00 | | 30-seconds OT with TC | 0.77 | 0.00 |
| | 60-seconds OT without TC | 0.52 | 0.02 | | 60-seconds OT without TC | 0.02 | 0.47 |
| | 60-seconds OT with TC | 0.40 | 0.08 | | 60-seconds OT with TC | 0.31 | 0.09 |
| TR | | | | TR | | | |
| | Protocols | ICC | SIG | | Protocols | ICC | SIG |
| ANKLE | 30-seconds OT without TC | 0.63 | 0.00 | ANKLE | 30-seconds OT without TC | 0.60 | 0.00 |
| | 30-seconds OT with TC | -0.09 | 0.58 | | 30-seconds OT with TC | 0.35 | 0.11 |
| | 60-seconds OT without TC | 0.67 | 0.00 | | 60-seconds OT without TC | 0.50 | 0.02 |
| | 60-seconds OT with TC | -0.13 | 0.61 | | 60-seconds OT with TC | -0.24 | 0.70 |
| HALLUX | 10-seconds OT without TC | 0.98 | 0.00 | HALLUX | 10-seconds OT without TC | 0.90 | 0.00 |
| | 10-seconds OT with TC | 0.94 | 0.00 | | 10-seconds OT with TC | 0.98 | 0.00 |
| | 30-seconds OT without TC | 0.95 | 0.00 | | 30-seconds OT without TC | 0.96 | 0.00 |
| | 30-seconds OT with TC | 0.82 | 0.00 | | 30-seconds OT with TC | 0.60 | 0.00 |
| | 60-seconds OT without TC | 0.91 | 0.00 | | 60-seconds OT without TC | 0.77 | 0.00 |
| | 60-seconds OT with TC | 0.77 | 0.00 | | 60-seconds OT with TC | 0.93 | 0.00 |
| OT = Occlusion Time; TC = Temperature Control at probe site | | | | | | | |

**Figure 1: Protocols used in the current study**

The image shows the 12 different protocols used in the current study based on 3 different occlusion times, 2 different occlusion sites and use of temperature control

**Figure 2: PORH graph showing various measures**

Rest Flow (RF), Biological Zero (BZ), Peak Flow (PF), Time to Latency (TL), Time to Recovery (TR), Time to Half Before Hyperemia (TH1), Time to Max (TM), Time to Half After Hyperemia (TH2), Area of Occlusion (AO), Area of Hyperemia (AH), Area of Hyperemia/Area of Occlusion and Hyperemia repayment ratio (AH/AO)

**Figure 3: Right Foot: Mean of perfusion measures RF, BZ and PF (PU) across 12 protocols**

Secs = Seconds; TC = Temperature Control

**Figure 4: Left foot: Mean of perfusion measures RF, BZ and PF (PU) across 12 protocols**

Secs = Seconds; TC = Temperature Control

**Figure 5: Right foot: Mean Time to max (seconds) categorized based on 12 protocols**

Secs = Seconds; TC = Temperature Control

**Figure 6: Left foot: Mean Time to max (seconds) categorized based on 12 protocols**

Secs = Seconds; TC = Temperature Control

**Figure 7: Right foot: Mean Time to Recovery (seconds) categorized based on 12 protocols**

Secs = Seconds; TC = Temperature Control

**Figure 8: Left foot: Mean Time to Recovery (seconds) categorized based on 12 protocols**

Secs = Seconds; TC = Temperature

## Appendix 4: University Ethics Committee Approval

**PROPORTIONATE REVIEW APPROVAL FEEDBACK**

| | |
|---|---|
| **Researcher name:** | Gayathri Balasubramanian |
| **Title of Study:** | Predicting the risks of diabetic foot ulcers using vascular, neurological and biomechanical parameters |
| **Status of approval:** | Approved |

Thank you for addressing the committee's comments. Your research proposal has now been approved by the School Ethics Panel and you may commence the implementation phase of your study. You should note that any divergence from the approved procedures and research method will invalidate any insurance and liability cover from the University. You should, therefore, notify the Panel of any significant divergence from this approved proposal.

You should arrange to meet with your supervisor for support during the process of completing your study and writing your dissertation.

When your study is complete, please send the ethics committee an end of study report. A template can be found on the ethics BlackBoard site.

**Date: 26.11.18**

**Signed:** Dr Peter Kevern

Acting Chair, LSE Ethics panel

## Appendix 5: Risk assessment form

| | | | | | |
|---|---|---|---|---|---|
| **Risk Assessment Title:** | **Predicting the risks of diabetic foot ulcers using vascular, neurological and biomechanical parameters** | | | | |
| **Date:** | 17/10/18 | **School/Service:** | LSE | **Staff/ Student Ratio:** | |
| **Review Date:** | | **Main Location:** | R004/05 | | |
| **Ethics Approval Date:** | | **Assessed By:** | Gayathri Balasubramanian - Mark Young | **Approved By:** | TechnicalTeam |

**Description \ Standard Operating Procedure**

Diabetes has been shown to lead to structural and functional changes to the tissues of the body due to glycation, which is the bonding of sugars to lipids and proteins in the blood. Complications such as vascular disorders, neuropathy, and diabetic foot syndrome have all been linked to glycation. One of the key complications of diabetes is diabetic foot syndrome that leads to ulceration and amputation of the lower limbs. There are risk stratification systems (RSS) that aid in predicting ulcer incidents, thereby, preventing adverse outcomes. Understanding the pathophysiology of the diabetic foot is a continuing area of research. However, the mechanisms that underpin interactions between macrocirculation, microcirculation and neuropathy, especially that involves small fibres is still not fully understood. Microcirculation of the feet is pivotal in maintaining the protective properties on the plantar aspect of the foot such as thermoregulation and responsiveness to plantar pressure. Microcirculatory dysfunction is also known to cause damage to the small fibres which is key in sensation to pain and thermal stimuli. Small fibre neuropathy is known to precede large fibre neuropathy. Therefore, diagnosis of microcirculatory and small fibre dysfunctions and understanding the role of neurovascular aspects on the biomechanical aspects of the feet may aid to predict the risks of diabetic foot ulcers. This will have practical implications for early diagnosis and devising improved clinical management and prevention strategies within primary care.

This study is aimed to determine the association between vascular and neurological aspects, specifically microcirculation and small fibres. Additionally, the aim is to explore the extent at which microcirculation, small and large fibre functions, plantar pressure are associated with diabetic foot ulcer. This will aid to inform if the addition of microcirculatory measurements to existing RSS that includes well-established parameters such as macrocirculation and peripheral neuropathy can better predict ulcer incidents.
Objectives:
1) To understand the association between microcirculation and small fibre neuropathy
2) To identify non-invasive and clinically viable tests to assess the microcirculatory function the feet
3) To investigate the association between neurovascular aspects and the biomechanical aspects of the feet.

**The vascular assessments will include:** 1) microcirculation - Ankial Brachial index (ABI), Toe Brachial Index (TBI) 2) Microcirculation – Post occlusive reactive hyperaemia (PORH), Pressure induced vasodilation (PIV) and sympathetic vasoreflex during deep inspiratory gasps
**Neurological assessments will include:** 1) Autonomic functions - Galvanic skin response (GSR) and Heart Rate (HR) 2) Sensory functions – Warm/cold sensation, hot/cold threshold limits and vibration perception 3) Motor function – Muscle control strength
**Biomechanical assessments will include:** 1) Plantar skin thickness 2)Plantar pressure during walking and standing 3) Stability or sway

**The informed consent, list equipment that will be used to collect the above measures and study flow is enclosed as Appendix 1, 2 and 3 respectively in this document.**

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| | Activity/Process/ Machines | Hazard | Persons in Danger | Severity 1-5 | Likelihood 1-5 | Risk Rate | Measures/Comments | Result |
|---|---|---|---|---|---|---|---|---|
| 1 | Moving around the lab whilst setting up and performing the tests | Slip/Trip/falling | Participants and Researchers | 2 | 1 | 2 | Lab will be checked and cleared of all hazards. All the cables will be taped and secured before the start of the test to prevent any risk of tripping. Any spillages will be cleaned up as soon as possible. | T |
| 2 | Performing all process's using all electrical equipment | Electrical shock | Participants | 3 | 1 | 3 | The equipment will be PAT tested, checked before and after use for safety, any defaults will be reported to the Technicians immediately and the equipment will not be used until repaired. | T |
| 3 | Setting up testing equipment (manual handling) | Back, Lower and Upper Body Muscle Joint Injuries | Researchers and technicians | 2 | 1 | 2 | The technicians and researchers involved in this project will receive training in manual handling and will adhere to the correct manual handling techniques concerned with lifting and moving a heavy object. | T |
| 4 | Getting on and off the couch **Process 1 and 10** And comfort breaks if needed | Falling off couch | Participants | 2 | 1 | 2 | Prior to the start of the activity couch will be checked to ensure it is clean and free of hazards to avoid the risk of falling incidents. There is enough space for participant to lie down in a balanced position. Assistance will be given for getting on and off the couch. | T |
| 5 | Getting up from a supine position – postural changes | Discomfort, rolling-down and effects of postural change | Participants | 1 | 1 | 1 | Laying in supine position for too long and standing up can cause difficulties and may have effects due to sudden postural changes. Therefore, breaks will be given to interrupt staying in prolonged supine position. Care will be taken that the participant will not fall off the couch. A head rest will be provided for support. Hand support and further assistance will be provided by the researcher to facilitate getting on and off the couch. | T |

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| | Activity/Process/ Machines | Hazard | Persons in Danger | Severity 1-5 | Likelihood 1-5 | Risk Rate | Measures/Comments | Result |
|---|---|---|---|---|---|---|---|---|
| 6 | Applying Electrode, and adhesive tape, Gloves | Allergy | Participants | 2 | 1 | 2 | Participants will be asked if they have any known allergy before starting any testing. If they specify any allergy, then other alternative measures will be taken i.e. Non-Latex Gloves. Failing this, they will be excluded from the trials. | T |
| 7 | Preforming **Process 2,4,5,6,7,8,9,10** | Cross Contamination | Participant & Researchers | 1 | 1 | 1 | The operator will wear protective gloves every time they are in contact with the participants' feet. All devices will be cleaned with Anti-Bacterial wipe before and after their use. | T |
| 8 | Pressure cuffs being applied to occlude blood circulation **Process 4,5** | Excessive Occlusion to blood circulation | Participant | 2 | 1 | 2 | Protocols based on literature evidence will be used. Occlusion time of 10 second, 1 minute and 3 minutes with a rest period of 3 to 5 minutes or until perfusion returns to baseline will be used. Although the participant will feel some pressure whilst taking measurements, they will be asked if they feel uncomfortable or find it too tight whilst applying the cuff/testing. | A |
| 9 | Performing data collection using Laser Doppler Flowmetry and Laser Speckle Contrast Imager **Process 3, 4, 5** | Damage to eyesight | Participants | 2 | 1 | 2 | A Class 2 laser is relatively weak. It normally would not harm an eye unless a person deliberately stared into the beam. Laser protective eyewear is normally not necessary. A Class 2 laser is not a skin or materials burn hazard. Participants will be requested not to look at the laser directly. | T |
| 10 | Measuring Thermal sensation and threshold limits Thermode heating and cooling. **Process 6,** | Thermode malfunctioning may burn the skin. | Participant. | 2 | 2 | 4 | The software program stops the heating/cooling if the thermode temperature exceeds the working limits; upper = 50.5°C, lower = 0°C. The software program halts the heating or cooling if any problems are detected in the thermode. If the thermode temperature reaches 57°C an analogue circuit overrides the system and lowers the temperature gradually. Participants will be made aware that they can remove or stop the test at any time their foot from the thermode at | T |



| | Activity/Process/ Machines | Hazard | Persons in Danger | Severity 1-5 | Likelihood 1-5 | Risk Rate | Measures/Comments | Result |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | any time they desire. A thermal imaging camera will be also used to measure the skin temperature around the sensor. | |
| 11 | Measuring Vibrating Sensation with either TSA probe or the Biothesiometer. **Process 7** | Damage to Bones in Feet. | Participants | 1 | 2 | 2 | Participants who have suffered recent fractures within 12 weeks prior to testing. Will be excluded from the trial. This will be declared on the pre-test consent form | T |
| 12 | Immobilising Foot for **Process 3,4, 9** | Anxiety Attach (due to being secured into device) | Participants | 2 | 1 | 2 | There is a binding mechanism that can be immediately released to avoid trapping the foot. Foot will only be secured from pressure induced vasodilation and skin thickness measurement. Participants will be made aware that they can remove or stop the test at any time | T |
| 13 | Preforming **Process 8** to measure Plantar and Dorsiflexion Moving testing equipment | Trapped fingers in the testing apparatus | Researchers | 1 | 2 | 2 | The testing will involve only the move (apart from the flexion action) of the apparatus from one position to the other using the main bar. Ensuring that hands and cables are clear of the device | T |
| 14 | Taking static, balance and Dynamic measurements using the RsScan Pressure mat **Process 9** | Slip/Trip E.g. Cables and Walkway | Participants and Researchers | 2 | 1 | 2 | Prior to the start of the activity the lab and walkway will be checked to ensure it is clean and free of hazards to avoid the risk of trip slip incidents. Where all cables will be secured away from the pressure mat whilst they are data is being collected. Additional hand rail will be set up to assist those who are unstable on their feet. A walkway will be added both before and after the pressure matt so that the participant is walking across the mat at the same level removing any form of trip hazard | T |

| Key to Results: | T = Trivial Risk<br>Hazards with risk ratings 1-4 can be considered | A = Adequately Controlled<br>Hazards with risk ratings 5-9<br>Adequately Controlled | N = Not Adequately Controlled<br>Hazards with risk ratings 10-14<br>Not Adequately Controlled | S = Stop<br>Hazards with risk ratings 15-25<br>Stop the Activity Immediately |
|---|---|---|---|---|
| NB: THE SEVERITY X LIKELIHOOD RATINGS ARE CALCULATED AFTER TAKING INTO ACCOUNT EXISTING PRECAUTIONS | | | | |

**Other special conditions specified as part of the permission to carry out the work/ procedure and actions needed to minimise risk.**

- All participants must complete a pre-test questionnaire and informed consent form excluding those medically unfit to perform the exercise test, before participating. Appendix 1
- A first aider will be available at all times during the activity.
- The experiment/procedure will cease immediately if a participant reports that they feel any discomfort, unwell, if an accident occurs, or if the fire alarm sounds.
- All accidents, near misses, and injuries, however slight, must be reported to the technician and supervisor immediately, appropriate forms must be completed.
- Operators will be told who to contact in an emergency, will be given a list of first aider contacts, and will be instructed to obey the fire orders.
- Operator will to be trained up to a competent level by an experienced member of staff before being allowed to use the equipment.
- Undergraduates may use the equipment on an individual basis i.e. dissertation work, thus they will receive training, assessment, and supervision.
- Operator must read relevant safety notes and guidelines in the operations manuals.
- Operators must report to the technician if they suspect that the equipment may be malfunctioning, faulty, or not working correctly.
- The equipment is PAT tested annually and a visual inspection of the cables and equipment should be carried out prior to use.

| | | | | | |
|---|---|---|---|---|---|
| I have read and understand the above: | Signed (Student) | | Date | Print Name: | Gayathri Balasubramanian |
| | Signed (Supervisor) | | Date: | Print Name: | Roozbeh Naemi |

## Appendix 6: Recruitment flyer

**Invitation email and message**

### Email Content:

Dear [Name],

Diabetes foot syndrome is an increasing public health concern. Diabetes foot syndrome results in ulcerations and amputations of the limbs. Facts show that every 20 seconds a limb is lost due to diabetic foot disease! Therefore, there is a need to understand the risk factors in order to prevent adverse complications.

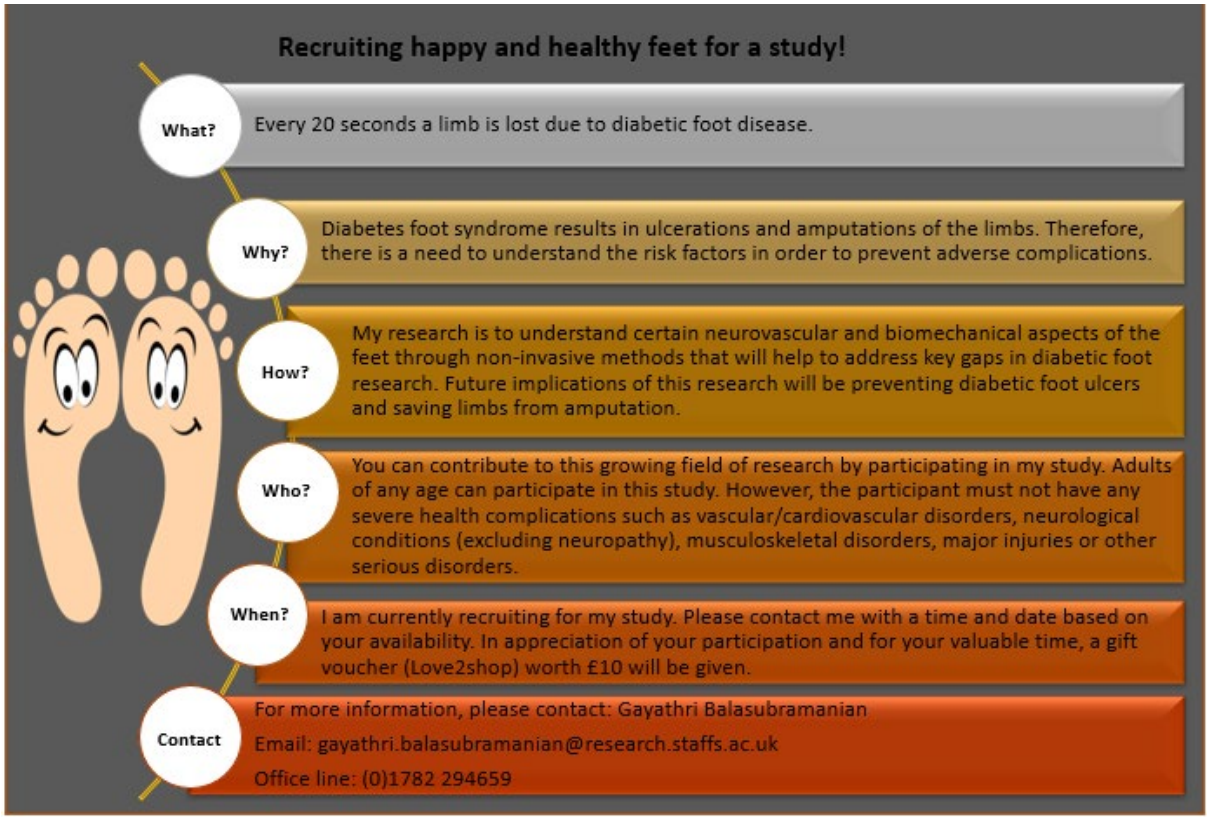
My research is to understand certain neurovascular and biomechanical aspects of the feet through non-invasive methods that will help to address key gaps in diabetic foot research. Future implications of this research will be preventing diabetic foot ulcers and saving limbs from amputation.

You can contribute to this growing field of research by participating in my study. Adults of any age can participate in this study. However, the participant must not have any severe health complications such as vascular/cardiovascular disorders, neurological conditions (excluding neuropathy), musculoskeletal disorders, major injuries or other serious disorders.

I am currently recruiting for my study and I request your participation. Please contact me with a time and date based on your availability. In appreciation of your participation and for your valuable time, a gift voucher (Love2shop) worth £10 will be given.

Best regards,
Gayathri Balasubramanian
(Contact: gayathri.balasubramanian@research.staffs.ac.uk, 01782 294659)

SMS/Whats app/Facebook message**:**




## Appendix 7: Participant Information sheet

**INFORMATION SHEET FOR PARTICIPANTS**

*Project Reference Number: [insert once provided by the university ethics committee]*

**Title of study**

A study into the relationship between neurovascular and biomechanical aspects of the soles of the feet

**Invitation Paragraph**

I would like to invite you to participate in this research project which forms part of my PhD studies and research. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask me if there is anything that is not clear or if you would like more information. Thank you for reading this.

**What is the purpose of the study?**

Blood circulation plays a vital role in the temperature control on the surface of the foot and provide nutrients to the local tissues. Impaired circulation leads to various complications including the tissue response to high pressure in the soles of the feet, injury or trauma. On the other hand, the nerves of the feet provide protective sensation to various stimuli such as pain, temperature and mechanical stress. Studies highlight the role of neurovascular disturbances in the lower limbs related to peripheral arterial diseases, neuropathy and diabetic foot complications. Therefore, neurovascular aspects in the feet and its interaction with the biomechanics of the foot is an increasingly important area in understanding diabetic foot syndrome and to investigate predictors of diabetic foot ulcers.

This research project aims to investigate and quantify the blood circulation, neurological aspects and certain biomechanical parameters at the soles of the feet in individuals in order to gain better understanding of the interplay between biomechanics and neurovascular aspect of the foot.

**Why have I been invited to take part?**

You are invited because you may be an adult of any age who can participate in this study and can visit Staffordshire University Biomechanics Lab located in the ground floor of Science Centre building for participation. However, as a participant you must not have any severe health complications such as vascular/cardiovascular disorders, neurological conditions (excluding neuropathy), musculoskeletal disorders, major injuries or other serious disorders.

**What will happen if I take part?**

You will be asked to visit the biomechanics lab located in the ground floor of the Science Centre building for data collection which will approximately take two hours and thirty minutes per session. You can choose to participate in one or more sessions. You must not consume caffeine or alcohol and should not engage in strenuous exercise from 2 hours prior to the study as it may interfere with the data collection. In addition, you may let us know your interest to participate in subsequent studies in the area.

**Do I have to take part?**

Participation is completely voluntary. You should only take part if you want to and choosing not to take part will not disadvantage you in anyway. Once you have read the information sheet, please contact us if you have any questions that will help you make a decision about taking part. If you decide to take part we will ask you to sign a consent form and you will be given a copy of this consent form to keep.

**Incentives**

In appreciation of your participation and for your valuable time, a Love2shop voucher worth £10 will be given.

**What are the possible risks of taking part?**

The data collection takes 2.30 hours approximately per session. You are invited to participate in multiple sessions of data collection, but the decision to participate in one or more sessions is solely yours. Participating in the research is not anticipated to cause you any disadvantages or discomfort. However, if as a participant you are experiencing any discomfort you can withdraw participation at any time. First Aiders will be always present at the facility. In case of any emergency university health and safety procedures will be followed.

**What are the possible benefits of taking part?**

Whilst there are no immediate benefits for those people participating in the project, it is hoped that this work will have a beneficial impact on continued research on the prevention of diabetic foot complications.

**Data handling and confidentiality**

Your data will be processed in accordance with the data protection law and will comply with the General Data Protection Regulation 2016 (GDPR).

**Data Protection Statement**

The data controller for this project will be Staffordshire University. The University will process your personal data for the purpose of the research outlined above. The legal basis for processing your personal data for research purposes under the data protection law is a 'task in the public interest' You can provide your consent for the use of your personal data in this study by completing the consent form that has been provided to you.

**What if I change my mind about taking part?**

You are free withdraw at any point of the study, without having to give a reason. Withdrawing from the study will not affect you in any way. You are able to withdraw your data from the study up until data analysis begins [exact date will be provided], after which withdrawal of your data will no longer be possible as the data recorded would have been anonymised and processed for the development of report/publication/thesis.

If you choose to withdraw from the study, we will not retain any information that you have provided us as a part of this study.

**How is the project being funded?**

Staffordshire University (UK)

**What will happen to the results of the study?**

Results of the research will be published. You will not be identified in any report or publication. Your institution will not be identified in any report or publication. If you wish to be given a copy of any reports resulting from the research, please ask us to put you on our knowledge dissemination list.

**Who should I contact for further information?**

If you have any questions or require more information about this study, please contact me using the following contact details:

Gayathri Balasubramanian

Email: gayathri.balasubramanian@research.staffs.ac.uk

**What if I have further questions, or if something goes wrong?**

If this study has harmed you in any way or if you wish to make a complaint about the conduct of the study you can contact the study supervisor or the Chair of the Staffordshire University Ethics Committee for further advice and information:

Supervisor: Dr Roozbeh Naemi
Email: r.naemi@staffs.ac.uk

**Thank you for reading this information sheet and for considering taking part in this research.**

## Appendix 8: Consent form

**CONSENT FORM**

**Title of Project:** Predicting the risks of diabetic foot ulcers using vascular, neurological and biomechanical parameters

**Study Title:** Neurovascular and biomechanical assessment on the plantar aspects (soles) of the feet

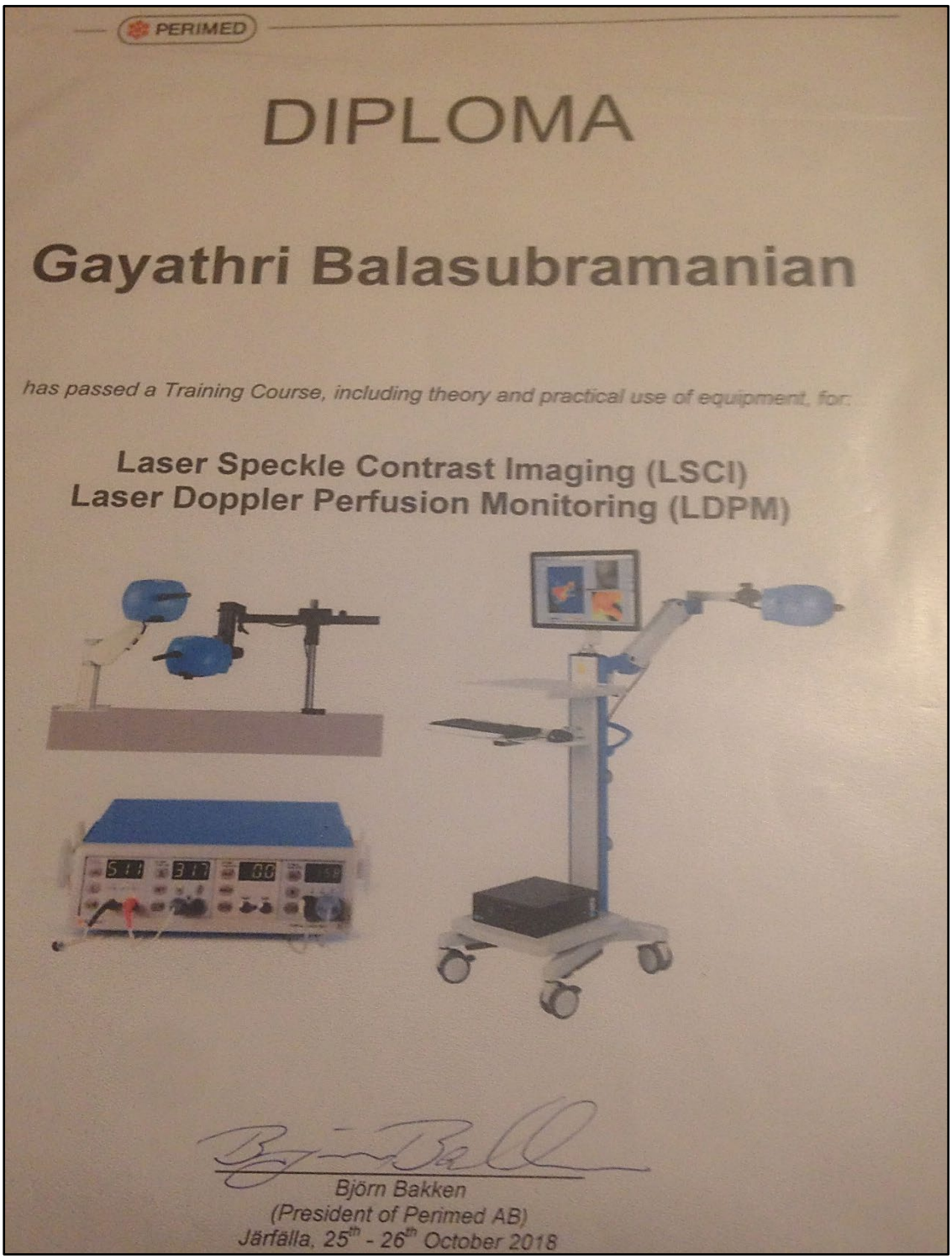
Name of Researcher: Gayathri Balasubramanian

| | Please initial box |
|---|---|
| 1. I confirm that I have read the information sheet dated..... (version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. | ☐ |
| 2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason. | ☐ |
| 3. I understand that the information collected about me will be used to support other research in the future, and may be shared anonymously with other researchers. | ☐ |
| 4. I agree to take part in the above study. | ☐ |

| Name of Participant | Date | Signature |
|---|---|---|
| | | |

| Name of Person taking consent | Date | Signature |
|---|---|---|
| | | |

## Appendix 9: Diploma certificate from Perimed® training course


PERIMED

DIPLOMA

Gayathri Balasubramanian

has passed a Training Course, including theory and practical use of equipment, for:

Laser Speckle Contrast Imaging (LSCI)
Laser Doppler Perfusion Monitoring (LDPM)

Björn Bakken
(President of Perimed AB)
Järfälla, 25th - 26th October 2018

## Appendix 10: Poster Presentation

Poster presented at the 14th International Symposium on Biomechanics in Vascular biology and Cardiovascular Disease 2019

# A systematic investigation into the measurement of skin microcirculation in the foot

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## Background and Aim:

The role of microcirculatory dysfunction has been linked to the development of foot complications such as peripheral arterial disease, diabetic foot complications, impaired tissue response to injury or trauma and poor wound healing. Moreover, an association between the presence of peripheral sensory neuropathy and altered microvascular reactivity in the lower limb has been established in people with diabetes.[1] One of the measures to assess microcirculatory function is reactive hyperaemia which is an indicator of the intrinsic ability of an organ or tissue to locally autoregulate its blood supply. Post Occlusive Reactive Hyperaemia (PORH) has been used to assess the microvascular function with/without the temperature control, while occlusion time for PORH based on previous studies ranged from 3 to 10 minutes.[2–5] However, with an increase in the occlusion time there will be an increase in pain at the site and risk of complications, especially in people with diabetic foot disease. Despite this, there is no standard protocol to test PORH.[3,6,7] The purpose of this study was to investigate the effect of systematically controlled changes in temperature and occlusion time on PORH measurements.

## Methods:

PORH was measured using Laser Doppler Flowmetry (Perimed PeriFlux, Sweden) (Figure 1) with the probes (size 1 $cm^2$ each) placed at the distal/plantar aspect of the hallux. The occlusion was at the ankle and hallux levels (one followed by other), while the cuffs were inflated to a suprasystolic pressure (200 mmHg). The occlusion times were 10, 30, and 60 seconds. Additionally, the same set of tests were performed with and without temperature control at 33°C at the probe site. For each participant, the PORH was measured in 12 different conditions (3 occlusion times with/without temperature control at ankle or toe level occlusion). Upon obtaining ethical approval data was collected from 20 healthy adults age ranging from 18 to 50 years. The participants laid in supine position and testing commenced following 15 minutes acclimatisation to room temperature (Figure 1).

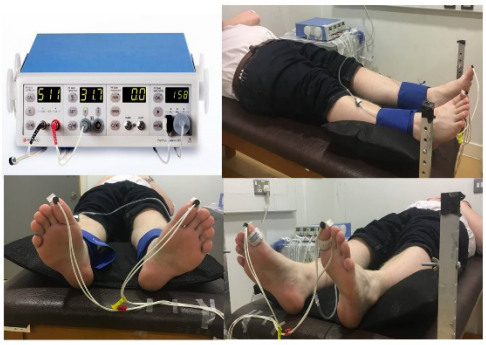
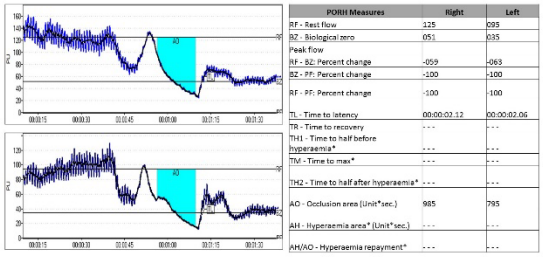


**Figure 1: Laser Doppler Flowmetry system and lab setting**

## Results and Discussion:

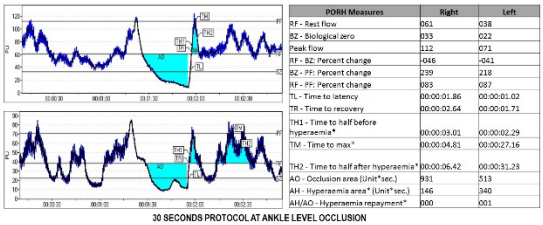
- Protocols developed within this study were practical to follow at both ankle and toe level occlusions
  - Pre-programming the protocols to be semi-automated aided with the data collection
  - More consistent, replicable and feasible
- The 30 seconds and 60 seconds protocol produced more consistent results with data available for most PORH measures reliably (Figure 2)
- **The rest flow to peak flow percent change** measure was low whilst using 10 seconds protocol (~ 60% of the tests using 10 seconds protocol or ~ 20% of the all protocols)
  - These findings imply that 10 seconds may not be enough to provoke enough hyperaemic response (Figure 2)
- **The rest flow** observed in protocols with temperature control was higher than (mostly above 50% higher) in protocols without temperature control in the observed data (~ 70%)
  - This was observed across all protocols and was not dependent on occlusion times and occlusion site
  - The thermal sensation of the small nerve fibres plays a significant role in skin microcirculatory responses

*Preliminary results and sample images are from one participant showing the differences across three different protocols



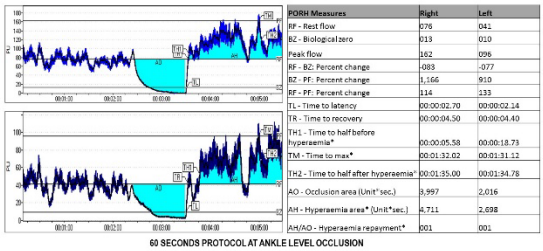
| PORH Measures | Right | Left |
|---|---|---|
| RF - Rest flow | 125 | 095 |
| BZ - Biological zero | 051 | 035 |
| Peak flow | | |
| RF - BZ: Percent change | -059 | -063 |
| BZ - PF: Percent change | -100 | -100 |
| RF - PF: Percent change | -100 | -100 |
| TL - Time to latency | 00:00:02.12 | 00:00:02.06 |
| TR - Time to recovery | - - - | - - - |
| TH1 - Time to half before hyperaemia* | - - - | - - - |
| TM - Time to max* | - - - | - - - |
| TH2 - Time to half after hyperaemia* | - - - | - - - |
| AO - Occlusion area (Unit*sec.) | 985 | 795 |
| AH - Hyperaemia area* (Unit*sec.) | - - - | - - - |
| AH/AO - Hyperaemia repayment* | - - - | - - - |

10 SECONDS PROTOCOL AT ANKLE LEVEL OCCLUSION



| PORH Measures | Right | Left |
|---|---|---|
| RF - Rest flow | 061 | 038 |
| BZ - Biological zero | 033 | 022 |
| Peak flow | 112 | 071 |
| RF - BZ: Percent change | -046 | -041 |
| BZ - PF: Percent change | 239 | 218 |
| RF - PF: Percent change | 083 | 087 |
| TL - Time to latency | 00:00:01.86 | 00:00:01.02 |
| TR - Time to recovery | 00:00:02.64 | 00:00:01.71 |
| TH1 - Time to half before hyperaemia* | 00:00:03.01 | 00:00:02.29 |
| TM - Time to max* | 00:00:04.81 | 00:00:27.16 |
| TH2 - Time to half after hyperaemia* | 00:00:06.42 | 00:00:31.23 |
| AO - Occlusion area (Unit*sec.) | 931 | 513 |
| AH - Hyperaemia area* (Unit*sec.) | 146 | 340 |
| AH/AO - Hyperaemia repayment* | 000 | 001 |

30 SECONDS PROTOCOL AT ANKLE LEVEL OCCLUSION



| PORH Measures | Right | Left |
|---|---|---|
| RF - Rest flow | 076 | 041 |
| BZ - Biological zero | 013 | 010 |
| Peak flow | 162 | 096 |
| RF - BZ: Percent change | -083 | -077 |
| BZ - PF: Percent change | 1,166 | 910 |
| RF - PF: Percent change | 114 | 133 |
| TL - Time to latency | 00:00:02.70 | 00:00:02.14 |
| TR - Time to recovery | 00:00:04.50 | 00:00:04.40 |
| TH1 - Time to half before hyperaemia* | 00:00:05.58 | 00:00:18.73 |
| TM - Time to max* | 00:01:32.02 | 00:01:31.12 |
| TH2 - Time to half after hyperaemia* | 00:01:35.00 | 00:01:34.78 |
| AO - Occlusion area (Unit*sec.) | 3,997 | 2,016 |
| AH - Hyperaemia area* (Unit*sec.) | 4,711 | 2,698 |
| AH/AO - Hyperaemia repayment* | 001 | 001 |

60 SECONDS PROTOCOL AT ANKLE LEVEL OCCLUSION

**Figure 2: Preliminary data analysis - PORH graphs and measures**

A practical implication of this study is to provide recommendations on protocols that are feasible, systematic and less time consuming to use in routine practice. Understanding the skin microcirculatory responses in healthy subjects and having a reference range will help to evaluate the importance of microcirculatory measurements in diabetic foot and further explore their role in ulceration. This study also highlights the association between microcirculation and function small nerve fibres. Further knowledge in this area may help to understand the relationship between skin microcirculatory dysfunction and small fibre neuropathy. As these dysfunction precedes most other complications, this in turn can aid in early diagnosis and prevention of diabetic foot and its adverse consequences such as amputations.

## Conclusions:

This study addresses the current gaps in existing literature, highlighting the importance of systematically measuring microcirculation of the feet. In developing protocols for reliable measurements of PORH, it is vital to consider temperature variation and occlusion time.

## References:

[1]. Barwick AL, Tessier JW, Janse de Jonge X, Ivers JR, Chuter VH. Peripheral sensory neuropathy is associated with altered postocclusive reactive hyperemia in the diabetic foot. *BMJ Open Diabetes Res Care*. 2016;4(1):e000235-e000235. doi:10.1136/bmjdrc-2016-000235

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[3]. Morales F, Graaff R, Smit AJ, et al. How to assess post-occlusive reactive hyperaemia by means of laser Doppler perfusion monitoring: Application of a standardised protocol to patients with peripheral arterial obstructive disease. *Microvasc Res*. 2005;69(1-2):17-23. doi:10.1016/j.mvr.2005.01.006

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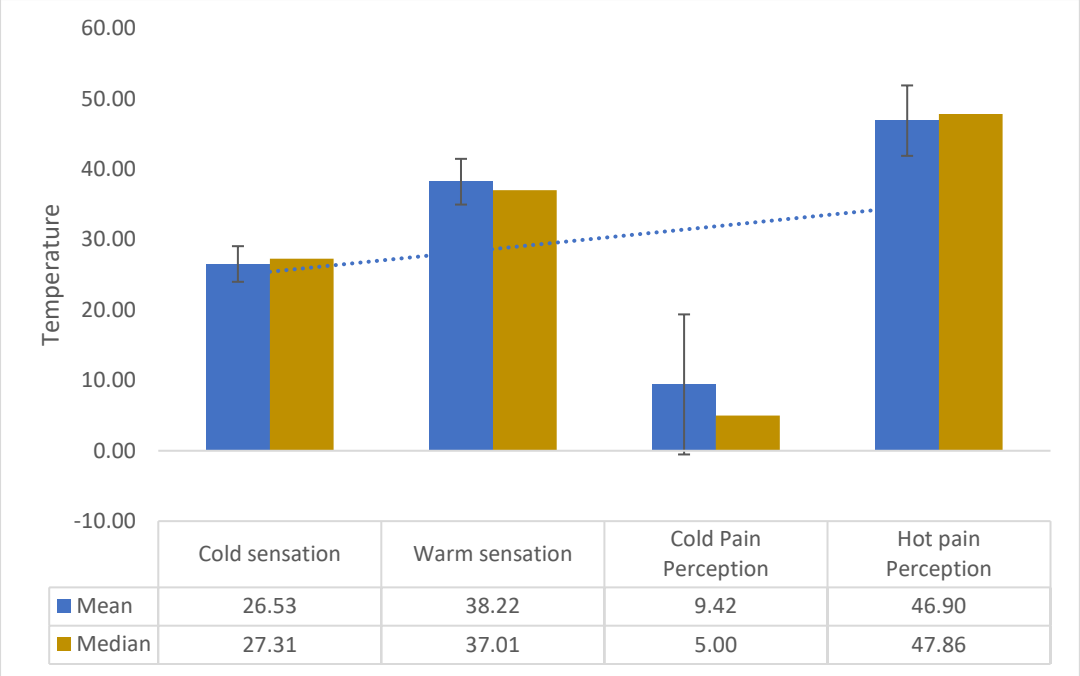
[5]. Clough G, Chipperfield A, Byrne C, de Mul F, Gush R. Evaluation of a new high power, wide separation laser Doppler probe: potential measurement of deeper tissue blood flow. *Microvasc Res*. 2009;78(2):155-161. doi:10.1016/j.mvr.2009.05.003

[6]. Babos L, Járai Z, Nemcsik J. Evaluation of microvascular reactivity with laser Doppler flowmetry in chronic kidney disease. *World J Nephrol*. 2013;2(3):77-83. doi:10.5527/wjn.v2.i3.77

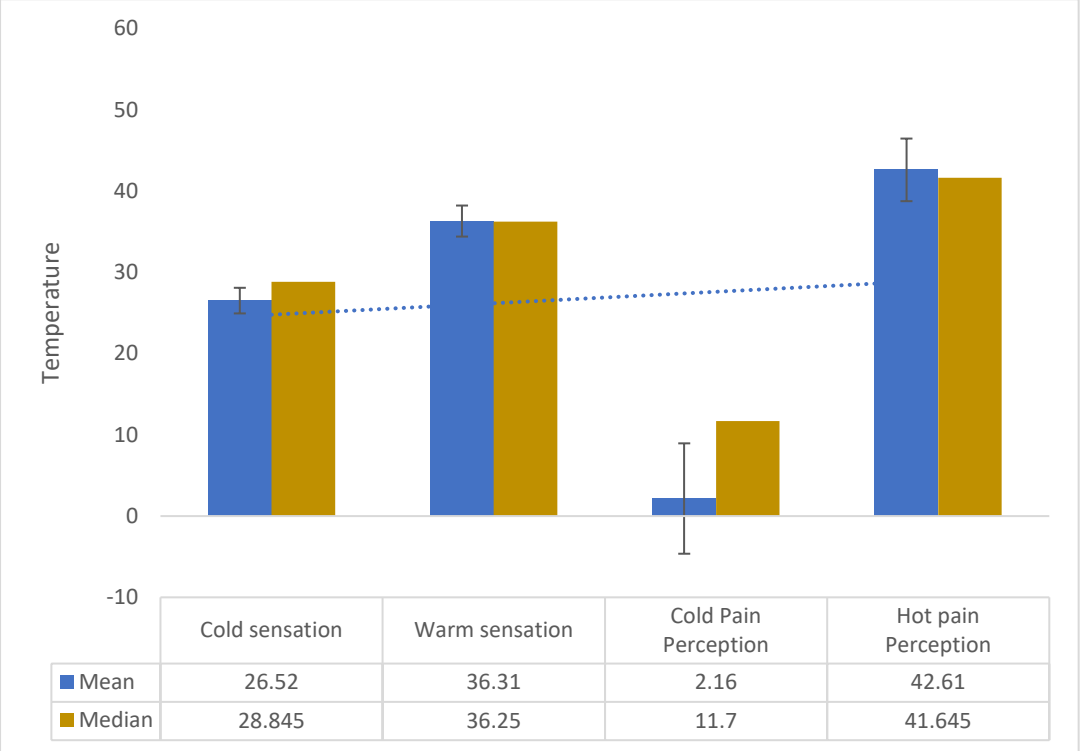
[7]. Lanting SM, Johnson NA, Baker MK, Caterson ID, Chuter VH. The effect of exercise training on cutaneous microvascular reactivity: A systematic review and meta-analysis. *J Sci Med Sport*. 2017;20(2):170-177. doi:10.1016/j.jsams.2016.04.002

## Appendix 11: QST Results



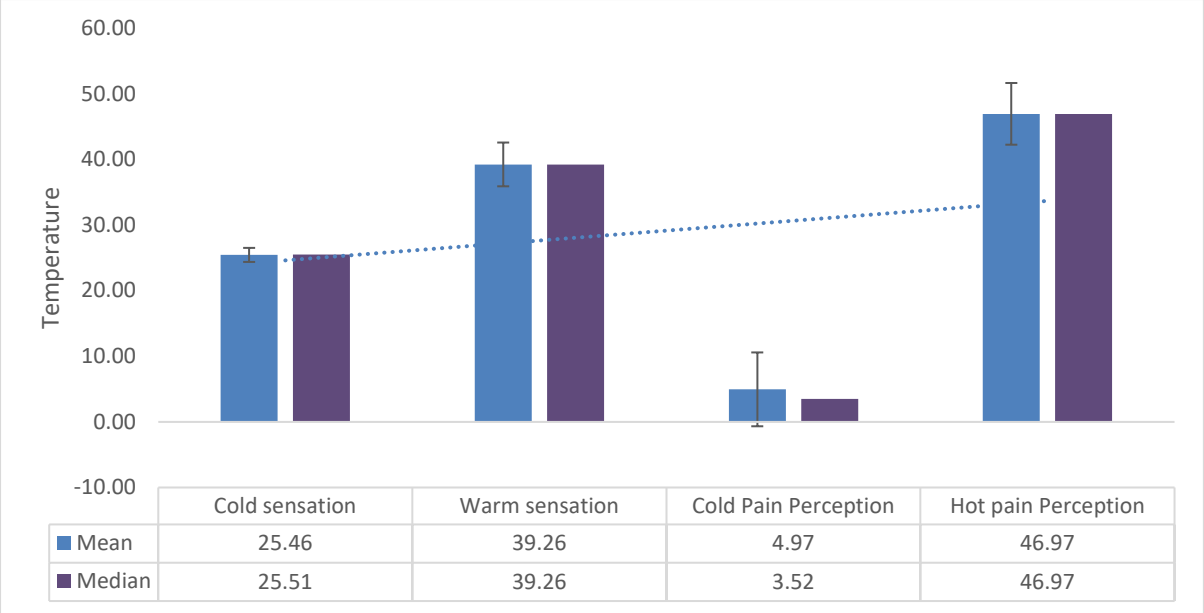

| | Cold sensation | Warm sensation | Cold Pain Perception | Hot pain Perception |
|---|---|---|---|---|
| Mean | 26.53 | 38.22 | 9.42 | 46.90 |
| Median | 27.31 | 37.01 | 5.00 | 47.86 |

*Figure 6.2 Right Foot - Plantar QST Testing (Group Average in ℃)*



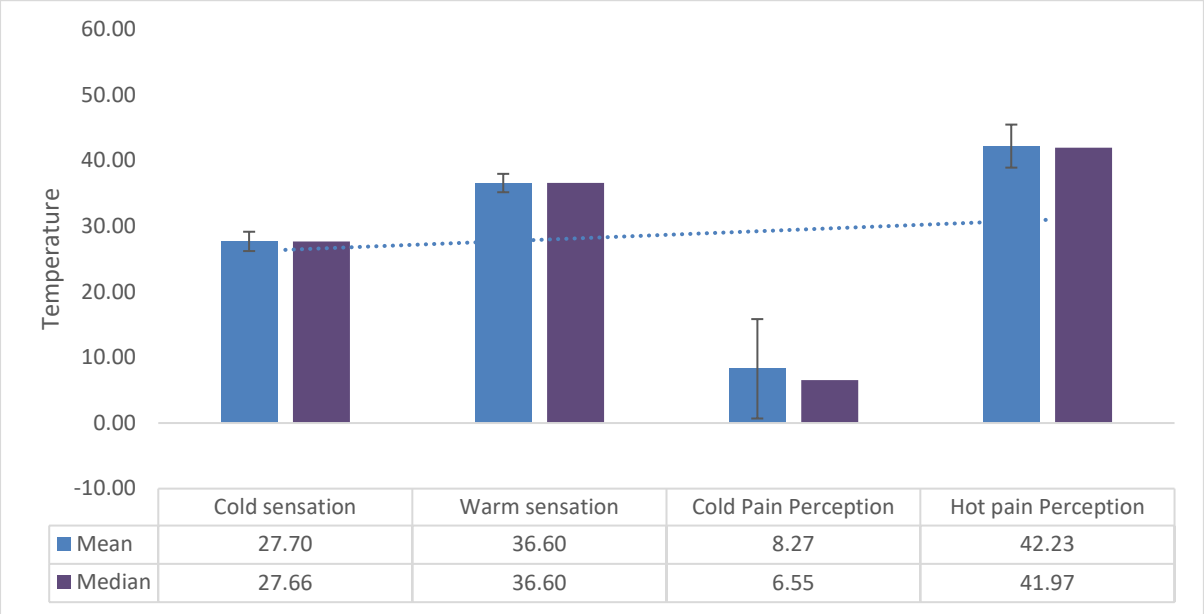

| | Cold sensation | Warm sensation | Cold Pain Perception | Hot pain Perception |
|---|---|---|---|---|
| Mean | 26.52 | 36.31 | 2.16 | 42.61 |
| Median | 28.845 | 36.25 | 11.7 | 41.645 |

*Figure 6.3 Right Foot - Dorsal QST Testing (Group Average in ℃)*




| | Cold sensation | Warm sensation | Cold Pain Perception | Hot pain Perception |
|---|---|---|---|---|
| Mean | 25.46 | 39.26 | 4.97 | 46.97 |
| Median | 25.51 | 39.26 | 3.52 | 46.97 |

*Figure 6.4 Left Foot - Plantar QST Testing (Group Average in ℃)*




| | Cold sensation | Warm sensation | Cold Pain Perception | Hot pain Perception |
|---|---|---|---|---|
| Mean | 27.70 | 36.60 | 8.27 | 42.23 |
| Median | 27.66 | 36.60 | 6.55 | 41.97 |

*Figure 6.5 Left Foot - Dorsal QST Testing (Group Average in ℃)*