Evaluating Non-Invasive Cytokine Sampling to Detect Diabetic Peripheral Neuropathy: *A Proof-of-Concept Study*

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Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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August 1, 2021

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Abstract

Diabetic Peripheral Neuropathy (DPN) is the most common form of peripheral neuropathy worldwide. However, there are limited treatments for DPN, thus early diagnosis and prevention are essential. A large component of the pathophysiology of diabetes and DPN is based on the presence of inflammation that consequently leads to the complications associated with DPN. The focus of this proof-of-concept study is investigating the link between local inflammatory factors on the skin and comparing them to the circulating cytokines in the blood. This will provide insight into whether the same cytokines associated with DPN that have been previously identified in the blood – mainly Interleukin-6 (IL-6), Interluekin-1 (IL-1), Interleukin-8 (IL-8), Tumour Necrosis Factor Alpha (TNF-α), C-Reactive Protein (CRP) and Interkeukin-10 (IL-10) – are also present on the skin of people with diagnosed peripheral neuropathy. Twenty-five participants with diabetes were recruited to undergo both functional and objective assessments to determine DPN diagnosis and severity: the Michigan Neuropathy Screening Instrument (MNSI) in conjunction with Quantitative Sensory Testing (QST), and nerve conduction testing. Sebum from the foot and blood samples were collected from participants and analysed by an external lab. While there were no statistically significant results due to the small sample size, this pilot study provided some direction for future research with a larger sample size. Finding an association between local inflammatory factors on the skin and those circulating in the blood might provide an opportunity for the development of a localised, skin sebum test that is non-invasive, inexpensive, and accessible for a more reliable, measurable, and standardised diagnostic tool for DPN.

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Abbreviations

ADP - Adenosine Diphosphate

AGE - Advanced Glycation End-products

APOE - Apolipoprotein E

ATP - Adenosine Triphosphate

ATPase - Adenosine Triphosphatase

BGLs - Blood Glucose Levels

BMI - Body Mass Index

CN - Charcot Neuropathy

CRP - C-Reactive Protein

DM - Diabetes mellitus

DPN - Diabetic Peripheral Neuropathy

EDTA - Ethylenediaminetetraacetic Acid

eNOS - Nitric Oxidesynthase

FDA - Food and Drug Administration

HbA1c - Glycated Haemoglobin Test

IFN-γ - Gamma Interferon

IL - Interleukin

IL-1 - Interleukin-1

IL-10 - Interleukin-10

IL12 - Interleukin 12

IL-1RA - Interleukin-1 Receptor Antagonist

IL-1β - Interleukin-1 beta

IL-6 - Interleukin-6

IL-8 - Interleukin-8

JUN - Jun Proto-Oncogene, AP-1 Transcription Factor Subunit

K+ - Potassium Ions

LEP - Leptin

LPS - Lipopolysaccharide

MNSI - Michigan Neuropathy Screening Instrument

Na+ - Sodium Ions

NAD^+ - Nicotinamide Adenine Dinucleotide

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NCS - Nerve Conduction Studies

NO - Nitric Oxide

NOS2 - Nitric Oxide Synthase 2

PBS - Phosphate Buffered Saline

PKC - Protein Kinase C

PPARG - Peroxisome Proliferator Activated Receptor Gamma

QST - Quantitative Sensory Testing

RNS - Reactive Nitrogen Species

ROS - Reactive Oxygen Species

SD - Standard Deviation

SERPINE1 - Serpin Family E Member 1

SNRI - Serotonin and Norepinephrine Reuptake Inhibitor

t1DM - Diabetes Type 1

t2DM - Diabetes Type 2

TCA - Tricyclic Antidepressants

TNF-α - Tumour Necrosis Factor-Alpha

UCP-2 - Uncoupling Protein 2

1. Introduction

Evaluating Non-Invasive Cytokine Sampling to Detect Diabetic Peripheral Neuropathy: *A Proof-of-Concept Study*

Peripheral neuropathy is a common condition encountered by the health professional in a clinical setting (Donofrio, 2012) that is characterised by dysfunction of the nerves, and usually presents as loss of sensation, weakness, pain or hypersensitivity along the damaged nerve fibres (Bromberg, 2018). Early symptoms that commonly present with sensory polyneuropathies include distal numbness, tingling and neuropathic pain (peripheral neuropathy usually starts distally from the extremities such as the feet and hands), while symptoms associated with motor neuropathies feature gait imbalances, and muscle and toe weakness (Donofrio, 2012; Hanewinckel, Ikram, & Van Doorn, 2016). Early neuropathy can be detected by neurological examination which can include distal sensory loss to cold, vibration and/or pressure, reduced or loss of the ankle reflex, Romberg's sign[1], impaired gait, and toe extensor weakness (Donofrio, 2012). As the condition progressively worsens over time, there is advancement of the tingling, numbness and neuropathic pain along a proximal path, as well as the deterioration of gait and muscle weakness which frequently leads to tripping and falls (Callaghan, Cheng, Stables, Smith, & Feldman, 2012; Donofrio, 2012). People affected may also complain of cramps in the legs, and in some cases, peripheral neuropathy can lead to restless leg syndrome (Donofrio, 2012; Memon et al., 2020). The most common complaint of neuropathic pain is that it is worse at night and can prevent individuals from sleeping, affecting their quality of life and resulting in disturbed sleep and lethargy (Donofrio, 2012).

[1] Clinical examination to test proprioception which involves the person standing, feet together and eyes close, while the examiner detects any postural sway that may indicate impaired proprioception and sensory ataxia (Khasnis & Gokula, 2003).

Diabetes mellitus (DM) is a chronic disease hallmarked by the dysfunction of insulin and hyperglycaemia, leading to cellular impairment (Wagner, 2015). Long-standing diabetes leads to the development of a variety of complications, such as diabetic peripheral neuropathy (DPN), retinopathy and nephropathy as well as different types of neuropathies (Forouhi & Wareham, 2014; Wagner, 2015). There are two types of Diabetes – type 1 and type 2. Diabetes type 1 (t1DM) occurs due to an autoimmune inflammatory response in the pancreas that leads to the destruction of the beta cells that produce insulin, thus the body loses its ability to produce insulin (Graves, Liu, Alikhani, Al-Mashat, & Trackman, 2006). Diabetes type 2 (t2DM) is often hereditary and associated with obesity and environmental risk factors. It develops as a result of insulin resistance in the body due to a progressive process of apoptosis of the beta cells in the pancreas (Graves et al., 2006). There is a large body of evidence that points to the role of inflammation in the development and pathogenesis of t2DM (Wang et al., 2013). A systematic review and meta-analysis analysing a total of 18 prospective studies (N = 19,709) concluded that inflammation was a crucial element within the pathophysiology of t2DM. Two main inflammatory factors, C-Reactive Protein (CRP)[2] and Interlekuin-6 (IL-6) were associated with the risk and development of t2DM (Wang et al., 2013); BMI was also found to play a role. Other studies have linked IL-6, as well as Tumour Necrosis Factor Alpha (TNF-α) to the progression of diabetes and its complications, such as DPN (Li, Zhu, Liu, Liu, & Zhang, 2017; Wang et al., 2013).

DPN is the most common type of peripheral neuropathy globally, with incidence increasing with the duration of diabetes (Donofrio, 2012; Iqbal et al., 2018). Approximately half of people with diabetes will develop DPN (Aring, Jones, & Falko, 2005; Iqbal et al., 2018), with polyneuropathies being the most common manifestation (Hanewinckel et al., 2016). These neuropathies associated with DM comprise many diseases; polyneuropathies

[2] CRP: a protein that increases with the presence of inflammation (Doupis et al., 2009).

and mononeuropathies associated with diabetes can present as both painful and debilitating conditions (Hanewinckel et al., 2016; Iqbal et al., 2018). DPN usually starts distally and progresses proximally, spreading in a stocking-like distribution, and most commonly affecting the hands and feet (Callaghan et al., 2012; Iqbal et al., 2018). Neuropathic symptoms include burning, pain, numbness, allodynia, and hyperalgesia (Snyder, Gibbs, & Lindsay, 2016). Interestingly, affected individuals have the complex paradox of experiencing numbness as well as hypersensitivity simultaneously (Callaghan et al., 2012). Generally, neuropathic pain is characterised by burning, stabbing, electrical sensations with numbness (sometimes without numbness as well) and is a frequent occurrence in people with DPN (Callaghan et al., 2012).

DPN affects both small and large peripheral nerve fibres (Jin & Park, 2018), usually involving small nerve fibres early on, which then progress to large nerve fibres (Vinik, Nevoret, Casellini, & Parson, 2013) . Accurate detection of small nerve fibre neuropathy is challenging (Vinik et al., 2013). Currently, tools to enable diagnosis include electro-diagnostic tests as well as clinical diagnostic tests such as vibration, reflexes and light pressure (Callaghan et al., 2012). Both approaches have limitations: electrodiagnostic tests are expensive and time-consuming, while clinical tests are inaccurate and are dependent on the clinician (Pop-Busui et al., 2017). Additionally, these tests cannot diagnose early onset of DPN and by the time it is diagnosed, the condition would have progressed to severe impairment and nerve damage, where both large and small nerve fibres have failed (Callaghan et al., 2012).

1.2. Epidemiology of Diabetes, its Complications and Peripheral Neuropathy

In 2019, it is estimated that 463 million people are living with diabetes, demonstrating 9.3% of the global adult population aged between 20–79 years (Saeedi et al., 2019). It is expected that number will rise to 578 million (10.2%) in 2030 and 700 million (10.9%) in

2045 (Saeedi et al., 2019). This increase is mainly attributed to the increase in the prevalence of world-wide diabetes risk factors including obesity and unhealthy lifestyle behaviours, such as poor diets and low levels of physical activity (Forouhi & Wareham, 2019).

Approximately 4.9% of Australians had diabetes between 2017-2018 - an estimated 1.2 million Australians - with prevalence tripling since the 1990s and current numbers growing (Australian Institute of Health and Welfare, 2020). Diabetes incidence increases with age, and in 2018, it accounted for 10.5% of deaths in Australia as an associated cause of death (Australian Institute of Health and Welfare, 2020)

DPN is the most prevalent type of peripheral neuropathy globally (Hicks & Selvin, 2019; Iqbal et al., 2018). Interestingly, recent studies on the prevalence of DPN are scarce. This may be due to the challenges in both diagnosis and undertaking a population-based studies. Furthermore, the broad range of diagnostic methods used to diagnose peripheral neuropathy lack consistency in defining DPN and are prone to clinician error (Martin, Albers, & Pop-Busui, 2014).

Historically, several studies reported on the epidemiology of peripheral neuropathy. One significant study to determine the prevalence of DPN was conducted over 25 years and following 4,400 adults in France diagnosed with DM found that about 50% of participants later developed DPN at the end of the 25 year follow up (Hicks & Selvin, 2019; Pirart, 1977). Other studies report a prevalence of DPN around 6–51%, commonly diagnosed through Quantitative Sensory Testing (QST) (Gregg et al., 2007; Hicks & Selvin, 2019; Kumar et al., 1994). The Pittsburgh Epidemiology of Diabetes Complications study examined t1DM and found that prevalence of DPN was 34% and increased significantly with age. However, that study only considered t1DM, limiting external validity and generalisability to t2DM and different age groups (Hicks & Selvin, 2019; Maser et al., 1989). The SEARCH for Diabetes

in Youth study revealed that prevalence of peripheral neuropathy was higher in t2DM compared to type 1; 26% of t2DM had peripheral neuropathy compared to 8% for t1DM (Jaiswal et al., 2013). It has been estimated that the prevalence of peripheral neuropathy is ~51% in adults with t1DM and t2DM, depending on the risk factors such as age, duration of diabetes, blood glucose levels and their control, the type of diabetes (Hicks & Selvin, 2019). Interestingly, there is evidence that DPN prevalence is higher amongst those with t2DM compared to type 1, and that DPN prevalence increases with age and duration of the diabetes (Cabezas-Cerrato, 1998; Hicks & Selvin, 2019; Young, Boulton, MacLeod, Williams, & Sonksen, 1993).

Foot ulcerations are devasting consequences of diabetes including amputation, and the risk in people with DPN is estimated to be around 25% (Hicks & Selvin, 2019). The risk of developing foot ulcerations is three times higher in people with DPN compared with those without DPN (Hicks & Selvin, 2019). Foot ulcerations occur due to a multitude of contributing complications as a result of diabetes, one of which is DPN as individuals lose their protective sensation and ability to feel pain, incurring injuries that they do not become aware of, which remain open and are subject to infection (Selvarajah et al., 2019). As a result of long-standing open wounds and impaired vascular and immune systems, these ulcerations do not heal and infections may penetrate deeper to bone (osteomyelitis), where limb salvage is not possible leading inevitably to amputation (Selvarajah et al., 2019). Foot amputations not only affect quality of life for the person, but also result in lower life expectancy (Selvarajah et al., 2019). Due to these alarmingly high rates, the emphasis is to ensure that diagnosis of DPN is made for early intervention and prevention of lower limb ulcerations and amputations (Cabezas-Cerrato, 1998). Hence, this is the aim of this proof-of-concept study; to determine whether there are more reliable, consistent methods of early diagnosis of DPN that are non-invasive and accessible for the health practitioner. Screening is extremely

important to detect the early signs of DPN before it leads to severe complications such as ulcers and amputation (Hicks & Selvin, 2019).

1.3. Risk Factors for DPN

There are many risk factors that lead to the development and progression of DPN, with the most common identified by the literature included older age, increased diabetes duration, smoking, hypertension, obesity, hyperlipidaemia, hypercholesterolemia, hyperglycaemia as determined by higher glycated haemoglobin test (HbA1c) (Jaiswal et al., 2017; Su et al., 2018) . As part of clinical diagnosis, management, and treatment of DPN, these risk factors must be identified and managed accordingly to prevent the progression of DPN. Understanding these risk factors and their relationship with DPN is important to determine the most appropriate management to reduce the progression of DPN.

Hyperglycaemia is known to be associated with peripheral neuropathy, particularly with fluctuating blood glucose levels (BGLs) (Nisar et al., 2015; Su et al., 2018; Ziegler & Fonseca, 2015). Development of DPN increases rapidly after insulin treatment commencement and is associated with longstanding diabetes, (Hanewinckel et al., 2016; Iqbal et al., 2018) however, proper glycaemic control can delay the development and progression of DPN (Su et al., 2018). Nerve damage occurs due to the formation of advanced glycation end products and ischemic damage, mainly as a result of inflammation and oxidative stress and injury to the blood vessels (Gustavsson et al., 2010; Su et al., 2018; Wu, Cao, He, & Xiong, 2012). A cross-sectional study demonstrated that diabetes and obesity are the main metabolic drivers of peripheral neuropathy and that the prevalence of DPN increased with poorly managed glycaemic control (Callaghan et al., 2018).

Hyperlipidaemia is defined as the malfunction of lipid metabolism hallmarked by increased levels of lipid plasma such as cholesterol, triacylglycerols, and lipoproteins (Wu et

al., 2012). Hyperlipidaemia is associated with diabetes and hypertension, and there is evidence that it contributes to peripheral neuropathy. Several studies have illustrated the association between hyperlipidaemia and DPN development, especially in the presence of hyperglycaemia (Al-Ani, Al-Nimer, & Ali, 2011; Smith & Singleton, 2013; Wiggin et al., 2009). The damage occurs due to oxidative stress in sensory neurons, or hypoxia due to damage to the microcirculation (Vincent, Hinder, Pop-Busui, & Feldman, 2009; Vincent, McLean, Backus, & Feldman, 2005; Wiggin et al., 2009). Hyperlipidaemia may also promote inflammation locally in the peripheral nerves that may lead to the development of DPN (Gustavsson et al., 2010). Further, body mass index (BMI) and obesity are associated with hyperlipidaemia and have been linked with diabetes development, DPN progression and onset (Callaghan et al., 2018).

The duration of diabetes contributes to the development of DPN, especially with prolonged hyperglycaemia (Nisar et al., 2015; Nishikawa et al., 2000; Ziegler & Fonseca, 2015). Oguejiofor, Odenigbo, and Oguejiofor (2010) found that the prevalence of DPN was higher in those with diabetes of more than 15 years, and lower in those with less than 5 years duration. The long duration of diabetes, especially with both fluctuating and uncontrolled BGLs, is associated with increased glycosylation end-product production, metabolic dysregulation, endothelial damage, and increased oxidative products (Oguejiofor et al., 2010). The risk of developing DPN also appears to increase rapidly after insulin treatment, although this is not due to the actual insulin treatments, but the fact that the person's hyperglycaemia can no longer be controlled by oral medications or lifestyle modifications, and therefore has warranted the use of insulin due to uncontrolled hyperglycaemia, which consequently leads to DPN development (Hanewinckel et al., 2016; Iqbal et al., 2018).

Advanced age is associated with duration of diabetes and is another risk factor to consider (Jaiswal et al., 2017; Popescu et al., 2016). Popescu et al. (2016) demonstrated for

every 1-year increase in a person's age, the risk of developing DPN increased by 11.2%. Older people have greater risk of requiring lower limb amputations and developing complications related to DPN (Popescu et al., 2016). The studied population also exhibited poorly controlled hyperglycaemia and hyperlipidaemia, as well as exhibiting a longer duration of diabetes, which further contributed to DPN progression (Popescu et al., 2016).

1.4. Pathophysiology and Aetiology

DPN is one of the major complications of diabetes that results in a series of events leading to ulcerations potentially progressing to lower limb amputations. There are many subtypes of DPN, however the most common type of DPN is diabetic symmetric polyneuropathy, or distal symmetric polyneuropathy, and accounts for approximately 75% of diabetic neuropathies (Albers & Pop-Busui, 2014). It is a chronic manifestation of both t1DM and type 2, and is usually a result of uncontrolled, long-standing hyperglycaemia.

With the impact of diabetes and DPN so high, there are many studies on the pathogenesis of the condition, yet to date, there is no clear pathophysiological explanation for DPN, only proposed mechanisms (Albers & Pop-Busui, 2014). Inflammation is one of the main components implicated in the development of DPN in people with diabetes and pre-diabetes (Jin & Park, 2018; Schreiber, Nones, Reis, Chichorro, & Cunha, 2015). Current hypotheses explore the theory that hyperglycaemia leading to glucotoxicity, coupled with the impairment of insulin function and the presence of other risk factors, activates a number of biological and biochemical pathways that affect cell functions, metabolism, and lead to cellular damage (Albers & Pop-Busui, 2014). The main pathogenic explanations that explore DPN pathogenesis include the Polyol Hyperactivity Pathway, which leads to oxidative stress and consequently inflammation. These have been described below.

1.4.1. Polyol Pathway Hyperactivity

Polyol pathway hyperactivity is one theory proposed to explain DPN pathogenesis (Schreiber et al., 2015). The metabolic changes in diabetes produces the cascade of cellular events that lead to DPN; primarily the effects of hyperglycaemia, produced by the reduction in insulin secretion or resistance, leading to the activation of the polyol pathway (Schreiber et al., 2015). The enzyme aldose reductase formulates sorbitol from glucose, oxidizing nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$. Further oxidation of sorbitol to fructose and reducing nicotinamide adenine dinucleotide (NAD^+) to NADH causes excessive intracellular osmotic stress (increases osmotic pressure (Kawano, 2014)) due to the accumulation of sorbitol. This impaired process induces physical and anatomical defects of tissues and cells (Kawano, 2014). Increases in sorbitol causes an increase in osmotic pressure and the accumulation also reduces the intracellular myoinositol content, which inhibits the metabolism of phosphoinositide and reduces the activity of protein kinase C (PKC) and sodium ions (Na^+), potassium ions (K^+) and adenosine triphosphatase (ATPase) in peripheral nerves, leading to the development of diabetic neuropathy (Kawano, 2014). Nerve damage occurs primarily due to an increased production of NADPH and NAD^+, reducing formation and regeneration of glutathione. The reduction in glutathione leads to an increase in advanced glycation end-product (AGE) formation and activation of diacylglycerol and PKC isoforms. Reduction in glutathione is the primary cause of oxidative dress to the nerve tissues through the accumulation of toxins (Charnogursky, Lee, & Lopez, 2014; Schreiber et al., 2015).

1.4.2. Oxidative Stress

The pathophysiology of peripheral neuropathy due to oxidative stress associated with hyperglycaemia is depicted in Figure 1 and described in detail below. The increase in oxidative stress due to hyperglycaemia occurs through the enhancement of NADPH oxidase expression and the endothelial nitric oxidesynthase (eNOS) uncoupling reaction in vascular

endothelial cells, leading to the excessive production of superoxide. As nitric oxide (NO) is vital for cell function, the excessive build-up of superoxide reduces the NO levels by binding to it. This causes the consequent synthesis of reactive oxygen species (ROS), specifically perioxynitrite and hydroxyl radials, which in turn lead to cytotoxicity and neurosis as a result of the increase in ROS (Kawano, 2014).

Consistent hyperglycaemia in diabetes causes the formation of ROS and nitrosative species (RNS), which are responsible for the structural damage of nucleic acids, carbohydrates, proteins and lipids that are essential for cellular function (Román-Pintos, Villegas-Rivera, Rodríguez-Carrizalez, Miranda-Díaz, & Cardona-Muñoz, 2016). RNS is a class of antimicrobial molecule derived from NO formed by the enzymatic action of nitric oxide synthase 2 (NOS2) (Iovine et al., 2008). NOS2 is predominantly expressed in macrophages following activation by cytokines and microbial materials, in particular gamma interferon (IFN-γ) and lipopolysaccharide (LPS) (Iovine et al., 2008).

ROS and RNS are a crucial component in DM macro-and micro-vessel damage and complications. The problem is not only the excessive production and presence of ROS and RNS, but also the decreased activity of antioxidant enzymes leading to endothelial dysfunction, insulin resistance, and DM complications (Román-Pintos et al., 2016).

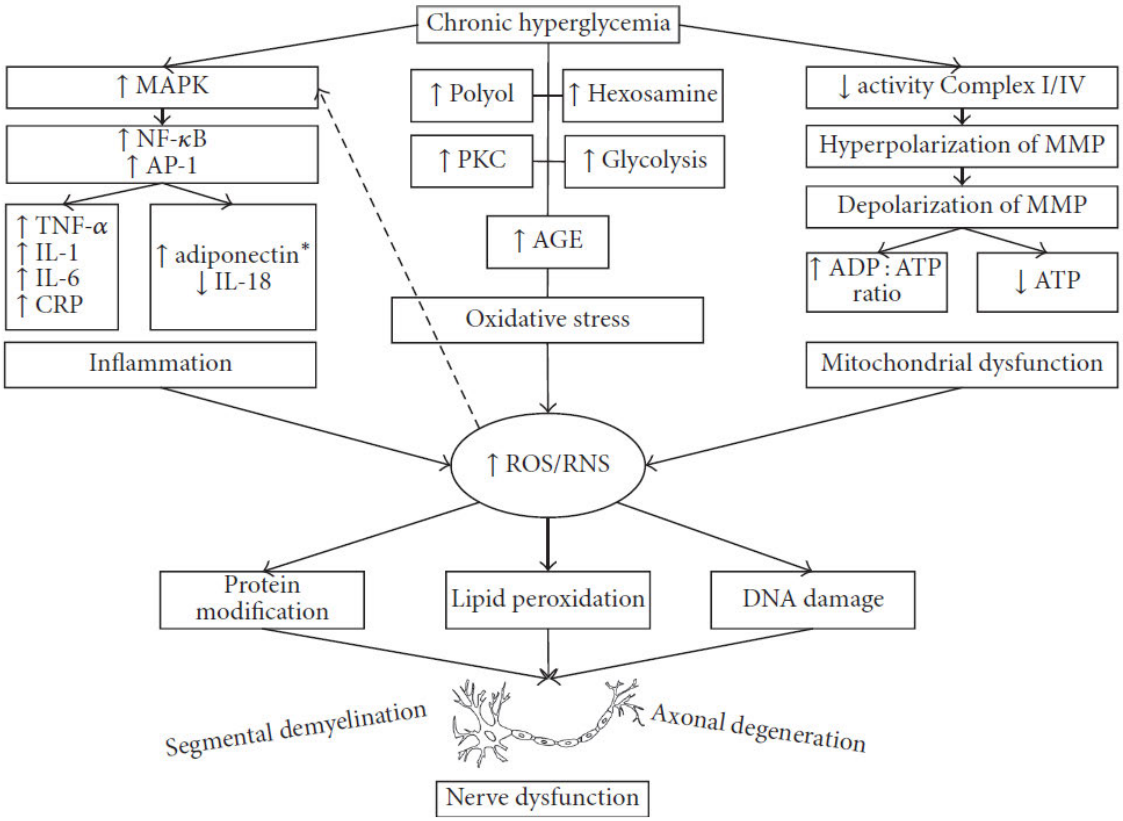
NO and superoxide have essential functions in vascular vessels (Guzik, West, Pillai, Taggart, & Channon, 2002). NO is a facilitator of important vascular functions such as platelet activation, vascular cell signalling, and regulation of smooth muscle tone and blood pressure (Guzik et al., 2002). NO reacts quickly with superoxide, causing its depletion and reduced bioavailability, and in turn limits its essential function, resulting in vascular complications and vascular endothelial dysfunction. The reaction between NO and superoxide also produces peroxynitrite, which adds another level of complication as it

produces further detrimental issues such as additional vascular dysfunction, oxidation of cellular lipids and proteins and direct cellular toxicity (Guzik et al., 2002). The above described oxidative stress also occurs in DM which later manifests as axonal degeneration and segmental demyelination of peripheral nerves leading to DPN (Román-Pintos et al., 2016).

Oxidative stress caused by enhanced free radial formulation due to malfunction of glucose metabolism and lack of antioxidants activates various signalling pathways, leading to the transcription of genes that are associated with diabetic complications and manifestations (Román-Pintos et al., 2016). Examples of genes associated with DPN include the Jun Proto-Oncogene, AP-1 Transcription Factor Subunit *(JUN),* Peroxisome Proliferator Activated Receptor Gamma *(PPARG*), Leptin *(LEP),* Serpin Family E Member 1 *(SERPINE1),* and Apolipoprotein E *(APOE)* genes (Prabodha, Sirisena, & Dissanayake, 2018). Proinflammatory proteins that are activated by gene expression generate an inflammatory response process, which plays a role in damage of peripheral nerves and manifestation of DPN (Ayepola, Chegou, Brooks, & Oguntibeju, 2013; Román-Pintos et al., 2016).

Figure 1

The pathophysiology of peripheral neuropathy due to hyperglycaemia.



Note. Depicted inflammation, oxidative stress, and mitochondrial dysfunction contributes to ROS/RNS formation and nerve damage (Román-Pintos et al., 2016).

RNS can lead to oxidative stress which can all contribute to the nerve damage leading to DPN (Schreiber et al., 2015). Moreover, microvascular impairment attributable to insufficient circulation leads to a reduction in peripheral nerve perfusion, resulting in nerve ischemia and damage. Hyperglycaemia causes nerve and skin hypoxia, reducing electrical stability and function, resulting in nerve loss in proximal and distal segments (Schreiber et al., 2015). Furthermore, oxidative stress prevents insulin release in pancreatic beta-cells by stimulating uncoupling protein 2 (UCP-2), which in effect decreases the ratio of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and thus decreases insulin function. The pancreatic dysfunction is triggered by glucose toxicity is the main DM pathophysiology (Román-Pintos et al., 2016).

1.4.3. Inflammation

Inflammation is one of the manifestations of DPN pathophysiology and there is evidence of a link between DPN presence and progression, and inflammation (Jin & Park, 2018). Low-grade inflammation is hallmarked by an increase in pro-inflammatory and anti-inflammatory cytokines that leads to the activation of the immune system (Jin & Park, 2018). Consequently, inflammation can lead to neurodegeneration and DPN (Jin & Park, 2018) due to axonal degeneration and segmental demyelination of peripheral nerves (Román-Pintos et al., 2016). There has been an established a link between inflammation, t2DM and DPN, with evidence demonstrating that an increase in circulating inflammatory markers can lead to development of t2DM (Jin & Park, 2018).

There are specific inflammatory markers that are elevated in DPN; the interleukin-1 (IL-1) family of cytokines and receptors are associated with the innate immune system and upregulated in acute and chronic inflammation (Dinarello, 2018). Interleukin-1 beta (IL-1β) is released with the presence of disease, can be found in blood plasma (Dinarello, 2018), and is associated with neuropathic pain; producing a pro-inflammatory effect that occurs in low-grade inflammation that leads to DPN development (Gui et al., 2016; Mika et al., 2008; Ren & Torres, 2009).

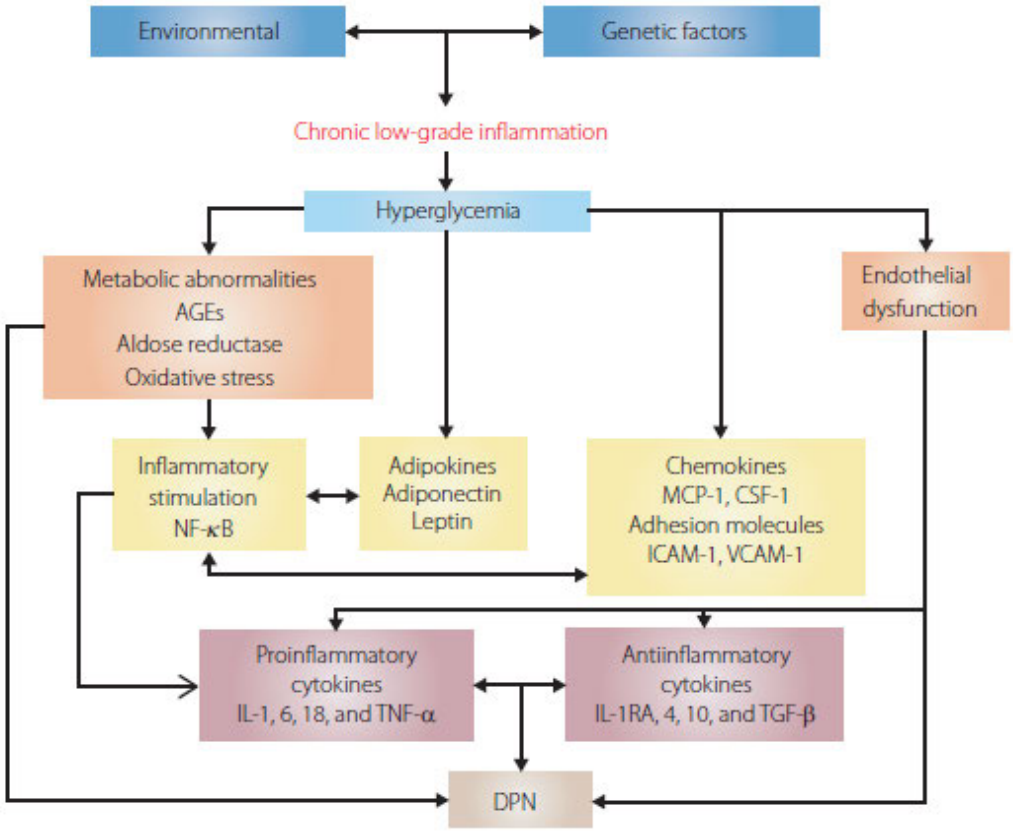
Other specific inflammatory cytokines that have been found to be associated with the development and progress of DPN include IL-6, interleukin 8 (IL-8) and interleukin 10 (IL-10). TNF-α is another cytokine with an established role in the development of DPN (Ge et al., 2016). Pro-inflammatory factors are usually associated with the activation of immune cells, local macrophages and adipocytes, specifically TNF-α, IL-1, IL-6 and IL-8 in conjunction with CRP (Jin & Park, 2018). When pro-inflammatory cytokines are elevated, inhibitory anti-inflammation markers (as antagonists) also become elevated in response to pro-inflammation (Gui et al., 2016). Such an example can be seen with IL-10, which is an anti-inflammatory

cytokine that is elevated to counteract the elevating of pro-inflammatory factors (Jin & Park, 2018).

A study by Doupis et al. (2009) reported participants with DPN had increased serum levels of inflammatory cytokines and growth factors. Individuals with painful neuropathy had a further increase in inflammation, indicated by higher levels of CRP and subsequent endothelial dysfunction (Doupis et al., 2009). Inflammation has always been regarded as a risk factor for macrovascular disease; the study reported impairment of skin microcirculation in people with diabetes and pre-diabetes due to the presence of inflammatory cytokines (Doupis et al., 2009; Herder et al., 2017). They concluded an association between inflammatory markers and development of DPN by dysfunction of microcirculation (Doupis et al., 2009).

Figure 2

Inflammatory pathway associated with DPN (Jin & Park, 2018).



Note. Summarises the inflammatory factors associated with the development and progression of DPN.

1.5. Treatments for DPN

There are limited treatments to reverse DPN, with only pharmacological options being available to alleviate symptoms and manage the disorder (Snyder et al., 2016). First line treatments have been approved by the U.S. Food and Drug Administration (FDA) for the management of DPN; pregabalin (Lyrica) and duloxetine (Cymbalta) (Snyder et al., 2016). Animal and preclinical studies have provided evidence of the reliability, safety, efficacy, and tolerance of pregabalin in painful DPN by providing an analgesic effect (Snyder et al., 2016; Verma, Singh, & Singh Jaggi, 2014). A recent systematic review and meta-analysis reported that gabapentin and pregabalin effectively reduced the pain associated with DPN (Davari et al., 2020).

Tricyclic antidepressants (TCAs) have also been proposed as appropriate first line treatments for DPN; drugs such as amitriptyline and nortriptyline (Callaghan, Price, & Feldman, 2015). Duloxetine, a serotonin and norepinephrine reuptake inhibitor (SNRI), has been shown to be a safe intervention for DPN (Ormseth, Scholz, & Boomershine, 2011). However, pregabalin is preferred due to its superior efficacy and evidence base (Khdour, 2020; Snyder et al., 2016).

Second- and third-line interventions include topical treatments with analgesic effects, for example, lignocaine, tramadol, and oxycodone (Snyder et al., 2016). These are only management options to reduce pain; these drugs deliver pain relief by providing an analgesic effect, so they do not actually treat DPN and pose significant side effects for the person affected (Snyder et al., 2016; Verma, Singh, & Singh Jaggi, 2014). The pharmacological

treatments of DPN have been summarised in Table 1 with all their side effects according to a recent review conducted for DPN pharmacological intervention guidelines (Khdour, 2020). It is important to note that these pharmacological treatments do not treat DPN, nor do they reverse any neurological damage caused by the condition. Other non-direct management strategies for slowing the progression of DPN include controlling diabetes through medication and insulin therapy, if warranted (Perkins, Olaleye, Zinman, & Bril, 2001)

Table 1

Summary of guidelines for recent treatments for DPN (Khdour, 2020).

Line of Treatment	Drug group	Mechanism of Action	Example of Medications	Side Effects
First line therapy	Tricyclic antidepressants	Block noradrenaline and serotonin reuptake, providing an analgesic effect	• imipramine • amitriptyline • desipramine • nortriptyline	Dry mouth, orthostatic hypotension, constipation, and urinary retention
First line therapy	Serotonin and noradrenaline reuptake inhibitors	Inhibits noradrenaline and serotonin reuptake, relieving pain associated with DPN	• Duloxetine • Venlafaxine	Nausea, dry mouth, dizziness, headache, excessive sweating,

				constipation, insomnia
First line therapy	Calcium channel α2-δ ligands (gabapentinoids)	Act on Calcium α2-δ channel, decreasing release of the neurotransmitter's glutamate and noradrenaline, providing relief from neuropathic pain	• Gabapentin • pregabalin	sedation, dizziness, gait instability,
Second/third line therapy	Opioids	Opioids bind to the opioid receptors in the brain, slowing down activity and causing depression of the central nervous system. They also promote the release of dopamine, providing an analgesic effect.	• Oxycodone, • morphine • methadone • tramadol (weak opioid)	Addiction, constipation, breathing problems, dry mouth, drowsiness, nausea, poor appetite.
Third line	Topical medications	Providing peripheral pain relief by reducing	• Lignocaine	Irritation, swelling,

	the transmission of pain signals	• Capsicum extracts	redness, numbness

Early diagnosis is important to prevent further nerve damage, and this is where reliable, quick, and accessible testing is vital in the clinical setting. Early detection and diagnosis of DPN provides better outcomes for individuals, however, it is often difficult to establish due to the silent nature of DPN progression over time (Brown, Pribesh, Baskette, Vinik, & Colberg, 2017). According to the American Diabetes Association position statement, prevention is the key as treatment is limited and only directed at symptom management (Pop-Busui et al., 2017). The current recommendations include ensuring that glucose levels are controlled to prevent hyperglycaemia and its effects in the progression of DPN, as well as adopting a multifactorial management strategy that involves treating all the risk factors that the person may present with, such as obesity, hyperlipidaemia, and cardiovascular conditions (Pop-Busui et al., 2017; The Diabetes Control and Complications Trial Research Group, 1993). Prevention is always better than cure, thus preventing the non-reversible damage of DPN is the key element and priority of any health professional (Pop-Busui et al., 2017). However, in order to apply this, early diagnosis of DPN is of utmost importance. Currently diagnosis of DPN is not standardised and each method poses disadvantages and challenges.

1.6. Diagnosis of DPN

Screening and early diagnosis for DPN is essential due to the severe complications of the condition (Pop-Busui et al., 2017). In addition to poor quality of life for the afflicted individual, with ongoing pain, numbness and risk of falls, there is also the risk of foot ulceration due to DPN and it is also a precursor to Charcot Neuropathy (CN) (Pop-Busui et

al., 2017). Both these complications have devastating effects on the individual, heightening the risk of gangrene and amputation (Pop-Busui et al., 2017).

Diagnosis of DPN based solely on symptoms or medical history yields poor diagnostic accuracy (Doughty & Seyedsadjadi, 2018). Currently the accepted testing for DPN in the clinical setting is QST, which involves both large nerve fibre and small nerve fibre testing, summarised in Table 2 below, which has been adapted from two articles (Doughty & Seyedsadjadi, 2018; Pop-Busui et al., 2017). QST has high sensitivity and specificity, and test both large and small nerve fibres. The 10g monofilament test (sensitivity 66.7% and specificity 72.0%), as well as the 128 Hz tuning fork (sensitivity: 50.0%; specificity: 75.0%) are usually used for diagnosis of DPN (Brown et al., 2017). However, they only yield results once the neuropathy has progressed significantly with moderate to major neurological damage already established (Iqbal et al., 2018). Furthermore, they are subjective, operator dependent, are not standardised, and therefore not reliable means of testing.

Table 2

QST sensory testing for different nerve fibres (Doughty & Seyedsadjadi, 2018; Pop-Busui et al., 2017).

Large Nerve Fibre Testing	**Small Nerve Fibre Testing**
Proprioception	Pain/Nociception sensation
Pressure	Temperature
Vibration Perception Threshold (VPT)	

The strengths and weaknesses of the various DPN diagnosis approaches will now be reviewed. Skin punch biopsy at the distal leg can be used to detect somewhat early stages of

DPN by visualising the thinning of the myelinated and non-myelinated nerve fibres (Iqbal et al., 2018). However, it is not a practical assessment for DPN in a clinical setting, very invasive and must be performed by a specialised health professional (Iqbal et al., 2018). Questionnaires are sometimes used clinically, and in research, to determine the presence of DPN and its severity by relying on the participants' recount of their neurological symptoms. However, these types of tests are always subjective, can be distorted by the presence of other neurological conditions, and are not standardised as each person will have different perceptions associated with their symptoms (Iqbal et al., 2018). Nerve conduction studies (NCS) are considered the gold standard for DPN diagnosis as they are sensitive, specific, and standardised (Iqbal et al., 2018). However, they must be performed by a trained health professional (e.g., neurologist), are expensive, and not easily accessible to all health professionals (Iqbal et al., 2018). Like QST, NCS cannot detect early onset of DPN that is sometimes associated with early diabetes or pre-diabetes, as they usually test large, myelinated nerve fibres. DPN initially starts affecting small nerve fibres, thus the NCS test will not reflect any changes occurring in the small nerve fibres and consequently yield normal results (Fuller, 2005; Iqbal et al., 2018). Additionally, DPN involves the loss of axons, meaning that electrophysiologically, there is reduced neural amplitude that can be detected in the presence of DPN, but no changes in conduction velocity are observed, which is what NCS measure and therefore may not be appropriate for early diagnosis and monitoring progression of DPN (Vinik et al., 2013). This poses a problem as it provides both practitioner and affected individual a false sense of security that there is no initiation of nerve damage, preventing the correct preventative measures to be put in place to slow progression of DPN. This is where diagnostic tests for early DPN diagnosis become a necessity.

At this stage, currently the main diagnostic testing techniques are not reliable for early detection of DPN. The lack of early detection of DPN poses great risks for people affected as

the symptoms and consequences of DPN, such as foot ulcers. These can have devastating consequences including amputations and falls, yet may be preventable if detected early and appropriate strategies are put in place.

Previous reviews have shown a strong inflammatory presence in the pathophysiology of DPN development and progression, yet to date, there have been no studies that have explored the presence of local inflammatory cytokines on the skin of people with DPN. Perkins, Osterhues, Farage, and Robinson (2001) developed a technique to assess inflammatory markers from human skin. Another study by Henshaw, Bostan, Worsley, and Bader (2020) used the same concept to extract inflammatory markers from the epidermis of the foot in people with diabetes and successfully sampled IL-1α and IL-1 receptor antagonist (IL-1RA). They noted that these cytokines behaved differently in people with diabetes compared to non-diabetic controls and were both upregulated in the plantar skin after prolonged weight bearing sitting. However, the authors did not test for neuropathy and therefore could not explore the relationship between neuropathy and inflammation. Currently, to our knowledge, there are no studies that have investigated the relationship between local, peripheral inflammatory cytokines and DPN.

DPN poses a significant impact on daily life, and with the lack of reliable diagnostic testing, and absence of proper treatment, it poses a challenge to manage the condition when, in most cases, diagnosis is made too late. This calls for the development of diagnostic testing that can offer more reliable, measurable, accessible, and standardised diagnosis of DPN.

The present study aimed to explore a potential novel diagnostic method for DPN. Skin sebum was sampled to determine whether peripheral inflammation can be measured on localised skin of people with DPN. The sebum inflammatory markers were compared to the circulating cytokines in blood plasma, which are associated with DPN (IL-6, IL-1, IL-RA,

IL-8, TNF-α, CRP, and IL-10) to determine whether the same inflammatory markers circulating in the blood are present on the skin in individuals diagnosed with DPN. If an association between skin and sebum were found, it might provide an opportunity for the development of a localised, skin sebum test that is non-invasive, inexpensive, and accessible. This could lead to a more reliable, measurable, and standardised diagnostic tool for DPN.

The current study aims to answer the following research questions:

1. Are localised inflammatory markers associated with DPN detectable on the skin of people with DPN?
2. Are those localised inflammatory markers, if detected, comparable to the inflammatory markers present in the blood plasma of people with DPN?
3. Is there a correlation between the presence and absence of DPN, and the levels of inflammatory markers present in the blood and on the skin?

2. Methods

2.1. Participants

Twenty-five participants aged 46–83 years with an existing diagnosis of t2DM were recruited from the Diabetes NSW annual cycle of care screening events held in Sydney, Australia between March 2019, and February 2020. Testing and data collection occurred at multiple locations including Mittagong, Seaforth, and Western Sydney University Campbelltown campus. All participants provided both verbal and written informed consent to participate in the study and were able to withdraw at any time without penalty. Participants that presented with inflammatory skin conditions (such as eczema, psoriasis, lichen planus), and those taking any anti-inflammatory medications (such as cortisone, non-steroidal anti-inflammatory drugs) on the day, as well as those on long-term cortisone (such as prednisone) or anti-inflammatory courses, were excluded from the study. Further exclusions from the study included those individuals with current foot ulceration, active CN or suffering from nerve injury, nerve impingements, or radiculopathy. This was screened through the initial participant information form asking about medications, medical history and a visual assessment was conducted by the researcher to ensure the participants did not present with any of the exclusion criteria.

2.2. Procedure

Experienced nurses from Diabetes NSW conducted blood glucose testing using a blood glucose monitor, as well as HbA1c and lipid profiles, which were obtained using the Afinion (AS100) Analyser (point-of-care device). These results were documented on the participant information form by the researcher after consent was provided by the nurses and participant. All the information sheets that contained the medical information and testing results were de-identified and labelled with a participant number. Participant demographic information including subject ID, age, and gender, were recorded as part of the participant's

demographic form. Medical history was also documented from Diabetes NSW participant records, as well as confirmed verbally with the participant. Information documented included duration of t2DM, past and current medical conditions, allergies, and current medications.

Participants completed the Michigan Neuropathy Screening Instrument (MNSI) questionnaire. QST was conducted on both feet bilaterally. The results were recorded as part of the MNSI physical examination. Participants were then acclimatised for 10 minutes on the plinth before any further testing was conducted.

Nerve conduction testing of the sural nerve was conducted using the NeuroMetrix NC-Stat DPN-Check. Following the nerve conduction testing, sebum samples were collected using Sebutape from both feet bilaterally, as well as the left arm. A subset of the sample, 13 participants, also underwent blood collection. This procedure was approved by the Western Sydney University Human Research Ethics Committee (*H11932)*.

2.3. Materials and Apparatus

2.3.1. MNSI and QST. The MNSI is a clinical tool used assess DPN (Herman et al., 2012). Several studies have demonstrated that it is a reliable, sensitive, and accurate tool for the diagnosis of peripheral neuropathy (Fateh, Madani, Heshmat, & Larijani, 2016; Feldman et al., 1994; Herman et al., 2012; Muntean, Bogdan, Tudorică, & Mota, 2016). The screening tool comprises two parts: a 15-item yes/no questionnaire that was provided to the participant to complete; and the physical exam, which was conducted by the researcher. The self-reporting questionnaire comprises 15 questions on neurological symptoms the participant has experienced. ‘Yes’ responses to questions 1–3, 5–6, 8–9, 11–12, 14–15, and ‘No’ responses to question 7 and 13 are regarded as one point to sum up a score out of 15. The higher the score, the more severe the peripheral neuropathy.

The second part of the MNSI screening tool incorporates QST and a physical assessment, including testing ankle reflexes and dermatological assessment of the foot (Herman et al., 2012). QST refers to a range of clinical tests used to assess somatosensory function, usually used for diagnosis and monitoring of peripheral neuropathies (Backonja et al., 2013). It involves presenting stimuli to the participant; mechanical pressure using a 10 g monofilament, vibration sensation utilising a 128 Hz tuning fork, and sharp/blunt testing using neuro-tips, to elicit a response (Backonja et al., 2013). The MNSI physical exam also incorporates assessment of any foot deformities, infections, and ulcerations. A score ≥ 2 (out of score of 10) is considered abnormal.

For this study, a modified version of the MNSI tool was used, which excluded the ankle reflex testing, and relied on the monofilament and tuning fork tests (Fateh et al., 2016; Feldman et al., 1994; Herman et al., 2012; Muntean et al., 2016) as they are relatively inexpensive, non-invasive, and yield high sensitivity and specificity as demonstrated by past studies (Backonja et al., 2013; Brown, Pribesh, Baskette, Vinik, & Colberg, 2017). The MNSI screening tool was used in conjunction with nerve conduction tests to determine each participant's level of DPN severity.

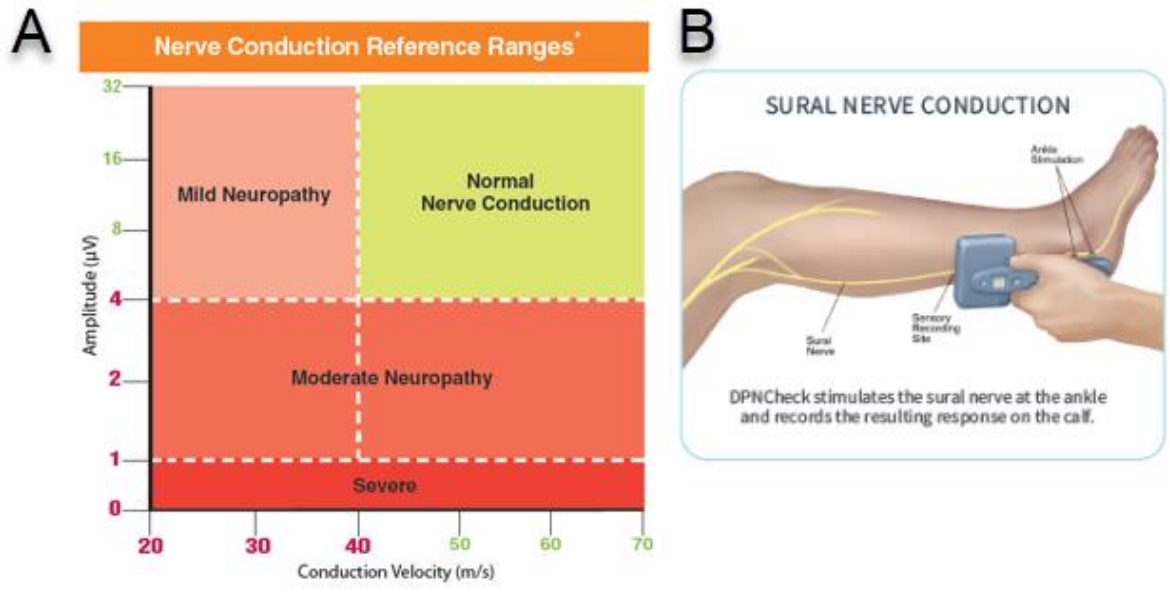
2.3.2. Nerve Conduction. Nerve conduction testing was completed using the NeuroMetrix NC-Stat DPN-Check, which is an accurate and verified nerve conduction technology that allows fast, non-invasive reliable diagnosis of peripheral neuropathy (Lee et al., 2014). It involves placing the probes with a small amount of conduction gel on the participant's leg (lateral aspect for sural nerve) to obtain the nerve amplitude and velocity (Lee et al., 2014), which can be cross-referenced with the device "*Sural Nerve Conduction Interpretation Guide*" to obtain DPN severity (normal, mild, moderate, severe). Figure 3 (A) depicts the reference chart created by the manufacturer to determine classify normal nerve conduction and DPN severity (NeuroMetrix, 2012). Lee et al. (2014) compared gold-standard

NCT to the DPN-Check, which demonstrated that sural nerve amplitude had 88% sensitivity and 94% specificity, while a sural nerve conduction velocity had 94% specificity and 82% sensitivity (Lee et al., 2014).

The procedure involved instructing the participant to lay on their left side, with the lateral aspect of the left leg exposed for the nerve conduction test using the DPN-Check machine on the sural nerve. The participant's skin was prepared with an alcohol swab. A new biosensor was applied to the machine biosensor port and aligned with the foam (indicated by illumination of a green light upon successful insertion) and the machine's stimulating probes were coated with conduction gel, enough to cover the whole head of the probe; care was taken to ensure that the gel did not smear between the two probes. The LED display indicates which leg to test (L=left, R=right). The anode (short probe on the distal aspect of the machine) and cathode (long, proximal probe) were aligned with the lateral malleolus in such a manner that the cathode was adjacent to the central prominence of the malleolus. The backing was removed from the biosensor and firm pressure was applied to ensure all areas were in contact with the skin, that is, that the biosensor had total contact with the skin on the lower calf. The centre button was then pressed, releasing a monophasic electrical current for 10–15 seconds to obtain a reading of the sural nerve's velocity (metres/second) and amplitude (microvolts). Figure 3 (B) (NeuroMetrix, 2021) depicts the process.

Figure 3

Nerve conduction references and procedure.



Note. (A) Nerve conduction reference ranges to classify normal nerve conduction, mild, moderate, and severe neuropathy, which is used as a reference guide after the process acquiring the values from the nerve conduction test. (B) Process of how the nerve conduction test is conducted on the sural nerve on the lateral aspect of the leg using the NeuroMetrix NC-Stat DPN-Check. From NeuroMetrix (2021).

2.3.3. Local Inflammatory Markers (Sebum). Sebutape (Evalulab) is a lipophilic polymeric film usually used for sebum extraction from the skin (Ashraf et al., 2011; Perkins, Osterhues, Farage, & Robinson, 2001) as demonstrated by previous studies (Piérard & Piérard-Franchimont, 1993). Perkins et al. (2001) developed a method using Sebutape to extract inflammatory markers successfully from sebum on the skin, specifically interleukins IL-1RA/ IL-1α. Not only is this a safe and non-invasive method, but it has been demonstrated that inflammatory markers were successfully sampled and analysed using sebutape and ELISA kits (Perkins et at., 2001).

Sebutape samples were obtained from the participant's skin between the 2nd and 3rd metatarsal phalangeal joints of the left foot, as well as on the participant's cubital fossa on their left arm. The sebum sample on the arm was considered a control; each participant served as their own control. This was to determine whether the inflammatory markers on the skin were localised to foot or were present on the arm as part of a more circulating inflammatory presence. Blood circulating inflammation is indicated if the inflammatory markers were present on both arm and foot.

The sebutape sample was left on the skin for 2 minutes, before it was removed and placed in an Eppendorf tube. Samples were stored at -80 ^{o}C within 1–2 hours and processed at a later date. All the sebutapes were processed according to the protocol developed by Perkins et al. (2001). Prior to biochemical analysis, the sebum was reclaimed from the tape. The frozen tapes were thawed at room temperature and then were adjusted using sterile forceps to ensure that the non-adhesive side faced outward in the Eppendorf tube; such that the entire skin sampling surface of each tape was facing inward and immersed in the phosphate buffered saline (PBS). Each Eppendorf tube had 1.2 ml of PBS pipetted to fully immerse the tapes. The sebutapes were then sonicated for 10 minutes at approximately 20 ^{o}C, vortexed for 1 min and then mixed vigorously with a pipette tip, after which they were removed from the Eppendorf tube. Labelled Eppendorf tubes then had 100 µL aliquoted into them before being stored in a labelled box at -80 °C. Additionally, 100 µL of plasma was aliquoted into labelled 600 µL Eppendorf tubes for transporting to Eve Technologies (Canada) at -80 °C for analysis.

2.3.4. Systemic Inflammatory Markers (Blood). To compare localised inflammation to circulating pro- and anti-inflammatory cytokines, a subset of participants underwent blood sample collection to analyse the inflammatory markers present in the plasma. A tourniquet was applied to the participant's arm, and after instructing them to pump

their fist 3 times, the prominent veins were located and palpated on the ante-cubital area. Warm packs were used if locating the veins proved difficult. A 23 G 19 mm butterfly needle with lure (Becton, Dickinson and Company, USA), 10 ml 18 mg Ethylenediaminetetraacetic acid (EDTA) Vacutainer collection tubes (Becton, Dickinson and Company, USA), and Vacutainer standard holder (Becton, Dickinson and Company, USA), were used to collect two 10 ml tubes of blood from each participant. The blood sample was then transported to the lab immediately within a 30-minute timeframe, weighed and centrifuged at 23 ^{o}C for 6 minutes at 4000 rpm to separate the plasma. Plasma was then aliquoted into 1500 µL Eppendorf tubes using 1000 µL pipette tips. These were all stored at -80 ^{o}C and processed later by aliquoting 100 µL of plasma into labelled 600 µL Eppendorf tubes for transporting to Eve Technologies (Canada) at -80 °C for analysis.

2.3.5. Multiplex Analysis of Cytokines by Eve Technologies. Eve Technologies lab (Canada) used Luminex xMAP technology for multiplexed quantification of 14 Human cytokines, chemokines, and growth factors. The multiplexing analysis was performed using the Luminex™ 200 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta). Fourteen markers were simultaneously measured in the samples using Eve Technologies' Human High Sensitivity 14-Plex Discovery Assay® (Millipore Sigma, Burlington, Massachusetts, USA) according to the manufacturer's protocol. The 14-plex consisted of GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17A, IL-23, TNF-α. Assay sensitivities of these markers range from 0.11–3.25 pg/mL for the 14-plex.

2.4. Statistical Analysis

Descriptive and inferential statistics were performed using IBM SPSS statistics V26 (IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant at $\alpha = .05$ ($p < 0.05$), 2-tailed. The data were inspected for normality. For data that did not

follow a normal distribution, non-parametric statistical analyses were conducted, specifically, the Mann-Whitney U test was performed to determine whether any statistically significant difference was present between the DPN and no DPN groups' demographic factors; Pearson's chi-squared was conducted to determine if there were any significant differences between DPN status and sex. Spearman's rank correlations were conducted to determine any associations between the inflammatory factors in foot and arm sebum, and blood plasma.

3. Results

Twenty-five participants with t2DM were recruited into the study; the sample comprised 11 females and 14 males with a mean age of 73 (*SD* = 8.3) years.

3.1. Demographics

A Pearson chi-squared test of association (2-sided) was run and there was no significant association between gender and DPN status ($p = 0.201$). All demographics did not yield any notable results, except BMI, where it was significantly higher in the DPN group ($p = 0.003$) compared to the no DPN group.

Table 3 presents the descriptive statistics of participant demographics enrolled in the study, featuring the mean, standard deviation, and range of each demographic factor. The average duration of diabetes was 16 years (SD = 12.64), with HbA1c slightly elevated above normal around the 6.91% (SD = 1.17), and elevated cholesterol with a mean of 4.18 (SD = 1.28) and majority of participants were classified as obese with a mean BMI of 31.8 (SD = 5.31).

Table 4 categorises the participants into those with and without DPN. Sixty-eight percent of participants that were recruited had DPN, with the DPN group significantly ($p = 0.003$) more overweight (M = 33.67, SD = 5.2) than the no DPN group (M = 27.82, SD = 2.9). HbA1c was higher in the DPN (M = 7.21, SD = 1.2) than the no DPN group (M = 6.28, SD = 0.8) with borderline statistical significance ($p = 0.060$). The DPN and no DPN groups did not differ on the other demographics including age, duration of diabetes, and cholesterol.

Table 3

Descriptive demographic statistics (N=25).

	Mean (SD)	**Range**
Age (years)	72.96 (8.29)	46–83
Duration of Diabetes (years)	16.20 (12.64)	1.0–2.0
HbA1c (%)	6.91 (1.17)	5.2–10.8
Cholesterol (mmol/L)	4.18 (1.28)	2.6–7.7
BMI	31.80 (5.31)	23.9–44.5

Table 4

Descriptive statistics according to DPN status (N=25).

	DPN Status			
	No DPN	DPN	Mann-Whitney Test	*p*-value
N (% total)	8 (32 %)	17 (68 %)		
Age, Mean Years (SD)	68.75 (10.8)	74.94 (6.3)	40.5	0.108
Sex, F:M	5:3	6:11		
BMI, Mean (*SD*)	27.82 (2.9)	33.67 (5.2)	19	**0.003**
Duration of diabetes, Mean years (*SD*)	13.63 (11.5)	17.41 (13.3)	59	0.628

Cholesterol, Mean mmol (*SD*)	4.39 (1.6)	4.09 (1.6)	64	0.842
HbA1c, Mean percentage (*SD*)	6.28 (0.8)	7.21 (1.2)	35.5	0.057

3.2. Clinical Markers

Non-parametric tests (Mann-Whitney U) were conducted to determine whether there was any difference between DPN status and presence of inflammatory markers on the foot, arm, and in the blood (Table 5). All results were non-significant between the two groups except for Interleukin 12 (IL12) foot sebum ($p = 0.038$), which was higher in the no DPN group compared to the DPN group. TNF-α trended toward statistical significance ($p = 0.07$) between the two groups, where it was noted to be lower in the DPN group compared to the no DPN group, or close to the same values. Comparisons between the arm and foot did not yield any differences, with inflammatory factor levels about the same, whilst blood plasma levels were higher compared to the foot and arm samples. Percentage normalisation of foot and arm sebum values that did not yield any significant differences between the two locations.

Table 5

Inflammatory markers analysed for the foot and arm sebum, and blood plasma samples using non-parametric tests to determine significance between DPN and no DPN groups.

Inflammatory factors	**Median in Picograms (IQR)**		***p*-value**
	No DPN	DPN	
		Foot Sebum	
GM-CSF	0.34 (0.06)	0.30 (0.07)	0.189

Inflammatory factors	**Median in Picograms (IQR)**		***p*-value**
	No DPN	DPN	
IFN-γ	0.14 (0.09)	0.11 (0.05)	0.228
IL-1β	0.16 (0.02)	0.14 (0.02)	0.055
IL-2	0.15 (0.05)	0.14 (0.05)	0.478
IL-4	0.88 (0.16)	0.78 (0.23)	0.122
IL-5	0.14 (0.04)	0.14 (0.02)	0.575
IL-6	0.05 (0.02)	0.05 (0.02)	0.856
IL-8	0.10 (0.06)	0.08 (0.06)	0.814
IL-10	0.50 (0.08)	0.47 (0.12)	0.381
IL-12	0.14 (0.02)	0.12 (0.02)	**0.038**
IL-13	0.35 (0.08)	0.30 (0.06)	0.218
IL-17A	0.11 (0.03)	0.11 (0.05)	0.894
IL-23	6.37 (2.98)	6.73 (2.31)	0.792
TNF-α	0.15 (0.02)	0.13 (0.03)	0.072
		Arm Sebum	
GM-CSF	0.35 (0.08)	0.32 (0.05)	0.209
IFN- γ	0.15 (0.05)	0.13 (0.07)	0.289
IL-1β	0.16 (0.03)	0.14 (0.03)	0.303
IL-2	0.14 (0.05)	0.13 (0.06)	0.507
IL-4	0.95 (0.34)	0.69 (0.23)	0.080
IL-5	0.15 (0.02)	0.13 (0.04)	0.301
IL-6	0.05 (0.02)	0.04 (0.02)	0.602
IL-8	0.08 (0.08)	0.09 (0.05)	0.360

Inflammatory factors	**Median in Picograms (IQR)**		***p*-value**
	No DPN	DPN	
IL-10	0.49 (0.16)	0.48 (0.12)	0.381
IL-12	0.14 (0.02)	0.12 (0.05)	0.375
IL-13	0.34 (0.06)	0.30 (0.10)	0.628
IL-17A	0.14 (0.03)	0.12 (0.05)	0.111
IL-23	7.40 (1.47)	6.59 (4.91)	0.726
TNF-α	0.14 (0.03)	0.13 (0.03)	0.303
	Blood Plasma		
GM-CSF	32.58 (8.54)	32.59 (34.22)	0.866
IFN-γ	9.25 (6.76)	8.94 (8.02)	0.735
IL-1β	0.68 (0.29)	0.62 (0.54)	0.735
IL-2	1.83 (0.21)	2.50 (1.91)	0.612
IL-4	9.13 (8.37)	8.26 (4.29)	0.395
IL-5	1.03 (1.12)	0.96 (0.94)	1.00
IL-6	0.79 (2.47)	0.80 (0.69)	0.735
IL-8	7.31 (11.88)	5.83 (8.30)	0.176
IL-10	5.45 (3.39)	5.14 (4.71)	0.799
IL-12	1.30 (0.43)	1.76 (1.36)	0.237
IL-13	2.97 (8.34)	2.43 (2.40)	0.128
IL-17A	5.22 (1.82)	5.26 (4.97)	0.933
IL-23	263.86 (162.39)	255.53 (294.06)	0.866
TNF-α	8.03 (4.80)	6.90 (4.00)	0.866

3.3. Correlations

Spearman's rank-order correlations were conducted to determine any relationship between the inflammatory factors and the demographics, as well as the inflammatory factors on the arm, foot, and in the blood.

Table 6 depicts Spearman's rank-order correlations of inflammatory markers between foot and arm sebum, as well as foot sebum and blood plasma. Results of the Spearman's rho correlation indicated that there was a significant positive association between the foot and arm sebum samples for IL-1β ($rs(24) = .51$, $p = 0.026$), IL-5 ($rs(24) = .40$, $p = 0.050$), IL-6 ($rs(24) = .44$, $p = 0.026$), IL-8 ($rs(24) = .65$, $p < 0.001$), IL-13 ($rs(24) = .63$, $p < 0.004$), and TNF-α ($rs(24) = .54$, $p = 0.017$). There was no significance noted between foot sebum and blood plasma correlations.

Table 6

Spearman's rank-order correlations of inflammatory markers between foot and arm sebum, and blood plasma

Inflammatory factor	Statistics	Correlation between the Foot and Arm Sebum	Correlation between the Foot Sebum and Blood Plasma
GM-CSF	ρ	0.35	0.12
	p	0.086	0.698
	N	25	13
IFN-γ	ρ	0.06	0.52
	p	0.805	0.233

Inflammatory factor	Statistics	Correlation between the Foot and Arm Sebum	Correlation between the Foot Sebum and Blood Plasma
	N	19	7
IL-1β	ρ	0.51*	0.23
	p	**0.026**	0.625
	N	19	7
IL-2	ρ	0.41	0.05
	p	0.081	0.908
	N	19	7
IL-4	ρ	0.31	0.40
	p	0.130	0.177
	N	25	13
IL-5	ρ	0.40	0.15
	p	**0.050**	0.635
	N	25	13
IL-6	ρ	0.44*	-0.18
	p	**0.026**	0.562
	N	25	13

Inflammatory factor	Statistics	Correlation between the Foot and Arm Sebum	Correlation between the Foot Sebum and Blood Plasma
IL-8	ρ	0.65**	0.10
	p	**0.000**	0.751
	N	25	13
IL-10	ρ	0.29	-0.24
	p	0.160	0.428
	N	25	13
IL-12	ρ	0.36	-0.22
	p	0.132	0.638
	N	19	7
IL-13	ρ	0.63**	0.20
	p	**0.004**	0.670
	N	19	7
IL-17A	ρ	-0.08	-0.04
	p	0.760	0.938
	N	19	7
IL-23	ρ	0.34	0.43

Inflammatory factor	Statistics	Correlation between the Foot and Arm Sebum	Correlation between the Foot Sebum and Blood Plasma
TNF-α	*p*	0.154	0.337
	N	19	7
	ρ	0.54*	0.04
	p	**0.017**	0.937
	N	19	7

*. Correlation is significant at the 0.050 level (2-tailed).

**. Correlation is significant at the 0.010 level (2-tailed).

Table 7 depicts Spearman's rank-order correlations of inflammatory markers between foot and arm sebum, grouped by DPN and no DPN groups. Results of the Spearman's rho correlation indicated that there was a significant positive association between the foot and arm sebum for IL-8 in both the no DPN groups ($rs(7) = .79$, $p = 0.020$) and DPN ($rs(16) = .62$, $p = 0.008$), while IL-13 was significantly positively correlated in the DPN group ($rs(12) = .57$, $p = 0.043$). IL-6 exhibited significant a positive correlation only in the normal group ($rs(7) = .83$, $p = 0.010$). TNF-α was borderline significantly positively correlated in the DPN group ($rs(12) = .51$, $p = 0.075$).

Table 7

Spearman's rank-order correlations of inflammatory markers between foot and arm sebum, grouped into DPN and no DPN groups.

Inflammatory factor	**Statistics**	**Correlation between the Foot and Arm Sebum**	
		No DPN	DPN
GM-CSF	ρ	0.32	0.27
	p	0.444	0.303
	N	8	17
IFN-γ	ρ	-0.18	0.00
	p	0.734	0.993
	N	6	13
IL-1β	ρ	0.06	0.48
	p	0.908	0.099
	N	6	13
IL-2	ρ	0.26	0.37
	p	0.612	0.208
	N	6	13
IL-4	ρ	0.52	0.04
	p	0.183	0.879
	N	8	17
IL-5	ρ	0.45	0.33

	p	0.260	0.191
	N	8	17
IL-6	ρ	0.83**	0.24
	p	**0.010**	0.349
	N	8	17
IL-8	ρ	0.79*	.62**
	p	**0.020**	**0.008**
	N	8	17
IL-10	ρ	0.57	0.22
	p	0.143	0.407
	N	8	17
IL-12	ρ	0.27	0.02
	p	0.599	0.946
	N	6	13
IL-13	ρ	0.32	.57*
	p	0.538	**0.043**
	N	6	13
IL-17A	ρ	-0.28	-0.12
	p	0.595	0.706

	N	6	13
IL-23	ρ	0.14	0.33
	p	0.787	0.266
	N	6	13
TNF-α	ρ	0.29	0.51
	p	0.577	**0.075**
	N	6	13

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 8 depicts the Spearman's rank-order correlations between foot sebum inflammatory markers and demographic factors across all participants in both groups. Results of the Spearman's rho correlation indicate that there was a significant positive association between duration of diabetes and GM-CSF ($rs(24) = .45$, $p = 0.025$), IFN-γ ($rs(18) = .74$, $p < 0.000$), IL-5 ($rs(24) = .53$, $p = 0.006$), and IL-23 ($rs(18) = .52$, $p = 0.021$), and a borderline effect was noted for TNF-α ($rs(24) = .41$, $p = 0.080$). A number of inflammatory factors were also positively correlated with HbA1c: GM-CSF ($rs(24) = .44$, $p = 0.027$), IL-1β ($rs(18) = .62$, $p < 0.000$), IL-12 ($rs(18) = .56$, $p = 0.013$), and IL-13 ($rs(18) = .47$, $p = 0.043$).

Table 8

Spearman's rho correlations between demographic variables and inflammatory factors from the foot sebum.

Inflammatory Factors in Foot Sebum	**Statistics**	**Age**	**Duration of Diabetes**	**HbA1c**	**BMI**	**Cholesterol**
GM-CSF	ρ	-0.36	-0.45*	-0.44*	-0.19	0.23
	p	0.078	**0.025**	**0.027**	0.363	0.264
	N	25	25	25	25	25
IFN-γ	ρ	-0.01	-0.74**	-0.33	-0.11	0.21
	p	0.956	**0.000**	0.164	0.644	0.384
	N	19	19	19	19	19
IL-1β	ρ	-0.30	-0.33	-0.62**	-0.27	-0.11
	p	0.205	0.165	**0.005**	0.272	0.645
	N	19	19	19	19	19
IL-2	ρ	-0.18	-0.35	-0.15	-0.15	-0.17
	p	0.473	0.140	0.551	0.533	0.476
	N	19	19	19	19	19
IL-4	ρ	-0.02	-0.31	-0.30	-0.30	-0.01
	p	0.939	0.125	0.141	0.143	0.964
	N	25	25	25	25	25
IL-5	ρ	-0.32	-0.53**	-0.29	0.00	-0.05
	p	0.122	**0.006**	0.163	0.985	0.806
	N	25	25	25	25	25
IL-6	ρ	-0.17	-0.24	-0.24	-0.14	0.09
	p	0.412	0.242	0.242	0.504	0.683
	N	25	25	25	25	25
IL-8	ρ	-0.14	-0.22	-0.17	0.11	0.05

	p	0.505	0.294	0.415	0.594	0.799
	N	25	25	25	25	25
IL-10	ρ	-0.21	-0.37	-0.29	-0.13	-0.12
	p	0.313	0.072	0.163	0.526	0.562
	N	25	25	25	25	25
IL-12	ρ	-0.33	-0.42	-0.56*	-0.31	0.03
	p	0.166	0.073	**0.013**	0.193	0.915
	N	19	19	19	19	19
IL-13	ρ	-0.30	-0.43	-0.47*	-0.07	0.03
	p	0.210	0.067	**0.043**	0.775	0.901
	N	19	19	19	19	19
IL-17A	ρ	-0.06	-0.24	-0.39	0.02	-0.13
	p	0.822	0.318	0.101	0.934	0.604
	N	19	19	19	19	19
IL-23	ρ	-0.26	-0.52*	-0.12	0.06	-0.21
	p	0.277	**0.021**	0.621	0.814	0.380
	N	19	19	19	19	19
TNF-α	ρ	-0.36	-0.41	-0.54*	-0.26	-0.11
	p	0.129	0.080	**0.017**	0.275	0.651
	N	19	19	19	19	19

*. Correlation is significant at the 0.050 level (2-tailed).

**. Correlation is significant at the 0.010 level (2-tailed)

4. Discussion

This study aimed to determine whether the presence of local inflammatory markers specific to DPN can be detected by extracting sebum from the skin, and whether they can be associated and correlated with inflammatory markers in blood plasma of people with DPN. While the research aims were not supported due to the small sample size, there were some notable findings that will be useful in guiding future research.

There were significant differences between the DPN and no DPN group in terms of BMI, where the people who had DPN were more obese than those without. BMI and obesity are also associated with hyperlipidaemia and have been linked with diabetes development, DPN progression and onset (Callaghan et al., 2018). The general trend that was demonstrated in the descriptive statistics, even though the results were not significant, showed that people with DPN tend to be older, have had diabetes for a longer duration of time, are more obese, and have a high HbA1c, which is consistent with the literature. A study investigating risk factors of DPN demonstrated that older age, increased diabetes duration, smoking, increased blood pressure, obesity, hyperlipidaemia, hypercholesterolemia, and high HbA1c all led to increased DPN prevalence within their study cohort (Jaiswal et al., 2017). Unlike previous research demonstrating an association between hyperlipidaemia and DPN development, especially in the presence of hyperglycaemia (Al-Ani et al., 2011; Smith & Singleton, 2013; Wiggin et al., 2009), the participants of this study depicted a similar cholesterol level across the DPN and no DPN groups, where the cholesterol level was slightly lower in people with DPN. This could be due to the small sample size with older participants that are most likely managing hypercholesterolemia pharmacologically.

There were no statistically or clinically significant differences between inflammatory marker levels at the various sample locations including arm, foot, and plasma. Most of the inflammatory factors were similar between the DPN and no DPN groups across all sample types. Interestingly, the cytokines were in fact slightly lower on average in the DPN group, compared to the no DPN group, which is contradictory to what has been demonstrated in the literature. Doupis et al. (2009) reported that people with DPN had increased plasma levels of inflammatory cytokines and growth factors compared to those without DPN. People with painful neuropathy have a further increase in inflammation, where the inhibitory anti-inflammatory markers become elevated in response to pro-inflammation as an antagonist (Gui et al., 2016). This was not demonstrated with the results obtained here, and that maybe due to the small sample size.

The correlations between the inflammatory factors on the arms and foot did not support the hypothesised effect. While there were some significant correlations, there was no indication that the inflammatory markers were higher on the foot (*cf.* arm) to indicate that the presence of DPN would cause the local foot inflammatory factors to increase. The fact that there were instances where inflammatory factors increased on the foot and the arm, and vice versa, means that the presence of the cytokines on the foot was not specific to DPN in the lower limb.

This study was a proof-of-concept research idea that has not been attempted previously in the literature, and therefore, there was no exact indication of what results to expect, with other studies focusing on inflammatory biomarkers in skin ulcerations (Bader & Oomens, 2018; Henshaw et al., 2020). A study evaluated the effect of sedentary behaviour on plantar skin by evaluating inflammatory marker change in people with and without diabetes, also using sebutape, revealed that there was a sustained inflammatory response highlighted by the release of sebum biomarkers after extended weightbearing, especially in people with diabetes (Henshaw et

al., 2020). IL-1α was elevated in the no diabetes group after mechanical irritation to the site, indicating that it is not specific to diabetes or the possible presence of DPN (Henshaw et al., 2020). This is somewhat consistent with the results observed here as there were no differences between the groups for sebum inflammatory marker levels, which suggests that there may be no localised inflammatory response on the superficial layers of the skin that can be collected via sebum to diagnose DPN. Such a test to measure any physiological change would be redundant as there are no disorder-specific changes in people with DPN. This study tested a novel concept, and while there were no significant results to indicate that there is a possibility that inflammatory factors could be harvested from skin sebum in the foot to diagnose DPN, the fact that it was a small sample size makes the study inconclusive and further research should be conducted with more participants.

The study sample comprised mainly older people with long standing diabetes, which limits its generalisability. That was also reflected in their duration of diabetes being approximately 17 years. The duration of diabetes is another contributing factor to the development of DPN, especially with prolonged hyperglycaemia (Nisar et al., 2015; Nishikawa et al., 2000; Ziegler & Fonseca, 2015), and as demonstrated here, the DPN group had a higher HbA1c compared to the no DPN group. A study by Oguejiofor et al. (2010) found that the prevalence of DPN was higher in those with diabetes of more than 15 years, and a lower prevalence in those with less than 5 years duration. This could then skew the data as there were more participants with DPN who had longer duration of diabetes. In future, an even distribution between the two groups that better represents the diabetes and population would be ideal.

4.1. Limitations

There are many limitations associated with small sample sizes in quantitative research, one of which is the detection of clinically significant differences between groups (Faber & Fonseca, 2014). In this case, it was anticipated that there would be differences between the DPN and no DPN groups, but due to the sample size, differences were not detected. Significant results (from a larger sample size) are essential to determine whether this proof-of-concept study is appropriate to apply clinically as a diagnosis tool for health practitioners.

One of the preventative ways to ensure that a proper sample size can be obtained is by conducting sample size and effect size calculations (Faber & Fonseca, 2014). The main issue associated with the conduct of this study was the COVID-19 pandemic that resulted in the study being paused for 12 months due to lockdown restrictions limiting face-to-face contact with research participants. Due to the limited timeframe of the Master of Research program, the recruitment of extra participants was not possible. There are other elements to consider, such as financial restraints, timeframes, difficulty of recruitment, and overall acceptability of the study methods (Hertzog, 2008).

Other limitations to the study included that the sample was excessively varied, there was not an even number between participants with DPN and those without, and the majority of participants were aged 60 years and above (except one who was 46 years). In addition to the sample size being small, it was not reflective of the general t2DM population, but rather a subset of older people with diabetes. Further, there was no control for diet or medications, and these elements can be pro-inflammatory or anti-inflammatory and may have impacted results accordingly.

The execution of the methods of this study was controlled thoroughly. Despite this, there are other elements and confounders that may have interfered with data collection, such as the amount of sebum removed from the skin. Older people tend to have dryer skin with reduced sebum production (Farage, Miller, Elsner, & Maibach, 2013), and that would impact the quantity/concentration of cytokines that could be removed from the skin. To counteract that, we normalised for protein content of the raw inflammatory marker data to ensure consistency. Standardising the protocol minimised potential measurement errors. Here, a reputable commercial laboratory (Eve Technologies, Canada) was contracted to analyse the samples. High sensitivity assays were used to measure cytokines to ensure minute amounts were detectable.

Recruitment was terminated after the COVID-19 pandemic struck in March 2020, and government stay at home and lockdown orders prevented any further participant recruitment or testing. The university was shut down and any planned research, as well as facilities, were also on hold for the majority of 2020, and now again in 2021. Due to this extended period of time affected by the pandemic, this research project recruitment was terminated, and the project did not resume until December 2020, in which case due to timelines, recommencing recruitment was not feasible.

4.2. Conclusion

Findings did not support the hypotheses for this study. However, the research questions were explored, providing direction for future research. That is, a localised diagnostic tool would not be possible to develop as the inflammatory response related to DPN is not specific to localised skin or sebum through sebutape. There is opportunity to direct further research into other modalities, such as improved sebum extraction procedures and higher sensitivity assays, that are more sensitive to obtain and analyse inflammatory markers on the skin. This could prove

to be a diagnostic tool for early DPN diagnosis and promote the implementation of prevention strategies that mitigate the foot complications arising from progressively damaging DPN.

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