“THE ROLE OF PRO-INFLAMMATORY CYTOKINES AND AUTOIMMUNE ANTIBODIES IN DIABETIC PERIPHERAL NEUROPATHY”

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Abstract

Introduction – The pathogenetic vision of diabetes mellitus has changed in the last few years, with inflammatory and autoimmunity pathways playing roles in the development and progression of diabetic complications. This study was conducted to investigate whether inflammation and/or autoimmunity are associated with the pathogenesis of human diabetic peripheral neuropathy.

Methods – A cross-sectional analysis was initially conducted to explore the population of patients with diabetes mellitus in the Kingdom of Bahrain. The demographics of patients diagnosed with diabetes mellitus in the Royal Medical Services-Bahrain Defence Force Hospital were randomly collected from 500 record cards. Case-control analysis included three groups: 30 patients with diabetic peripheral neuropathy, 30 patients with diabetes mellitus without neuropathy, and 30 healthy controls. Blood analysis was conducted to compare the levels of pro-inflammatory markers and autoimmune markers between the three groups. Secondary analysis investigated the correlations between the level of markers and sample demographics and neurological manifestations. Due to the limited time and financial resources available, this research was considered as a pilot/exploratory study encouraging further investigations to take place in the near future.

Results – From the 500 sample initially selected, 48% were male (n=242) and 52% (n= 258) were female. The mean age was 55 ± 14 years and the mean BMI was 35 ± 9 kg/m². Type I DM was present in 8% (n=38) only as opposed to 92% (n=462) who had type II DM. From the sample randomly selected, 76% of the patients had other medical complications, the commonest being peripheral neuropathy; 26% (n=186) followed by vascular insufficiency; 20% (n=141). Case control analysis demonstrated very highly significant differences between the three groups in the levels of IL-6, IL-8 and IL-1β (p<0.001), highly significant differences in the levels of TNF-α, IFN-γ (p<0.01), and a significant difference in the levels of CRP (p<0.05). Highly significant differences between the percentages of positive and negative autoimmune antibodies (ANA) between the three groups were observed. The odds of positive values of ANAs in the neuropathy group were 50 times higher when compared to control groups. Secondary analysis detected a number of significant relationships between the levels of pro-inflammatory markers and sample demographics. Highly significant correlations were found to be associated with neurological characteristics in the neuropathy group at the levels of CRP, IL-8, and IL-1β.

Conclusion – The present study demonstrated that human peripheral diabetic neuropathy is associated with increased biochemical markers of inflammation and autoimmunity. Furthermore, painful neuropathy may be associated with further increase in inflammation. These results indicate that inflammation and autoimmunity may be important contributors in the development of peripheral neuropathy in diabetes mellitus. The new pathogenic factors may lead to the consideration of new management plans involving new therapeutic approaches and disease markers.
Abbreviation

The following abbreviations have been used throughout the text.

AAEM - American Association of Electrodiagnostic Medicine
AAN - American Academy of Neurology
AAPMR - American Association of Physical Medicine and Rehabilitation
ABI - Ankle Brachial Index
ACEI - Angiotension Converting Enzyme Inhibitor
ADA - American Diabetes Association
AGE - Advanced Glycation End products
AHA - America Heart Association
ALA - Alpha lipoic acid
ANA - Antinuclear Antibodies
ANOVA - Analysis Of Variance
ATP - Adenoside Triphosphate
BMI - Body Mass Index
BP - Blood Pressure
CBC - Complete Blood Count
CIDP - Chronic Inflammatory Demyelinating Polyneuropathy
CINHAL - Cumulative Index to Nursing and Allied Health Literature
cm - Centimetre
CNS - Central Nervous System
COX - Cyclooxygenase
CRP - C Reactive Protein
DCACT - Diabetes Control and Complication Trial
DAN - Diabetic Autonomic Neuropathy
DLRPN - Diabetic Lumbosacral Radiculoplexis Polyneuropathy
DM - Diabetes Mellitus
DN - Diabetic Neuropathy
DNA - Deoxyribonucleic Acid
DPN - Diabetic Peripheral Neuropathy
dsDNA - double stranded DNA
DSPN - Distal Symmetric Polyneuropathy
ELISA - Enzyme-linked immunosorbent assay
IDDM - Insulin-dependent Diabetes Mellitus
FIELD - Feno-fibrate Intervention and Event Lowering in DM
FU - Foot Ulceration
GABA – Gamma Aminobutyric Acid
GAD - Glutamate decarboxylase
GCC - Gulf Cooperation Council
HbA1c - Glycosylated Haemoglobin
HDI - Human Development Index
HDL - High Density Lipoprotein
HLA - Human Leukocyte Antigen
HRT - Hormone Replacement Therapy
hsCRP - high sensitivity C Reactive Protein
IDF - International Diabetes Federation
ICAM-1 - Intercellular adhesion molecule-1
IFN-γ - Interferon-gamma
IgG - Immunoglobulin G
IGT - Insulin Glucose Tolerance
IL - Interleukin
imCRP - Immunoturbidimetric assay
IRMM - Institute for Reference Materials and Measurements
IVIg - intravenous immunoglobulin
JNC7 - Joint National Committee on prevention of hypertension
K⁺ - Potassium
kg - Kilogramme
kg/m² - Kilogramme/metre square
LDL - Low Density Lipoprotein
Md¿ - Median
MENA - Middle East and North Africa
MER - Middle East Region
mmHg – Millimetre of mercury
MMP - Multiple Motor Polyneuropathy
MNCV - Motor Nerve Conduction Velocity
MNMM - Mononeuritis Multiplex
MNSI - Michigan Neuropathy System Index
mRNA - messenger RNA
Na⁺ - Sodium
NAD/NADH - Nicotanamide adenine dinucleotide ratio
NATHAN - Neurological Assessment of Thiocetic Acid in Diabetic Neuropathy
NARV - Nerve Axon Reflex-related Vasodilation
NCV - Nerve Conduction Velocity
NDS - Neuropathy Disability Score
Nf-Kβ - Nuclear factor Kappa
NGF - Nerve Growth Factor
NHANES - National Health and Nutrition Examination Survey
NHS - National Health Service
NO - Nitric Oxide
NOS - Nitrous Oxide System
nNOS - neuronal NOS
NP - Neuropathic Pain
NSS - Neuropathy Symptom Score
OCEBM – Oxford Centre of Evidence Based Medicine
OMC – Other Medical Complications
p - Probability level
PAD - Peripheral Arterial Disease
PASW - Predictive Analytic Software
PGE2 - Prostaglandin E2
PICO - Population Intervention Complication Outcome
PKC - Protein Kinase C
PPARs - Peroxisome proliferator activated receptors
PVD - Peripheral Vascular Disease
QMU - Queen Margaret University
QOL - Quality Of Life
RAGE - Receptor for Advanced Glycation End products
RBG - Random Blood Glucose
RCT - Randomised Controlled Trial
RMS-BDF - Royal Medical Services Bahrain Defence Force
ROS - Reactive Oxygen Species
RPPHs - Reference preparation for protein in human serum
RR - Relative Risk
sICAM-1 - Soluble intercellular adhesion molecule-1
SNCV - Sensory Nerve Conduction Velocity
SPSS - Software Package for Statistical Analysis
SWF - Semmes-Weinstein monofilament
TCAs - Tricyclic Antidepressant
TLR - Toll-Like receptor
TNF-α - Tumour Necrosis Factor-alpha
WHO - World Health Organisation
VCAM-1 - Vascular Cell Adhesion Protein
VEGF - Vascular endothelial growth factor
VPT - Vibration Perception Threshold
VSC 4.1 - Ventral Spine cord 4.1
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The Late Ali Bin Abdulla Bin Khalid Al-Khalifa Research Fund is an agency for supporting medical research in the Kingdom of Bahrain. The aim is to lead the way towards health research that improves the individual’s health and quality of life.

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To my Father

“I am who I am today because you believed in me”
Chapter One: Introduction

Diabetic peripheral neuropathy (DPN) is one of the major complications of diabetes mellitus (DM) and is the most common type of diabetic neuropathy (Boulton et al. 2004). Its clinical manifestations include painful neuropathic symptoms and insensitivity, which increases the risk of traumatic burns (hot or cold), wounds and foot ulceration (Tesfaye et al. 2010). Multiple possible risk factors have been implicated to be associated with the development of peripheral neuropathy in patients with DM. The complete pathophysiological process by which this complication is developed is, however, not fully understood.

Complications affecting the lower limbs are among the most common manifestations of DM. Targeting organ complications, secondary to DM, is therefore thought to be one of the most important medical concerns of the coming decades.

The goals of this chapter are to:

- Present an overview of the issue of diabetic peripheral neuropathy as a complication of diabetes mellitus worldwide;
- Outline the pathogenesis of diabetic peripheral neuropathy and possible relationship to inflammation and autoimmunity;
- Discuss the background of the research question and the significance of the problem;
- Specify the research question, aims and objectives of the study;
- Present the research hypotheses;
- Focus on some areas of contribution to the body of knowledge and to clinical practice;
- Provide an outline of the report.
1.1. Diabetes mellitus and diabetic peripheral neuropathy

Diabetes mellitus (DM) is a common health problem which has reached epidemic proportions due to the rapidly increasing rates of this disease worldwide (IDF 2013). Both the World Health Organisation (WHO) and the International Diabetes Federation (IDF) consider diabetes mellitus the 21st century’s leading health care challenge (Wilson and Wright 2011). In fact the overall burden of diabetes mellitus is immense with a worldwide population of approximately 382 million in 2013 (IDF 2013). According to the WHO (2011) evidence suggest that, without effective prevention and control programmes, DM will likely continue to increase globally.

Diabetes mellitus has been recognized as a group of heterogeneous disorders with the common elements of hyperglycaemia and glucose intolerance, due to insulin deficiency, impaired effectiveness of insulin action, or both (IDF 2011). Although there are several distinct forms of DM, for classification purposes they have been divided on the basis of aetiology and clinical presentation of the disorder into two main categories: Type I DM (immune mediated diabetes), which is characterized by a marked reduction in the amount of insulin produced by the beta cells of the pancreas, and type II DM in which insulin resistance in peripheral tissues is a common feature ranging from primarily insulin resistance with relative insulin deficiency to predominantly an insulin secretory defect with insulin resistance (ADA 2010). Other types of DM include gestational DM, and less commonly drug-induced DM and idiopathic DM (ADA 2010).

Type I DM usually accounts for only a minority of the total burden of DM in a population (IDF 2013); it is more common in younger age groups in most high income countries and low income countries (WHO 2011). Type II DM constitutes about 85 to 95% of all DM in high income countries, and accounts for an even higher percentage in low and middle income countries (IDF 2013).
Persistently elevated levels of glucose are thought to trigger long term complications of DM. Complications of DM are common and often occur if the disease is not diagnosed or treated soon enough (Tolfaye et al. 2005). These complications include heart and vascular disease, eye disease (retinopathy), kidney disease (nephropathy), as well as nerve damage (neuropathy) that may eventually predispose to loss of a limb (amputation) (Tolfaye et al. 2005).

Diabetic neuropathy is described as damage to nerves in the body that occurs due to high concentration of glucose in the blood. Diabetic neuropathies affect different parts of the nervous system which result in the presence of diverse clinical manifestations (Boulton et al. 2005). Most common among the neuropathies are chronic sensorimotor distal symmetric peripheral neuropathy, commonly known as diabetic peripheral neuropathy, and the autonomic neuropathies (Tolfaye et al. 2010).

Diabetic peripheral neuropathy has been recognised since the 5th century B.C., when the Hindu Sustruta physician described what today we would call “painful neuropathy”. In 1848, Claude Bernard suggested that DM was a neurological disease with secondary metabolic manifestations (Skljarevski 2007). Marchal de Calvi however clarified at a later stage that the neuropathy was a consequence and not the cause of diabetes mellitus (Skljarevski 2007).

Diabetic peripheral neuropathy involves somatic sensory and motor nerves, as well as autonomic nerves (Yagihashi et al. 2010). Numerous classifications of the variety of syndromes affecting the peripheral nervous system in DM have been proposed in previous years (Boulton et al. 2004). The first classification of DPN was proposed in 1893 (Tracy and Dick 2007), which was commonly used in clinical neuropathy (Yasuda et al. 2003). With the advances of knowledge in the pathophysiological aspects of DM and DPN, the classification system advanced to include pathophysiological bases (Sinnreich 2005). This will be further discussed in Chapter two.
Several studies have implicated poor glycaemic control (UKPDS 1998), duration of DM, hyperlipidaemia (particularly hypertryglyceridaemia) (Yeung et al. 2009), elevated albumin excretion rates (Thomas et al. 2004) and obesity (Ylitalo et al. 2011) as risk factors for the development of DPN. Although there is conclusive evidence demonstrating that hyperglycaemia is clearly important in the development of DPN, and that intensive DM management reduces the incidence and progression of the disease (Tesiaye and Selvarajah 2012) the pathophysiological processes by which hyperglycaemia causes neuropathy are not fully understood.

1.2. Research focus

Diabetic peripheral neuropathy is the most common complication of DM, where population-based studies have suggested more than half of the patients with either type I or type II DM will develop DPN, and as much as 30% of those manifestations are symptomatic (Harati 2007; Ramos et al. 2007; Farmer et al. 2012).

Defining a precise cause for diabetic peripheral neuropathy is difficult. Numerous hypotheses have been proposed individually, but the pathogenesis of DPN remains uncertain, and it is likely that the overall mechanism may ultimately be complex and multifactorial (Forbes and Cooper 2013).

Until recently, the pathophysiology of DPN was constructed around four theories (discussed in detail in Chapter two). Studies proposed that axonal loss was the end result of microangiopathy (damage to capillaries and arterioles) of the vasa nervorum, combined with, axonal enlargement, impaired axonal transport, and destruction of the myelin sheath (Olmos et al. 2012).

This traditional approach, however, may be too general and does not explain the great variability of clinical presentations of DPN, with sensory, motor and autonomic manifestations. Moreover, Forbes and Cooper (2013) stated that most of the clinical
trials that have prospectively evaluated various classes of pharmacological agents for treating DPN have failed to show therapeutic benefit.

The mechanism by which hyperglycaemia is linked to peripheral nerve damage has been a matter of debate. On the one hand, some investigators have suggested that vascular problems may lead to peripheral nerve damage. Since nerve function depends on adequate blood flow and blood flow is known to be diminished in patients with DM, the vascular hypothesis was proposed as a possible mechanism for DPN (Dyck et al. 1986; Tesfaye et al. 2005, Zochodne 2007). In line with this theory, therapeutic interventions to promote vasodilatation have been shown to increase nerve perfusion in diabetic rats (Ziegler et al. 2004) and DM subjects (Stevens 2000; Amitov et al. 2003), which have led some investigators to conclude that vascular abnormalities contribute to the causes of neuropathies (Betul and Gondogdu 2006).

On the other hand, some investigators have associated neuropathy with metabolic mechanisms related to hyperglycaemia, among which the polyol pathway has been stated as being the most important (Crook 2004). DM selectively damages cells, like endothelial cells and mesangial cells, whose glucose transport rate does not decline rapidly as a result of hyperglycaemia (Brownlee 2005). Most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycaemia, in order to maintain a constant level of their internal glucose concentration (Brownlee 2005). In Contrast, the cells of the retina, kidney and nervous tissue (which are the parts of the body commonly associated with diabetic complications) will use the required concentration of glucose for energy, and any glucose not used for energy will enter the polyol pathway and be converted into sorbitol (Kaiser et al. 1993; Heilig et al. 1995; Forbes and Cooper 2013).

Activation of the polyol pathway is dependent on the enzyme aldose reductase and there are suggestions that this metabolic cascade contributes to the development of neuropathy (Yagihashi et al. 2011). However, the mechanism of how the polyol pathway contributes to the development of neuropathy remains elusive.
In addition to the polyol pathway, other hyperglycaemia-dependent metabolic pathways such as advanced glycation end-products or increased oxidative stress have also been proposed as possible mechanisms of the disease (Cameron et al. 1994; Figueroa-Romero et al. 2008).

In 1998, a hypothesis was proposed suggesting that long-term innate immune system activation, resulting in chronic inflammation, has led to the development of type II DM (Pickup et al. 1998). Forbes and Cooper (2013) noted that within the last few years, numerous studies have demonstrated that low-grade inflammation is associated with the risk of developing type II DM. Furthermore, Nguyen et al. (2012) stated that chronic subclinical inflammation is considered a part of the insulin resistance syndrome, which is strongly related to features of the metabolic syndrome.

The mechanisms by which chronic inflammation may evoke type II DM are not clear. However, it has been suggested that adipose tissue can synthesize and release the main pro-inflammatory cytokines; tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) (Navarro and Mora 2005). Pro-inflammatory cytokines and acute phase reactants are implicated in multiple metabolic pathways relevant to insulin resistance; including insulin regulation, reactive oxygen species, lipoprotein lipase action and adipocyte function (Singh et al. 2010). Therefore, activated innate immunity and inflammation are believed to be relevant factors in the pathogenesis of DM, with considerable data that type II DM involves an inflammatory component (Crook 2004; Pickup 2004; Singh et al. 2010).

Inflammation, more specifically pro-inflammatory cytokines, may play a determinantal role in the development of micro-vascular diabetic complications (Forbes and Cooper 2013). It has also been proposed as a major factor in the development of diabetic peripheral neuropathies in animal models (Yamakawa et al. 2011). Similarly, autoimmunity has been identified in a significant number of neuropathies, such as, proximal neuropathy, and autonomic neuropathies associated with DM (Vinik et al. 2005). However, possible correlations between DPN and
inflammation and/or autoimmunity have not been fully investigated. This will be further discussed in Chapter two.

Based on this perspective, an appropriate question would be whether inflammation and/or autoimmunity are related to the development of diabetic peripheral neuropathy.

1.3. Significance and Rationale of the study

1.3.1. Health problems associated with diabetic peripheral neuropathy

Diabetic neuropathy, especially painful DPN, is associated with a high degree of functional impairment which affects health-related quality of life and activities of daily living (Jensen et al. 2007; O’Conner 2009). Approximately 15% of people with DM develop at least one foot ulcer during their lifetime (Boulton 2000; Kantor and Margolis 2001), and while vascular disease plays a role in the pathogenesis of diabetic foot ulcers, approximately, 60–70% of diabetic foot ulcers are primarily neuropathic in origin (Gonzalez and Oley 2000).

Peripheral neuropathy is believed to be an independent risk factor for foot ulceration and amputation (Veves et al. 1992; Shaw and Boulton 1997). Potter et al. (1998) investigated the incidence of peripheral neuropathy in the contralateral limb of 38 individuals with DM with unilateral amputations. Evidence of neuropathy in the contralateral limb was found in 97% of the patients at the time of the amputation. McGill et al. (2005) carried out a follow up study on 477 patients with diabetes mellitus of which 250 were diagnosed with DPN and 222 were not. They observed that during the follow up period, 34 new ulcers occurred in the neuropathy group and 3 ulcers in the control group, resulting in an annual incidence of 6.3% and 0.5%, respectively. These figures highlight the impact foot complications have on DPN subjects.
Commonly, ulceration in patients with peripheral neuropathy is triggered by a cascade of events. A minor trauma in the presence of neuropathy (sensory loss), if unattended, is believed to be the starting point. Changes in gait characteristics are considered a common mechanism by which tissue damage may occur in DM patients with peripheral neuropathy. This population shows changes in gait characteristics compared to non-neuropathic counterparts (Kwon et al. 2003), which result in higher stress on the plantar surface and, consequently, higher risk of tissue damage. Thereafter, a lack of adequate blood supply at this critical phase contributes to the risk of significant infection and hence, further tissue breakdown and risk of amputation. Microcirculatory impairments are thought to play a role in the diminished blood flow responses under stressful conditions (Tooke 1995; Veves et al. 1998), which may prevent the diabetic neuropathic foot’s response to injury and infection in the usual manner.

It seems clear that a combination of altered foot biomechanics and microcirculatory changes are responsible for the increased risk of foot ulcerations observed in subjects with peripheral neuropathy. Figure 1.3.1 demonstrates how DPN contributes to the development of foot ulcers in patients with DM (Meana-Esteban 2011).
Figure 1.3.1: An overview of health problems related to DPN. The continuous arrow lines represent a strong correlation; the dashed arrow lines represent a weaker correlation (Although DM may lead to some changes in gait, neuropathy is the main factor explaining gait changes in DPN. This diagram was sourced from Meana-Esteban (2011)

In addition to gait and microcirculatory impairments, diabetes mellitus in general and neuropathic complications in particular have also been associated with diminished quality of life (QOL). It has been noted that there is a positive correlation between DM and its complications and reduced QOL, with subjects with more severe complications showing the poorest QOL (Price and Harding 2000).

Painful symptoms such as burning, tingling, shooting (like electric shock) or lancing (stabbing) are present in around a third of patients with DPN (Tesfaye et al. 2012). These symptoms are generally worse at night and can disturb sleep (Quattrini and Tesfaye 2003). Together with painful symptoms during the day, this may often lead to a reduction in the individual’s ability to perform daily activities (Quattrini and Tesfaye 2003). Gore et al. (2005) stated that the burden of painful DPN resulted in a
persistent discomfort despite poly-pharmacy use and high resource use, resulting in limitations in daily activities and poor satisfaction with treatments that were often considered to be inappropriate.

Neuropathy can trigger severe psychological effects (Gore et al. 2005). When a patient loses foot sensation, the feet are regarded as “unreal”, as are any associated problems (e.g., ulcerations). The patient then may become detached from his or her foot problems to the point where a serious complication often is neglected. In addition, any time a person is in chronic pain, there is a psychological profile associated with that, including depression. Chronic persistently painful DPN may be extremely distressing and might be associated with profound depression together with anxiety (Gore et al. 2005).

It is generally upheld that effective prevention of DPN requires in-depth knowledge of the pathogenesis and clinical manifestations of the complication (Ziegler et al. 2008); neither of which is well understood so far. Targeting patients at increased risk for developing DPN is therefore believed to constitute a cost effective approach to control disease progression to end stage complications.

Prevention is said to be the best treatment (Ziegler et al. 2008). Despite efforts to make an early diagnosis and to halt the progression of DPN, currently there is no effective treatment available at a global level, except for tight control of blood glucose (Yagihashi et al. 2010). This might be as a result of the complicated clinical picture that does not necessarily reflect the actual pathological mechanism of the disease and insufficient clarification of the pathogenesis of DPN (Yagihasi 2010). There might also be a possibility that the development of a therapeutic drug might not be based on genuine causative factors (Yagihasi 2010).

When it comes to the treatment of painful neuropathy, many of the current medications, such as, gabapentin (Neurontin®), pregabalin (Lyrica®), serotonin–norepinephrine reuptake inhibitors, tricyclic antidepressants or analgesics provide pain relief but do not affect the actual disease process. Treatment of the risk factors
for the development of neuropathy and offering treatment for the specific type of pain may eventually help alter the development of further complications of neuropathy.

Podiatric management of the neuropathic foot is a challenging task. It is one that requires ongoing patience, determination, and periods of trials and errors that often predisposes both the clinician and the patient to a feeling of hopelessness.

1.3.2. The burden of DM and its complications

As previously mentioned, diabetes mellitus is now one of the most common non-communicable diseases globally (IDF 2013). It is the fourth or fifth leading cause of death in most high-income countries and there is substantial evidence that it is endemic in many low- and middle-income countries (IDF 2013). Diabetes mellitus has a significant impact on health because of its micro-vascular and macro-vascular complications. Complications from DM, such as peripheral vascular disease, cardiac complications, diabetic neuropathy, amputations, renal failure and blindness are resulting in increasing disability, reduced life expectancy and enormous health costs worldwide (IDF 2013).

Over the last 10 years Bahrain has faced a rapid transition in its socio-economic status, which resulted in great changes both in life-style and in patterns of health and disease (Al Zubra 2001). These social advances have been accompanied by certain cultural and lifestyle changes that have led to unhealthy nutritional habits and sedentary lifestyles, increasing the prevalence of obesity (Al-Mahroos and Al-Roomi 2007). The estimated prevalence of DM in Bahrain in 2011 was 19.8% according to the International Diabetes Federation estimates (IDF 2013). Section 2.2 in Chapter two presents a thorough review of the prevalence of DM and its complications worldwide, especially in the Arab world.
Although epidemiological studies have persistently shown a high prevalence of DM in Arab countries, the control of DM is still poor and complications of DM are common in Bahrain (Al-Mahroos and Al-Roomi 2007). Approximately 25 – 50% of DM patients in developed countries have peripheral neuropathy (Harris et al. 1993; Boulton et al. 2004). Studies conducted in the Middle East region report higher rates of painful diabetic peripheral neuropathy (DPN), ranging from 35% to 65% of DM population in their region (Motlagh et al. 2009). Patients with DPN almost account for more hospital admissions than all other DM complications combined, and are responsible for 50 – 75% of non-traumatic amputations (Vinik and Mehrabyan 2004).

### 1.3.3. Economic costs of DM and DPN

Diabetes mellitus imposes a large economic burden on individuals and their families, national health services, and countries (IDF 2013). According to the IDF (2013) health expenditure on DM and its complications accounted for 10.8% of total health expenditure worldwide in 2013. The global annual total cost to treat DM and manage DM complications was estimated to be at least USD 548 billion in 2013 (IDF 2013). By 2035, this number is projected to exceed USD 627 billion (IDF 2013).

The management of DPN and its complications is likely to form a large proportion of this total expenditure, because treatment is often resource intensive and long term. In 2003, the total annual cost of DPN and its complications in the United States was estimated to be between USD 4.6 and USD 13.7 billion for type I and type II DM (Gordois et al. 2003).

Peripheral neuropathy, especially DPN, reportedly results in significantly higher healthcare costs when compared with age and sex-matched DM patients without DPN (Dwarkin et al. 2010). Annual healthcare costs in two separate databases were 24 – 38% higher even after adjusting for differences in co-morbid medical conditions such as cardiovascular illness (Dwarkin et al. 2010). Up to 27% of the direct medical
cost of DM may be attributed to DPN (Gordois et al. 2003). These staggering figures cover the annual cost of DPN only, which is believed to represent only 30-40 percent of the prevalence of overall peripheral neuropathy (Gordois et al. 2003).

The estimated predicted global prevalence of DM according to IDF (2013) is 592 million by 2035. Together, not only can DM and other causes of peripheral neuropathy lead to tremendous debilitating complications, such as amputations, pain, numbness, loss of balance, sleep, strength, quality and length of life, and polypharmacy use, but they may also account for significant overall morbidity and healthcare costs. Studies have shown that the costs of caring for the DM patient with neuropathy can be as much as USD 7,000 more per year as caring for the DM patient without neuropathy. Sadly, most of this cost is directed to symptom management and control only (Gordois et al. 2003).

From these epidemiologic data, Gordois et al. (2003) stated that diabetic peripheral neuropathy and the associated foot ulceration and neuropathic pain are far from rare and far from benign, posing a major healthcare challenge to the medical profession and to the society.

1.4. Major areas of contributions

The study is expected to contribute in a number of ways to the body of knowledge to enhance the understanding of diabetic peripheral neuropathy and its pathogenesis, and to improve clinical practice as described below:

1.4.1. Improve the management of patients with DPN through understanding the course of the disease

The early recognition and appropriate management of neuropathy in patients with DM is important for a number of reasons. Loss of protective sensation allows
unperceived trauma and pressures of daily movement to cause foot ulcers, which can lead to hospital stays, prolonged immobilization or even amputation of the lower limb (Vileikyte et al. 2005). Peripheral neuropathy may also affect the muscles and result in muscle weakness and loss of reflexes, leading to changes in the pattern of gait (Katoulis et al. 1994). Foot deformities may consequently develop and result in areas of increased plantar pressure. Blisters and sores may appear on numb areas of the foot because pressure or injury goes unnoticed. If foot injuries are not treated promptly, the infection may spread to the bone, which may eventually lead to amputation.

Furthermore, diabetic painful peripheral neuropathy is estimated to affect approximately 15 – 25% of cases of DPN, and is associated with excruciating disabling pain (Harris et al. 1993; Boulton et al. 2004). Understanding the mechanism of developing this disease in patients with DM is expected to improve the management of such patients, the patient’s health, quality of life, and acceptance. This might eventually aid in reducing the health cost burden of this disease as described earlier.

1.4.2. Aid future research to improve the pharmacological management of DPN

Treating diabetic neuropathy is a difficult task for the physician and the patient. Most of the medications currently used do not lead to complete symptom relief. Clinical trials are under way to help find new ways to treat symptoms and delay disease progression. Researching the topic of diabetic neuropathy and investigating whether other elements such as inflammation and autoimmunity might contribute to the pathogenesis of this disease aids in gaining a wider knowledge and a better understanding of diabetic peripheral neuropathies and how they progress. Besides providing new clues for how the immune system works, this study may aid in identifying new therapeutic agents that either block the development of neuropathies or reduce the severity of neuropathic symptoms in such patients, as well as
improving the use of previously discovered agents to produce better outcomes under such circumstances.

In addition, if increased levels of pro-inflammatory markers and/or autoimmune antibodies were confirmed, this could contribute to the adoption of new markers for the activity of the disease, and to the development of effective new therapies in the future. This will subsequently highlight possible gaps in the literature and improve the management of DPN patients.

1.4.3. Control the use of ineffective drugs for managing symptoms of DPN

Despite the fact that there is no definite answer to whether inflammation and autoimmunity are associated with the pathogenesis of diabetic peripheral neuropathies, a number of anti-inflammatory and autoimmune agents have been prescribed to improve the symptoms of peripheral neuropathy in patients with DM (Cohen and Harris. 1987; Sharma et al. 2002). This study will therefore investigate whether circulating concentrations of pro-inflammatory markers and autoimmune antibodies differ among patients with DM with and without peripheral neuropathy for the purpose of improving the knowledge and the understanding of the pathogenesis of peripheral neuropathy in DM subjects, and perhaps aid the development of effective new therapeutic agents to control the disease in the future. If the concentration levels of pro-inflammatory cytokines and autoimmune antibodies are not elevated in the blood serum of patients with diabetic peripheral neuropathies, this might suggest that inflammation and autoimmunity are not involved in the pathogenesis of DPN. Pharmacological agents that target Inflammation and autoimmunity should subsequently be discontinued in DPN management.
1.4.4. Improve the Podiatric management of patients with DPN

Treating patients with DPN often requires a multidisciplinary approach of diabetologists, neurologists, podiatrists, and physiotherapists. Podiatrists are commonly aware of the potential complications associated with diabetic peripheral neuropathy. They are also trained to assess, recognize and diagnose the sensory component of this disease. Interestingly, despite the fact the DPN is a neurological disease, a large number of patients with DPN present to the podiatry clinic for initial evaluation.

It is well accepted that peripheral neuropathy is a risk factor for developing foot ulceration and that early detection leading to adequate foot care can reduce amputation rates by about 50 to 80 percent (Tesfaye et al. 2012). This emphasizes the need for investigating this complication in terms of pathogenesis, clinical features, assessment tools, prevention and management.

It is mandatory for podiatrists to appreciate the large scope of diabetic peripheral neuropathy, specifically how the motor and autonomic components contribute to the pathogenesis in addition to the sensory dysfunction. It is also important that consistent, objective and reliable clinical examinations are provided for these patients in all hospitals worldwide.

Developing a deeper understanding of this condition directs the podiatrist to treat the patient with more confidence, supports the potential of new assessment tools to be discovered in the future, as well as, detecting markers in the blood of the patients with DPN which might be used to predict the development of this complication and establish ways of controlling it. Widening the scope of DPN prevention and management from the podiatric prospective not only advances the control and management of patients with DPN but it will also expand the scope of the podiatry profession to include wider aspects of patient care.
1.5. Outline of the Thesis

1.5.1. Chapter Two:

A review of the literature constitutes Chapter two. The review of literature is designed to explore the theoretical foundation underpinning the diverse clinical classifications, pathogenesis, and current pharmacological managements of DPN worldwide. This chapter will also demonstrate the increased prevalence of this problem in the Arab countries and the possible relationships between the ethnicity of the Arab world and prevalence and incidence of DM and DPN.

The comprehensive review of previous studies is organised to discuss the existing phenomenon pertaining to the pathogenesis of diabetic peripheral neuropathies that led to questioning the possible relationship between inflammation and autoimmunity and the development of this disease. This problem identification follows a conceptual framework of theory and literature to test empirically the proposed hypotheses.

1.5.2. Chapter Three:

The methodology of the study is covered in Chapter three. This section covers all the relevant issues of the quantitative research approaches followed in this study. It also provides the justification for the quantitative approaches adapted throughout the study. It includes details on sample recruitment, inclusion and exclusion criteria, and unit of analysis and research instruments, data collection tools and data analysis tool.

1.5.3. Chapter Four:

Chapter four comprises two major sections; primary data analysis and secondary data analysis. Primary data analysis is further divided into results of the cross sectional
analysis (population demographics), and case control analyses (hypotheses testing). The secondary data section represents results of further analysis conducted to explore potential associations between variables.

1.5.4. Chapter Five:

Chapter five analyses and contextualises the findings of the study, the findings from the literature reviewed and from the theories that underlie the research question. This chapter is divided into: Part one (cross-sectional analysis), Part two (case-control analysis (hypotheses testing), and Part three (discussing the results of the secondary analysis). It then presents a summary of the discussion, followed by the implications of the study and how it can contribute to the body of knowledge and to the clinical practice. The chapter is concluded with a section discussing the limitations of the research methodology and highlighting the possibility of generalizing the findings, and the need for further research in the future.

1.5.5. Chapter Six:

This chapter (the conclusion) summarises the main findings emerging from the study to answer the research question in the light of the research limitations. It highlights the contributions to the theory and the body of knowledge, discusses the research implications for researchers and the application of such findings to clinical practice. The chapter concludes with a section reflecting on the thesis journey and demonstrating how this journey has met the aims of the Professional Doctorate programme.
Chapter Two: Literature Review

Diabetic peripheral neuropathies: an update on prevalence, classifications, pathogenesis and current management

In diabetes mellitus, the resulting complications are grouped under “micro-vascular disease” (due to damage to small blood vessels) and “macro-vascular disease” (due to damage to the arteries) (Forbes and Cooper 2011). Micro-vascular complications include retinopathy, nephropathy and neuropathy. The major macro-vascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes (Forbes and Cooper 2011). Other chronic complications of DM include depression (Nouwen et al. 2011), dementia (Ceukirman et al. 2005), and sexual dysfunction (Tesfaye et al. 2005; Adeniji et al. 2011). This review of the literature will focus on the micro-vascular complication of DM, mainly diabetic neuropathies.

The goals of this chapter are to:

- Present the prevalence and incidence of DM and DPN in the Arab world through reviewing studies that discussed epidemiology;
- Outline the different classifications of diabetic neuropathies in order to gain a better understanding of the pathogenesis of DPN;
- Build up a comprehensive understanding of the different theories of the pathogenesis of diabetic peripheral neuropathies in type I and type II DM;
- Discuss systematically the relationship between DM and inflammation, and its possible contribution to the pathophysiological mechanism of DPN;
- Review articles investigating the relationship between DPN and inflammation;
- Describe the autoimmune factor in DM and its complications;
- Review articles discussing the possible relationship between autoimmunity and DPN;
2.1. Literature search and search engines

The generation of literature search and citation of references was conducted in accordance with the PICO principles (Population-Intervention-Comparison-Outcome) as described by Sackett et al. (2000). The clinical scenarios was analysed in accordance with Evidence Based Practice (EBP) in order to develop a focused search strategy.

Generation of the search strategy was based on the concepts of the PICO principals:

• **Population group**: Patients with diabetes mellitus, patients with diabetic peripheral neuropathy, DM patients in the Arab world and DPN patients in the Arab world.

• **Intervention/prognostic factor/exposure**: Pathogenesis and pharmacological management of diabetic peripheral neuropathy/Inflammation and autoimmunity and their association with DPN/Prevalence of DM complications in the Arab world.

• **Comparison**: Theories relating to DPN pathogenesis and management/theories describing relationship between inflammation and autoimmunity and DM or DPN.

• **Outcomes**: Relationship between pro-inflammatory cytokines and pathogenesis of DPN/relationship between autoimmune antibodies and pathogenesis of DPN/The burden of DM complications in the Arab world.

The online databases; Cochrane Library, Medline, CINAHL, Up To Date and Clinical Evidence were selected to enable comprehensive review of the medical literature. The search strategy described below specifically relates to the Ovid
Medline Database. Medline was chosen as it covers a wide range of medical and nursing literature and is one of the most comprehensive databases for biomedical literature (Sackett et al. 2000). This topic has relevance to medical, nursing and allied health practitioners.

The structure of the literature search was split into four stages. The first stage of the process was to identify the prevalence and incidence of DM and DPN in the Arab world. The second stage was to present the different classification systems used to categorise the different types of diabetic neuropathies. The third stage concentrated on identifying the theories related to the pathogenesis of DPN. The fourth stage was designed to review the available studies investigating the relationship between inflammation and/or autoimmunity and DM and the relationship between inflammation and autoimmunity and DM complications, specifically diabetic peripheral neuropathy.

A list of key search terms was generated based on the breakdown of the PICO principals. Consideration at this point was given to synonyms, abbreviations and spelling variants. The key terms used were all in English language and included: diabetes; diabetic complications; diabetes mellitus; diabetes mellitus in the Arab world; diabetic peripheral neuropathy in the Middle East; neuropathy; neuropathies; peripheral neuropathy; painful peripheral neuropathy; sensory peripheral neuropathy; sensory neuropathy; diabetic neuropathy; neuropathy pathogenesis; inflammation and neuropathy, inflammation and diabetes mellitus, autoimmunity and peripheral neuropathy; autoimmunity and diabetes mellitus; pharmacological management of diabetic peripheral neuropathy; pharmacological agents; and drug therapy.

The study designs applicable to the research question were considered for reviewing. The study designs of the selected papers were ranked according to an evidence hierarchy to include randomised controlled trials (RCTs), meta-analysis, systematic reviews, cohorts and case control studies respectively.
2.2. Part one: Epidemiology of diabetes mellitus and its complications in the Arab world

A remarkable number of studies exist that describe the epidemiology of DM over the last 20 years. It has now been recognized that it is the developing countries that presently face the greatest burden of DM (WHO 2011). Diabetes mellitus has reached pandemic proportions within the Middle East and North Africa (MENA) and Gulf Cooperation Council (GCC) countries specifically. Figure 2.2.1 demonstrates the increased prevalence of DM (presented in dark red colour) in the MENA region in 2013 (IDF 2013).

Figure 2.2.1: Prevalence (%) of diabetes mellitus in adults (20-79 years) for the year 2013. Prevalence of DM in the MENA region is estimated to be > 12%. This figure is sourced from IDF (2013)
In the MENA region, 32.8 million people have been diagnosed with the disease and it has been estimated that the number of DM will increase by 94 percent between the years 2010 and 2030 (Figure 2.2.2) (WHO 2011). By the year 2030, it is estimated that nearly 4 percent of all deaths in the MENA region will be caused by DM and its complications (IDF 2011; WHO 2011).

![Figure 2.2.2: Number of patients with DM in the MENA region is estimated to increase by 94% between 2010 and 2030. This figure is sourced from WHO (2011).](image)

The incidence of DM was found to have increased over the previous decade and has been correlated with the presence of obesity (Motlagh et al. 2009). In December 2011, another alarm awakened the Arab governments when The International Diabetes Federation announced that six of the top ten countries with the highest prevalence of diabetes mellitus (in adults aged 20 to 79 years) are in the Middle East: Kuwait (21.1%), Lebanon (20.2%), Qatar (20.2%), Saudi Arabia (20.0%), Bahrain (19.8%) and UAE (19.2%) (IDF 2013).
As mentioned previously, in Chapter one, profound changes of those living in the Arabian Peninsula during the last 30 years have been associated with the distribution of DM. The increase in the incidence of type II DM, especially in developing countries, is suggested to follow the trend of urbanization and lifestyle changes, perhaps most importantly a “Western-style” diet with associated obesity (Wild et al. 2004).

In the Arab region, diabetes mellitus prevalence appears to increase with human development (Al-Mahroos and Al-Roomi 2007). To test this hypothesis, Boutayeb et al. (2012) carried out a linear regression to investigate the relationship between DM prevalence in the MER and human development index (HDI). The HDI values used were those published by the United Nations Development Program (UNDP) for the year 2011. It measures average achievements in three basic dimensions of human development; a long and healthy life, knowledge and a decent standard of living (UNDO 2011). The results of their study demonstrated a relatively high correlation (R = 0.81) between the two variables. This suggests that environmental influences are important contributors to this complication, which has a strong genetic component. It remains unlikely that genetic factors or ageing alone can explain this dramatic increase in the prevalence of type II DM. It remains to be fully determined as to how increased caloric and dietary fat intake in the context of reduced exercise with an associated increase in body weight could ultimately lead to this disease.

Al-Mahroos and McKeigue (1998) performed the first cross sectional survey in Bahrain to investigate the prevalence of DM among Bahraini natives. A total of 2128 Bahrainis, aged between 40 and 69 years were enrolled in their study. Diabetes mellitus and impaired glucose intolerance (IGT) were estimated to be prevalent in 18% and 30% of the population, respectively. The highest numbers of DM were seen in the 55-59 years age group (32% males, 36% females). The results further demonstrated that about 35% of Bahraini patients with DM between the ages of 40 and 69 years were previously undiagnosed with DM.
A cross-sectional study was further carried out by Al-Mahroos and Al-Roomi (2007) to investigate the prevalence of diabetic peripheral neuropathy, foot ulceration (FU) and peripheral vascular disease (PVD), and potential risk factors for these complications among patients attending primary care DM clinics in Bahrain. They included 1477 DM patients (of which 93% was type II DM) on routine visits to six specialized DM clinics in the country. Patients aged 18 to 75 years, who had already been diagnosed with DM, were enrolled in the study. Exclusion criteria included patients with other diseases known to cause neuropathy, such as pernicious anaemia and alcoholism.

Diabetic peripheral neuropathy was present in 36.6% of the population, foot ulceration in 5.9%, and PVD in 11.8%. Patients with DPN were older than patients without neuropathy, and had been diagnosed with DM for a longer period. Patients with DM who presented with foot ulcers had more severe neuropathy and higher vibration perception thresholds values than patients without foot ulcers (Al-Mahroos and Al-Roomi 2007).

Older age, poor glycaemic control, longer duration of DM, elevated cholesterol levels, current smoking, obesity defined by body mass index, large waist circumference, elevated triglycerides levels and hypertension - but not gender, were all considered significant risk factors for DPN in both the univariate and the multivariate analyses conducted by Al-Mahroos and Al-Roomi (2007). Diabetic peripheral neuropathy and PVD also remained significant risk factors for foot ulceration in the multiple logistic regression analysis (Al-Mahroos and Al-Roomi 2007).

As one would imagine considering the increase prevalence of DM, studies conducted in the MER report high rates of diabetic peripheral neuropathy (DPN), ranging from 35% to 65% (Sulaimani et al. 1991; Halawa et al. 2010). The prevalence of painful DPN was evaluated in type I and type II DM patients attending outpatient clinics across the Middle East (Jambart et al. 2013). Overall, 53.7% of 3989 patients with DM met the criteria for painful DPN. Significant predictors of painful DPN included
long history of DM (≥ 10 years), age ≥ 65 years, body mass index ≥ 30 kg/m² and
gender (Jambart et al. 2013).

In summary, DM and its complications within the MER region are rising. Previous
clinic-based studies on Bahraini patients with DM demonstrated that a large
proportion of the DM patients have neuropathic complications and were therefore, at
substantial risk of developing foot complications. Poor glycaemic control, older age,
long duration of DM, tall stature, smoking and high cholesterol levels are considered
important risk factors in the development of diabetic peripheral neuropathy in the
Arab population (Al-Mahroos and Al-Roomi 2007). Strategies to reduce the risk of
such complications among DM patients must therefore be studied and implemented.

2.3. Part two: Diabetic Neuropathies; classification and clinical
characteristics

Diabetic neuropathies (DN) encompass a wide range of nerve abnormalities and are
common, with prevalence rates reported between 5–100% depending on the
diagnostic criteria (Zochodne 2007). The neuropathies developing in patients with
DM are known to be heterogeneous by their symptoms, pattern of neurological
involvement, risk covariates, course of disease, pathological alterations, and
underlying mechanisms (Llewelyn et al. 2005).

Numerous classifications of the variety of syndromes affecting the peripheral
nervous system in DM have been identified in recent years (Boulton et al. 2005).
Classifying the types of diabetic neuropathy has been hindered by an incomplete
knowledge of the pathogenic mechanisms involved in this disease, the diverse
clinical neuropathic manifestations, and the fact that mixed forms of neuropathy are
often encountered. Nevertheless, classifications are clinically useful because
syndromes occur with sufficient frequency, and because classification may help to
better predict the natural history and outcome of diabetic neuropathy and, therefore, guide treatment (ADA 2010).

Although various classifications have been proposed, a simple system based on the symmetry of the neuropathy and the type of nerves involved is shown in Table 2.3.1. The classification shown in this table is based on that originally proposed by Thomas (1997). Thomas initially separated diabetic neuropathies into generalized polyneuropathies, focal neuropathies (e.g. median neuropathy at the wrist from carpal tunnel syndrome) and multifocal varieties (e.g. multiple mononeuropathy, lumbosacral, thoracic and cervical radiculoplexus neuropathies) (Dyck et al. 2001; Thomas 2003; Boulton et al. 2005).

Table 2.3.1: Classification of diabetic neuropathy based on the symmetry of the neuropathy and the type of nerves involved (Dyck et al. 2001; Thomas 2003; Boulton et al. 2005)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Clinical manifestation</th>
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<tbody>
<tr>
<td>Symmetric</td>
<td>Sensory or sensorimotor polyneuropathy</td>
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<tr>
<td></td>
<td>Autonomic neuropathy</td>
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<tr>
<td></td>
<td>Proximal lower limb motor neuropathy</td>
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<tr>
<td>Focal or multifocal</td>
<td>Cranial neuropathy</td>
</tr>
<tr>
<td></td>
<td>Trunk and limb mononeuropathy</td>
</tr>
<tr>
<td></td>
<td>Asymmetric lower limb neuropathy</td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
</tr>
</tbody>
</table>

Although the classification proposed by Thomas (1997) has been accepted by many authors, later evidence further classified the generalized varieties of diabetic neuropathy into at least two major subgroups; typical and atypical DN (Thomas
The definition of typical DN, distal symmetric polyneuropathy (of which DPN is a member), from the American Academy of Neurology (AAN), American Association of Electrodiagnostic Medicine (AAEM), and American Academy of Physical Medicine and Rehabilitation (AAPMR) report, and based on a formal review of the medical literature, suggests that the highest likelihood of neuropathy occurs with a combination of neuropathic symptoms, multiple signs, and abnormal electrodiagnostic studies (England et al. 2005). Whereas this definition seems acceptable and intuitively correct, it does not separate typical DN from atypical DN.

According to that, Dyck et al. (2011) proposed separate definitions for typical DN and atypical DN. They defined typical DN as a symmetrical length dependent sensorimotor polyneuropathy resulting from chronic hyperglycaemia, associated metabolic imbalance, cardiovascular risk covariates, and micro-vessel alterations (Tesfaye 2005; Dyck et al. 2006; Zochodne 2008). An abnormality of nerve conduction studies which may be subclinical (asymptomatic and without signs or symptoms of neuropathy) appears to be the first objective and quantitative indication of DPN and is a necessary symptom for the confirmed diagnosis of DPN according to Dyck et al. (2011). The occurrence of other DM complications, such as, diabetic retinopathy and nephropathy in a given patient strengthens the case that a patient’s peripheral neuropathy is associated to diabetes mellitus (Tesfaye et al. 2010). However, the association among these complications is not strong enough to allow diagnosis of DPN from knowing that diabetic retinopathy or nephropathy occurs in the same patient (Dyck et al. 2011).

Atypical DN has not been as well characterized and studied as have typical DNs, and it is possible that atypical DN is actually not a single entity but several varieties (Dyck et al. 2011). This condition appears to be an inter-current and monophasic or fluctuating disorder, tending to favourably involve small sensory and autonomic nerve fibres, by not being closely associated with chronic hyperglycaemia or associated with the micro-vessel abnormalities present in DPN (Dyck et al. 2011).
The atypical DNs are different from typical DNs in several important features, for example, onset, course, manifestations, associations, and perhaps putative mechanisms (Archer et al. 1983; Thomas 1997; Boulton et al. 2005). They appear to be developing at any time during the course of a patient’s DM (Boulton et al. 2005). Onset of symptoms may be acute, subacute, or chronic, but the course is usually monophasic or fluctuating over time. Pain and autonomic symptoms are typical features, and altered immunity has been suggested (Thomas 1997). Studies have suggested that impaired fasting glucose or impaired glucose tolerance (IGT) is more common in chronic idiopathic axonal polyneuropathy (Dyck et al. 2012), but other studies do not support this hypothesis (Singleton et al. 2001).

The atypical focal and multifocal neuropathies associated with DM can be broadly subdivided into those in which repeated, mild, mechanical trauma, compression, or entrapment is causative and others possibly related to inflammation with or without associated vascular abnormalities (Dyck et al. 2011). The first group may include median neuropathy at the wrist, ulnar neuropathy at the elbow, and peroneal neuropathy at the knee. The second group may include mononeuropathy, e.g. cranial nerve III and multiple mononeuropathies, and radiculoplexus neuropathies of the lumbosacral (also called diabetic amyotrophy), thoracic, and cervical segments (Dyck et al. 2011). There is increasing evidence that inflammation, micro-vascular disease, and ischaemia are involved in this type of radiculoplexus neuropathies (Dyck et al. 1999b; Kelkar et al. 2000).

Neuropathies may also be classified according to the size of the nerve fibres involved (Kiernan and Rajakumar 2014). This classification system can be divided into small fibre neuropathy, large fibre neuropathy, or mixed (small and large) fibre neuropathy (Kiernan and Rajakumar 2014). Each fibre type is responsible for different functions and when injured, causes different symptoms (Table 2.3.2). Large fibre neuropathies, for example, are associated with abnormalities in vibration and touch from a sensory perspective, and weakness and atrophy from a motor perspective. Small fibre neuropathies however mostly affect sensory and autonomic nerves as detailed in Table 2.3.2.
Table 2.3.2: Classification of nerve fibres according to type and size in peripheral neuropathy (Sourced from Kiernan and Rajakumar 2014)

<table>
<thead>
<tr>
<th>Fibre class</th>
<th>Fibre type</th>
<th>Size</th>
<th>Functional class</th>
<th>Fibre dysfunction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myelinated nerve fibres</strong></td>
<td>Large fibres</td>
<td>Aα motor neurons 12-20 μm</td>
<td>Motor</td>
<td>Weakness, atrophy, cramps, fasciculations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aβ fibres 5-15 μm</td>
<td>Sensory</td>
<td>Abnormal proprioception, vibration and touch sensation</td>
</tr>
<tr>
<td>Small fibres</td>
<td>Aγ fibres 3-8 μm</td>
<td>Sensory</td>
<td>Deep and lancinating pain, abnormal cold and pressure sensation</td>
<td></td>
</tr>
<tr>
<td><strong>Unmyelinated nerve fibres</strong></td>
<td>Small fibres</td>
<td>C fibres 0.2-1.5 μm</td>
<td>Sensory</td>
<td>Burning pain and abnormal heat sensation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C fibres 0.2-1.5 μm</td>
<td>Autonomic</td>
<td>Abnormal sweating, bowel, bladder, and sexual function, abnormal blood pressure control</td>
</tr>
</tbody>
</table>

Autonomic neuropathy is another type of diabetic neuropathy that could be associated with DM (Tesfaye et al. 2010). Diabetic autonomic neuropathy (DAN) is a disorder of the autonomic nervous system in the setting of diabetes mellitus or metabolic imbalance of pre-diabetes mellitus after the exclusion of other causes (Tesfaye et al. 2010). Diabetic autonomic neuropathy may affect cardiovascular, gastrointestinal (GI), and urogenital systems, and sudomotor function. It may result in a variety of signs and symptoms or may be subclinically detectable by specific tests (Tesfaye et al. 2010)

Diabetic autonomic neuropathy results in significant morbidity, and may lead to mortality in some patients with DM (Tesfaye et al. 2010). The symptoms of autonomic dysfunction should be elicited carefully during the history, particularly since many of these symptoms are potentially treatable. Major clinical manifestations of autonomic neuropathy include resting tachycardia, exercise intolerance, orthostatic hypotension, constipation, gastroparesis, erectile dysfunction, sudomotor
dysfunction, impaired neurovascular function, and hypoglycaemic autonomic failure (Tesfaye et al. 2010). Those types of diabetic autonomic neuropathies are beyond the scope of this research.

2.4. Part three: Diabetic Peripheral Neuropathy

Diabetic peripheral neuropathy is a chronic distal symmetric peripheral neuropathy with predominantly sensory deficits and a variable degree of autonomic involvement (Tesfaye et al. 2010). It is mainly axonal with a mixed large and small fibre involvement. The presentation is frequently insidious with burning or lancinating pain, numbness, paresthesia, and hyperalgesia in both feet and lower limbs (symmetrical) (Tesfaye et al. 2010). Typically, at a more severe stage, the hands become affected (Treede et al. 2008).

The prevalence of painful neuropathy may be difficult to estimate as definitions have varied enormously among studies (Tesfaye et al. 2010), however, it is estimated that between 3 and 25% of patients with DPN might experience neuropathic pain (Boulton et al. 2004). Similarly, Boulton et al. (2004) in his review stated that there are limited data on the natural history of painful DPN with some studies suggesting that painful symptoms improve with the worsening of the sensory loss and others reporting no appreciable occurrence of remissions.

Approximately 10–20% may experience severe sensory symptoms that require treatment (Boulton et al. 2004). Up to 50 percent of patients with DPN, however, may be asymptomatic and the DPN is only detected by neurological examination (Thomas 1997) or by a nerve conduction study (Fedele et al. 1997). In some clinical practices DPN is diagnosed when patients present with a painless, often infected, foot ulcer (Boulton et al. 2004), which is often the case in podiatry practices.
2.4.1. Risk factors for DPN

Diabetic peripheral neuropathy is related to the duration of DM and the quality of glycaemic control (Boulton et al. 2005). Independent of age or duration of DM, studies suggested no major differences in the prevalence of DPN between patients with type I or type II DM (Zochodne 2007). Increasing age, longer duration of DM and poor glycaemic control are well recognized risk factors for DPN, while cigarette smoking, retinopathy, hypertension, obesity, hyperlipidaemia and microalbuminuria have also been implicated as potential risk markers (Boulton et al. 2005). The Kumamoto study showed that intensive insulin treatment for 7 years in type II DM patients improved nerve conduction velocity (NCV) compared with those conventionally treated (Ohkubo et al. 1995). In contrast, the UK prospective diabetes study (UKPDS) on 3876 type II DM patients did not find the effects of improved glycaemic control (to the extent of a 0.09% decrease in HbA1c) on the prevalence of neuropathy, whereas there was significant reduction in the risk for retinopathy and nephropathy.

The EURODIAB IDDM Complications Study, which involved 3250 patients with type I DM from 31 centres in 16 European countries, found that DPN was related to both glycaemic control and duration of disease (Tesfaye et al. 1996). Although the 28% baseline prevalence of DPN was significantly related to glycosylated haemoglobin (HbA1c), the prevalence varied from 17 to 41% after data were adjusted for duration of DM, with lower HbA1c levels associated with lower prevalence rates and higher levels associated with higher prevalence rates (Tesfaye et al. 1996). However, even those with good glycaemic control (HbA1c<5.4%, equivalent to Diabetes Control and Complications Trial HbA1c of 7%) still developed micro-vascular disease, suggesting that factors other than glycaemic control and disease duration are involved (Tesfaye et al. 2005).

Follow-up data from the EURODIAB cohort of patients with type I DM revealed that over a 7-year period, approximately one-quarter of type I DM patients developed DPN; with age, duration of DM and poor glycaemic control being major factors
The development of DPN was also associated with potentially modifiable cardiovascular risk factors, such as hypertension, hyperlipidaemia, obesity and cigarette smoking, as illustrated in Figure 2.4.1 (Tesfaye et al. 2005).

![Figure 2.4.1: Risk factors for incident neuropathy (The EURODIAB Prospective Complications Study) sourced from Tesfaye et al. 2005.](image)

The figure illustrates odds ratios for the various risk factors for diabetic peripheral neuropathy in a cohort of 1101 type 1 diabetes mellitus patients followed for 7.3±0.6 years (Tesfaye et al. 2005). BMI: body mass index; CVD: cardiovascular disease

Recently, other studies have also implicated cardiovascular risk factors, such as obesity (Ziegler et al. 2008) and triglyceride levels (Vincent et al. 2009) in the pathogenesis of DPN. Moreover, Wiggin et al. (2009) found that elevated triglycerides correlated with myelinated fibre loss independent of disease duration, age and DM control (Wiggin et al. 2009). These data support the evolving concept that hyperlipidaemia might be instrumental in the progression of DPN.

Current smoking was also positively related to neuropathic rates in the population investigated by Tesfaye et al. (2005), which provides evidence to existing literature
that current smoking is a risk factor for diabetic neuropathy in DM patients (Barbosa et al. 2001) and emphasizes the need for patients to quit smoking. The presence of neuropathy was also related to dyslipidaemia, hypertension and obesity (Tefsaye et al. 2005). Earlier studies have reported that high blood pressure is a significant risk factor for DPN, both in type 1 and type 2 DM patients (Boressen et al. 1990). However, the role of hypertension in the pathogenesis of neuropathy remains controversial, and most experts consider high blood pressure as a risk marker for neuropathy rather than a risk factor (Tefsaye et al. 2005).

Within the Arabic population, Al-Mahroos and Al-Roomi (2007) suggested that the development of neuropathy was related to the level of glycaemic control. This confirms the need for optimal care of DM patients in preventing complications. The risk of neuropathy and peripheral vascular disease increased steadily with elevated glycated haemoglobin (AL-Mahroos and Al-Roomi 2007). They proposed that the increased risk of neuropathy associated with hyperglycaemia is likely to account for the high proportion of cases of foot ulcerations in DM population in Bahrain. The strong association of the presence of DPN with high levels of glycated haemoglobin suggests that poor glycaemic control is an important risk factor for DPN and foot ulcerations among Bahrainis (and other Arabian Gulf countries) (Al-Mahroos and Al-Roomi 2007). However, this was not the case in a Japanese cohort where no significant influence of the blood concentrations of glycated haemoglobin on the prevalence of neuropathy was detected (Yagihashi et al. 2010). It is clear after all that high blood glucose leads to peripheral nerve injury through a downstream metabolic cascade (Yagihashi et al. 2010).

2.4.2. Anatomy and vascular supply of the peripheral nervous system

Understanding the anatomy of the peripheral nervous system might explain why the pathogenesis of DPN is distinct from other micro-vascular complications in DM (Kihara et al. 1991). Figure 2.4.2 illustrates the anatomical structure of the peripheral
nerve covered by perineurium, where only a few transperineurial arterioles penetrate into the endoneurium (Yagihashi et al. 2010).

Figure 2.4.2: Vascular supply of the peripheral nervous system showing transperineurial arteriole penetrating into endoneurium. Autonomic nerve endings contact with the wall of arterioles, but vascular autoregulation is lacking in peripheral nerves as a result of inadequate innervations. This figure is sourced from Yagihashi et al. (2010).

Peripheral nerves are vulnerable to ischaemia (Yagihashi et al. 2010) due to the fact that the vascular supply in peripheral nerves is inadequate and blood flow is likely to be compromised due to the lack of autoregulation (Smith et al. 1977). Alterations of the blood-nerve barrier may affect the microenvironment of the peripheral nerve. Endoneurial microvessels are tightly connected with endothelial cells on their inner surface; however, when the vessels are destroyed they become leaky and may affect the endoneurial tissue components (Sima et al. 1991). In experimental animal models of DM, perturbation of the blood-nerve barrier was demonstrated even in
early stages of the disease (Rechtland et al. 1987). Similarly, disruption of the blood-nerve barrier with disintegration of the endothelial cell junction was detected ultrastructurally in the endoneurial microvessels in human DM patients (Sima et al. 1991). These endoneurial vascular abnormalities are associated with increased permeation of plasma albumin or immunoglobulins in the peripheral nerves of human DM patients. Such an enhanced vascular permeability is relevant to increased nerve water content or interstitial oedema (Sima et al. 1991). These changes further elicit microcirculatory disturbances by physical compression of the perineurial and transperineurial vessels and widening of intercapillary distances, resulting in reduced tissue oxygen tension (Wadhwani et al. 1989). Permeable vessels are mainly located in the ganglion with fenestrated vessels, and nerve terminals on the distal side are directly exposed to environments not covered by perineurium and are thus susceptible to traumatic injury (Yagihashi et al. 2010).

Innervation of epineurial microvessels is involved in DM, resulting in impaired blood supply in DM nerves (Beggs et al. 1992). Endoneurial microvessels show thickened and multilayered basement membranes, cell debris or pericytes, as well as disrupted endothelial cells, and thus constitute prominent structural changes in DM nerves (Yagihashi et al. 2010).

Independent of vascular supply, the dimensions of neuronal architecture specific to the peripheral nervous system might account for the reason why the most distal side is susceptible to nerve damage (Yagihashi et al. 2010). Ganglion cells have extensively long axons covered by Schwann cells. The neuronal cell body is relatively small compared with the extremely long distance of axonal neuritis, and thereby distal axons are innately too weak to support themselves for the long transport of nutrients, nerve trophic factors, as well as other signals (Yagihashi et al. 2010).
2.4.3. Theories in the pathophysiological mechanism of DPN

Numerous hypotheses relating to the pathogenesis of DPN have been proposed individually, but it is likely that the overall mechanism involved may be multifactorial. In order to understand the overall mechanism of this complex disease, a review of the previously proposed theories is valuable in understanding the suggested pathophysiology of DPN, and to evaluate the different treatment approaches available in managing and preventing this complication.

Until recently, there were two directions of thoughts regarding the aetiology and pathogenesis of DPN: metabolic versus vascular. A study by Cameron et al. (2001), however, demonstrated that both vascular factors and metabolic interactions are involved at all stages of diabetic peripheral neuropathies. Van Dam et al. (2013) stated that while the metabolic insult of DM may directly affect neural tissue, it is likely that neurodegenerative changes are precipitated by compromised nerve vascular supply.

This was in line with earlier work that showed pathological changes in vasa nervorum epi/perineural and dendoneural vessels (Fagerberg 1957) and established good correlations between vascular parameters, nerve structural damage and measures of function such as nerve conduction velocity, vibration perception threshold, and thermal discrimination (Giannini and Dyck 1995).

An important contributory factor, revealed by photography of epi/perineural vessels, was an increase of arterio-venous shunting in patients with neuropathy, bypassing the nutritive endoneural circulation (Tesfaye et al. 1993). Other morphological studies showed denervation of perineural vessels, suggesting that diabetic autonomic neuropathy could potentially contribute to the impaired control of nerve perfusion in DM (Beggs et al. 1992). Whether the resultant hypoxic stress on nerve fibres is sufficient to account for diabetic neuropathy or whether it acts in conjunction with direct metabolic stress on nerve fibres resulting from the diabetic milieu cannot be answered by the limited number of investigations carried out to date in patients with
DPN. Nevertheless, data from animal models provides insight into the pathogenetic mechanism underlying DPN, and pharmacological intervention studies have revealed numerous potential therapeutic targets, a small number of which have advanced clinical trials (Forbes and Cooper 2013).

The pathophysiology of DPN (summarized in Figure 2.4.3) has been associated with increased oxidative stress producing advanced glycosylated end products (AGEs), polyol accumulation, decreased nitric oxide/impaired endothelial function (Cameron et al. 1997), impaired sodium/potassium (Na⁺/K⁺) ATPase activity (Kathleen and Head 2006), and homocysteinaemia (high levels of naturally occurring amino acid homocysteine) (Ambrosch et al. 2001). Not only are nerve cells more likely to be destroyed in a hyperglycaemic environment, but repair mechanisms are also defective (Kathleen and Head 2006). Reduced levels of neurotrophic agents, including nerve growth factor and insulin like growth factor, have been noted in experimental DM (Pittenger et al. 2003)

![Figure 2.4.3: Pathophysiological Factors in Diabetic Peripheral Neuropathy. This figure is sourced from Pittenger et al. 2003](image)
There is an emerging concept that insulin resistance or deficiency is a more critical mediator of the disorder before hyperglycaemia develops (Betul and Gundogdu 2006). This concept is supported by animal studies demonstrating that peripheral nerve abnormalities increase in parallel with the decrease in insulin (Betul and Gundogdu 2006). In a cross-sectional study using a combination of physical examination and focused history, neuropathy was reported in 26% of DM patients, 11% of patients with impaired glucose tolerance, and 4% of age matched healthy control (Singleton et al. 2005).

Hyperglycaemia is thought to induce neurotoxicity through different pathways: 1) metabolic, 2) vascular, 3) neurotrophic growth factor–deficiency, and recently 4) an immunologic–autoimmune pathway have been suggested. The different theories associated with the pathogenesis of diabetic peripheral neuropathy are reviewed in separate sections below.

2.4.3.1. Metabolic Hypothesis

Hyperglycaemia is evidently linked to the development of neuropathy (DCCT 1993). Intravenous infusion of glucose has been shown to increase neuropathic pain in patients with DPN (Morely et al. 1984). The Glucose Control and Diabetes Complications Trial demonstrated that intensive control of blood glucose concentration decreases the incidence of neuropathy by 60% in those without neuropathy, and slows its development and progression in those with neuropathy (DCCT 1993). Follow-up data from the EURODIAB cohort of patients with type I DM revealed that over a 7-year period, approximately one-quarter of type I DM patients developed DPN; with age, duration of DM and poor glycaemic control being major factors (Tesfaye et al. 2005). Hyperglycaemia is therefore strongly linked to DPN. The exact mechanism of this action is however not fully understood.

In hyperglycaemia excess of glucose is believed to convert into sorbitol via the enzyme aldose reductase (through the polyol pathway) leading to accumulation of
sorbitol and depletion of myo-inositol in nerve tissue (Pittenger et al. 2003). Hyperglycaemic activation of the polyol pathway may lead to changes in the NAD/NADH (Nicotinamide adenine dinucleotide ratio), causing direct neuronal damage and/or decreased nerve blood flow (Boulton et al. 1990). In experimental studies, these metabolic changes induce osmotic swelling and alteration in sodium-potassium ATPase activity in diabetic nerves (Raccah et al. 1998). A fall in sodium-potassium ATPase activity has been linked to slowing of nerve conduction velocity in diabetic rats (Pittenger et al. 2003).

Based on this hypothesis, aldose reductase inhibitors have been used to prevent DPN in patients with DM. However, their success has been mediocre at most (Yamagishi et al. 2003). Sorbinil (one type of aldose reductase inhibitors) resulted in only a small increase in nerve conduction velocities in a study by Judzewitsch (1983), while tolrestat (another type of aldose reductase inhibitor) showed some clinical benefit (Boulton et al. 1990). Both studies however included only patients with mild DPN.

Histological studies of sural nerve biopsy specimens on the other hand noted no significant correlations between sorbitol content and neuropathy (Theriault et al. 1997). Moreover, dietary myoinositol replacement demonstrated no improvement in neuropathy symptoms (Dyck et al. 1980; Yamagishi et al. 2003). In fact, protein kinase C subunits in peripheral nerve are diffused and behave in such a manner as to make it uncertain whether their inhibition is to be counteracted or encouraged (Yamagishi et al. 2003).

In addition, hyperglycaemia has been linked to inducing the production of advanced glycation end products and free radicals, which are neuro-toxic (Pittenger et al. 2003). Concurrent with the generation of free radicals during the glycolytic process, mitochondria have a crucial role in cellular death by activation of specific signals and endonuclease system (Edwards et al. 2010). It has also been suggested that oxidative stress may target mitochondria and their injury may release cytochrome-c, initiating apoptosis (Pittenger et al. 2003). Accumulation of reactive oxygen species (ROS)
through each of these mechanisms causes apoptosis of neurons and Schwann cells (Russell et al. 2002).

The activation of polyol pathway also results in taurine depletion through sorbitol accumulation (Betul and Gandogdu 2006). Taurine is a nonessential amino acid important in cellular osmo-regulation. Its deficiency results in problems with cellular volume regulation resulting in increased glycation, AGE, and reactive oxygen species (ROS) (Betul and Gandogdu 2006). An elevated tension of ROS and subsequently decreased endothelial nitric oxide has been shown to increase matrix metalloproteinase activity (Uemura et al. 2001; Tyagi et al. 2003). This is thought to increase extracellular matrix remodelling and contribute to arterial vessel wall thickening. Giannini and Dyck (1995) reported that endoneurial capillary basement membrane thickening is a histological hallmark of diabetes mellitus.

2.4.3.2. **Vascular Hypothesis**

Vascular factors have also been implicated in the pathogenesis of DPN (Betul and Gandogdu 2006). Nerve ischaemia caused by small vessel disease is another hypothetical cause for diabetic peripheral neuropathy proposed by many authors (Pavy-Le Traon et al. 2010). Pathological studies of proximal and distal segments of nerve in diabetic neuropathy have demonstrated multifocal fibres loss along the length of the nerves, suggesting ischaemia as a pathogenetic contributor (Dyck et al. 1980). It has been demonstrated that blood flow is reduced and endoneurium lack oxygen in diabetic neuropathy (Tuck et al. 1984; Nerwick et al. 1986). Low et al. (1988) stated that oxygen supplementation and hyperbaric oxygenation were found to be able to prevent and reverse electrophysiological and biochemical abnormalities in experimental DPN. This was further supported by Kihara et al. (1995) who suggested that hyperbaric oxygen therapy was effective in reducing neuropathic pain.
Microangiopathy with endothelial dysfunction of the vasa nervorum is considered the vascular factor causing ischaemia and hypoxia in nerves of DM patients (Zochodne 2007). Pathological studies of sural nerves of patients with DM have illustrated a number of alterations, including thickening of the endoneurial capillary basement membrane, capillary closure, endothelial cell hypertrophy and hyperplasia, and pericyte degeneration (Dyck and Giannini 1996). Zochodne (2007) suggested that impaired vasodilatation of the vasa nervorum may develop early and signify microangiopathic changes, subsequently leading to narrowing of the capillary lumen, resulting in reduced capillary blood flow, hypoxia, and progression of neuropathy. Rheological alterations of the red blood cells may additionally contribute to microcirculatory disturbances (Koltai et al. 2006). Thrainsdottir et al. (2003) noted that degeneration of myelinated and unmyelinated nerve fibres may develop at a later stage and accompany the peripheral nerve. The relationship between the severity of hyperglycaemia and microangiopathy is not yet established.

Ischaemia may thus contribute to the development of neuropathy, and the benefit of vasodilators in improving nerve conduction velocities seems to be explainable (Feldam et al. 2003). Moreover, in support of this hypothesis the EURODIAB Prospective Complications study (Tesfaye et al. 2005) indicated that, apart from hyperglycaemia, the incidence of neuropathy is associated with modifiable cardiovascular risk factors such as raised triglyceride level, body mass index, smoking and hypertension.

Additional studies stated that metabolic and vascular mechanisms are likely to interact. Hyperglycaemia has been shown to have deleterious effects on blood vessels (Feldam et al. 2003). For example, the polyol pathway has been shown to consume nicotinamide adenine dinucleotide phosphate, reducing its availability for nitric oxide (NO) synthase; reduced bioavailability of NO can cause nerve ischaemia in animal models of DM (Feldam et al. 2003). Hyperglycaemia can increase oxidative stress with production of super oxides and NO which can act interactively leading to lipid peroxidation, protein nitration or nitrosylation, DNA damage, and endothelial and neuronal cell death. Super-oxides also may lead to activation of
protein kinase C (PKC), which induces vasoconstriction and reduces neuronal blood flow (Feldam et al. 2003).

One such study examined nerve blood flow and nitric oxide syntheses (NOS) activity in the microvasculature serving peripheral nerves in DM rats (Kihara and Low 1995). Hyperglycaemia resulted in a significant diminution of nerve blood flow compared to controls. An animal study also found disruptions in neuronal nitric oxide synthase (nNOS) in experimental DM. Decreased nNOS expression was shown to be associated with increased neuropathic pain (Sasaki et al. 1998).

Nitric oxide plays an important role in controlling (Na⁺/K⁺)-ATPase activity (Gupta et al., 2002), which has been implicated in the pathogenesis of DPN (Stevens et al. 1994). Experimental analysis revealed hyperglycaemia results in an excess of endothelial superoxide radicals that result in reduced stimulation of NO on (Na⁺/K⁺)-ATPase activity (Gupta et al. 2002). Another animal study, however, did not find a relationship between altered NO activity and the development of sensory neuropathy (Thomsen et al. 2002).

Conflicting findings gathered from different studies investigating the pathophysiology of DPN results in an incomplete picture. Blood flow to sural nerve in one study, for example, was found to be normal with the progression of DPN, with a slight increase in more severely affected patients (Theriault et al. 1997). Sympathectomy and vasodilation were found to be associated with progression rather than improvement of neuropathy (Zochodne and Ho 1992). Moreover, nerve blood flow was noted not to show any decrease in a number of diabetic neuropathies, using various blinded examiners and measurement techniques (Zochodne 2007). Although diabetic neuropathy is believed to be strongly associated with micro-vascular abnormalities, the evidence for its particular role is still questionable (Cade 2008).
2.4.3.3. **Neurotrophic and growth factor-deficiency pathway**

Betul and Gandogdu (2008) suggested that the repair and regeneration of the damaged neuronal unit is activated by a number of neurotrophic factors such as nerve growth factor (NGF), neurotrophins such as neurotrophin-3, vascular endothelial growth factor, insulin, and insulin-like growth factors. In DPN, as the Schwann cell becomes dysfunctional or undergoes apoptosis due to redox stress, the local production of neurotrophic growth factors becomes impaired, which in turn affects repair and restoration of the damaged neuronal unit (Betul and Gandogdu 2006). Other than deficiency of these factors, there is also an impairment of neurotrophic retrograde transport from target organs to neuronal cell bodies (Leinninger et al. 2004). The general depression of axonal transport in DM inhibits normal neuronal function by blocking access to signalling molecules and structural proteins from the axon terminal. Because the cell is unable to receive growth factor-mediated signals, it does not produce a regenerative response (Ginty and Segal 2002). As such, the rescue attempts are inhibited by depressed axonal transport, thus further compromising the already impaired neurons (Leinninger et al. 2004).

2.4.3.4. **Immunological Hypothesis**

Immunological mechanisms have been suggested to be involved in the pathogenesis of diabetic peripheral neuropathy by the circulating anti-neuronal antibodies detected in a number of patients with DM (Vinik et al. 2005) and the inflammatory cells observed in the nerves of DM patients with neuropathy (Younger et al. 1996). This will be discussed in detail in the following section.
2.5. Diabetic peripheral neuropathy and the immunological aspects of its pathogenesis

2.5.1. Understanding Inflammation

The acute inflammatory response is an important part of innate immunity, which is triggered in response to a real or perceived threat to tissue homeostasis (Forbes and Cooper 2013). While the innate immune response is relatively nonspecific, adaptive immunity allows the human body to recognize and remember different pathogens. This results in the ability to produce an immediate inflammatory response following re-exposure to a particular pathogen (Forbes and Cooper 2013). In brief, acute inflammation occurs with the primary aim being the removal of perceived pathogens and initiation of wound healing in the damaged tissue. This inflammatory process then resolves via apoptosis and subsequent clearance of activated inflammatory cells as soon as the threat of infection subsides and sufficient repair to the damaged tissue is achieved (Forbes and Cooper 2013).

Inflammation is carefully orchestrated by a cascade of factors such as pro-inflammatory cytokines, chemokines, and adhesion molecules that initiate the interaction between leucocytes and the endothelium and guide directional leucocyte migration towards infected or injured tissue (Forbes and Cooper 2013). Pro-inflammatory cytokines, for example, tumour necrosis factor (TNF-α) and interleukins and chemokines released further activate the endothelium to increase the expression of the adhesion molecules E-selectin, intercellular adhesion molecule (ICAM-1), and vascular cell adhesion molecule (VCAM-1) (Forbes and Cooper 2013).

While acute inflammation as part of innate and adaptive immunity is beneficial, excessive or uncontrolled inflammation can promote tissue damage (Pickup 2004). Indeed, chronic inflammation is thought to be a characteristic feature seen at sites of diabetic complications (Forbes and Cooper 2013). In clinical studies, circulating
inflammatory markers are increased in patients with type I and type II DM (Younger et al. 1996), and the levels of these markers appear to predict the onset and progression of DM complications (Betul and Gundogdu 2006).

2.5.2. Cytokines response to inflammation

Cytokines are small glycoproteins produced by a number of cell types, predominantly leukocytes. They regulate a number of physiological and pathological functions including innate immunity, acquired immunity and a plethora of inflammatory responses. (Dinarello 2000). There are presently over 30 cytokines with the name interleukin (IL) (Uceyler at al. 2010). Other cytokines have retained their original biological description, such as tumour necrosis factor (TNF) (Dinarello 2000). Some cytokines clearly promote inflammation and are called pro-inflammatory cytokines, whereas other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines. For example IL-4, IL-10, and IL-13 are potent activators of B lymphocytes. However, IL-4, IL-10, and IL-13 are also potent anti-inflammatory agents. They are anti-inflammatory cytokines by virtue of their ability to suppress genes for pro-inflammatory cytokines such as IL-1, TNF-α and the chemokines (Dinarello 2000).

The concept that some cytokines function primarily to induce inflammation while others suppress inflammation is fundamental to cytokine biology and also to clinical medicine (Akdis et al. 2011). This concept is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. For example, genes that are pro-inflammatory are phospholipase A2, cyclooxygenase (COX)-2, and inducible NO synthase. These genes code for enzymes that increase the synthesis of platelet activating factor and leukotrienes, prostanoids, and NO (Akdis et al. 2011). Another class of genes that are pro-inflammatory are chemokines, which are small peptides that facilitate the passage of leukocytes from the circulation into the tissues. The prototypical chemokine is the neutrophil chemo
attractant IL-8 (Dinarello 2000). IL-8 also activates neutrophils to degranulate and cause tissue damage. IL-1 and TNF-α are inducers of endothelial adhesion molecules, which are essential for the adhesion of leukocytes to the endothelial surface prior to emigration into the tissues (Akdis et al. 2011). Taken together, pro-inflammatory cytokine mediated inflammation is a cascade of gene products usually not produced in healthy persons. Whether induced by an infection, trauma, ischemia, immune-activated T cells, or toxins, IL-1 and TNF-α usually initiate the cascade of inflammatory mediators by targeting the endothelium (Uceyler et al. 2010).

Interferon (IFN)-γ is another example of the pleiotropic nature of cytokines. Like IFN-α and IFN-β, IFN-γ is also an activator of the pathway that leads to cytotoxic T cells. However, IFN-γ is considered a pro-inflammatory cytokine because it augments TNF-α activity and induces nitric oxide.

2.5.3. Inflammation and DPN

Pro-inflammatory cytokines are produced locally by resident and infiltrating cells. These molecules exhibit pleiotropic effects on homeostasis of glia and neurons in the central, peripheral and autonomic nervous systems (Pickup 2004). Changes induced by chronic hyperglycaemia may lead to dysregulation of these cytokines. It has been demonstrated that endogenous TNF-α production is accelerated in micro-vascular and neural tissues, which may undergo increased micro-vascular permeability, hypercoagulability and nerve damage, thus initiating and promoting the development of characteristic lesions of diabetic peripheral neuropathy (Satoh et al. 2003).

Inflammation plays an essential role in the progression of diabetic micro-vascular complications, mainly diabetic retinopathy and nephropathy (Forbes and Cooper 2013). In chronic hyperglycaemia, cytokines infiltrate vascular tissues and inhibit function and repair (Nguyen et al. 2012). C-reactive protein, tumour necrosis factor TNF-α, and interleukin (IL)-6 all have exhibited increased expression in DM (Peters
et al. 1986; Ford 1999; Festa et al. 2000; Müller et al. 2002; Temelkova-Kurktschiev et al. 2002).

Moreover, obesity, as previously noted, is a major risk factor for DM and can induce inflammation by Toll-like receptor (TLR) activation to recruit pro-inflammatory cytokines and chemokines (Kwon et al. 2012). With the onset of DM, adipokines such as TNF-α and IL-6 may contribute to insulin resistance (Rajala and Scherer 2003; Suganami et al. 2005). Adiponectin is initially upregulated to increase glucose uptake, and nitric oxide (NO) production; however, continued obesity may reduce adiponectin leading to complications observed in type II DM (Berg et al. 2001; Matsuzawa 2005).

Obesity is also associated with hyperlipidaemia with elevated levels of cholesterol and triglycerides which may contribute to inflammation and diabetic complications (Dodson et al. 1981). The Feno fibrate Intervention and Event Lowering in Diabetes (FIELD) study, however, found no relationship between serum lipid levels and retinopathy (Keech et al. 2007; Chew et al. 2010). Feno fibrate is known to lower lipid levels, but it can also activate peroxisome proliferator-activated receptors (PPARs) and suppress inflammation by inhibiting nuclear factor kappa B (NF-κB; Tomizawa et al. 2011). As metabolic syndrome and inflammation persist, oxidative stress, hypoxia, and advanced glycation end-products receptor (RAGE) converge to exacerbate the problem (Brownlee 2005; Vincent et al. 2011). A schematic summarizing the pathogenesis of diabetic micro-vascular complications illustrated by Forbes and Cooper (2013) is presented in Figure 2.5.2.1. This figure demonstrates how oxidative stress, hypoxia, and advanced glycation end-products activate the inflammatory cascade producing inflammation.
Studies have shown that inflammation, and more specifically pro-inflammatory cytokines, play a determinant role in the development of micro-vascular diabetic complications (Wang et al. 2001; Kellogg et al. 2007). For instance, it has been observed that DM patients with higher plasma and mRNA levels of TNF-α have a greater risk of developing cardiac complications (Kampoli et al. 2011; Purwata and High 2011). Moreover, compared with patients who have painless DPN, those with painful DPN have been shown to have higher levels of C-reactive protein (Doupis et al. 2009). C-reactive protein is a pro-inflammatory protein that is released by the liver in response to inflammation. CRP along with TNF-α have been shown to be directly involved in the production of pain in several models of nerve injury (Purwata
The effect of these markers on neurons seems to be mediated, directly and/or indirectly, by the phosphorylation of extracellular-regulated kinases (Schafers et al. 2003), translocation of nuclear factor kappa B to the nucleus, and activation of COX-2 dependent prostanoid release (Dinarello 2000). TNF-α also activates NFκB for initiation of NOS and NO production whereas NO is a pain transmitter (Lowenste 1994).

The mechanisms by which chronic inflammation may induce DM complications are not clear. Some suggested that low-grade inflammatory reactions may be triggered secondary to the oxidative stress generated by reactive oxygen species (Dominiczak 2003). Others proposed that since inflammatory markers such as interleukin (IL-6, IL-2), and TNF-α are elevated in hyperglycaemia, this suggests that a chronic, low-grade inflammatory state may exist in patients with DM (Bailey 2007; Neihoff et al. 2007; Sjoholm & Nystrom 2007; Kampoli et al. 2011).

Tumour necrosis factor-α has also been implicated in contributing to insulin resistance in obesity due to its increased expression in adipose tissue. Obese mice with a TNF-α mutation displayed improved insulin sensitivity and lowered circulating fatty acids, improving obesity-induced glucose tolerance (Uysal et al. 1997). Increased plasma TNF-α and macrophages are also associated with the progression of diabetic nephropathy, suggesting continued expression of these cytokines contribute to diabetic micro-vascular complications (Purwata and High 2011).

Similar experiments evaluating TNF-α in null mice showed that they are less susceptible to developing diabetic complications (Gao et al. 2007). Targeting TNF-α through pharmacological means thus may potentially reverse the adverse effects in diabetic neuropathy. Infliximab, for example, a monoclonal anti-TNF-α antibody approved for treatment of autoimmune diseases such as rheumatoid arthritis and psoriasis has been explored (Lin et al. 2008). Administration of infliximab into type I DM mice showed significant improvement in neural function comparable to non-diabetic controls (Yamakawa et al. 2011).
The use of non-steroidal anti-inflammatory compounds such as cyclooxygenase-2 (COX-2) inhibitors or high-dose aspirin given to DM individuals for other indications have also provided evidence in reducing the development of complications related to inflammation such as retinopathy (Kanamori et al. 2007), nephropathy (Cherney et al. 2009; Nasrallah et al. 2009), and macro-vascular disease (Ross 1999). However, not all studies have shown benefits following the use of these anti-inflammatory agents (Kohner 2003), and some of these agents cannot be considered as long-term treatment for diabetic neuropathy because non-steroidal anti-inflammatory drugs often have nephrotoxic side effects (Cheng and Harris 2005).

Studies related to investigating inflammation in DPN were mostly based on animal studies and very little information is available regarding human DPN. One study by Doupis et al. (2009) investigated the presence of inflammatory cytokines along with growth factors in painful and painless DPN patients and the results were both suggestive of a significant link and contribution to the pathology of DPN. Their conclusion stated that DPN is associated with increased biochemical markers of inflammation and endothelial dysfunction, and that painful neuropathy is associated with further increase in inflammation and markers of endothelial dysfunction and preservation of the nerve axon reflex.

Doupis et al. (2009) investigated the association between inflammation, micro-vascular reactivity, and the development of DPN in three groups: 55 healthy control subjects, 80 non neuropathic DM patients, and 77 neuropathic DM patients. They then subdivided the neuropathic patients into a subgroup of 31 subjects with painless neuropathy and 46 with painful neuropathy. Their study measured the foot skin endothelium-dependent and -independent vasodilation, the nerve axon reflex-related vasodilation (NARV), and inflammatory cytokines and biochemical markers of endothelial function.

Compared to the other two groups, the neuropathic group in their study had higher serum levels of CRP, TNF-α, and fibrinogen. Patients with painful neuropathy had higher CRP levels when compared to painless neuropathy. Nerve axon reflex-related
vasodilation was further reduced in the subgroup of painless neuropathy when compared to painful neuropathy. The endothelium-dependent and –independent vasodilation and NARV were lower in the neuropathic group. No major changes in the above results were observed in 78 DM patients who were seen for a second visit 21 months after the first visit.

Although this study investigated inflammatory cytokines in peripheral neuropathy, they only looked at one cytokine (TNF-α), and concentrated more on growth factor levels. Including a control group with healthy non DM subject was a positive point for this study as the results showed no significant difference between the control and the DM group with no neuropathy suggesting that the raised pro-inflammatory cytokines were more related to neuropathy rather than DM itself. However, the duration of DM in the neuropathy group was much longer than the non-neuropathy group which could indicate that the raised levels may be influenced by the duration of diabetes mellitus and not just the neuropathy.

Another limitation of this study was the fact that there was no mention of other types of neuropathies associated with DM that have proven to be related to inflammation or autoimmunity and there was no mention of the exclusion of such patients when selecting the sample for the neuropathy group.

Another study by Herder et al. (2009) suggested that subclinical inflammation is associated with DPN and neuropathic impairments. They measured markers of subclinical inflammation in 227 type II DM patients with diabetic peripheral neuropathy. The results of their study demonstrated that high levels of C-reactive protein and interleukin (IL)-6 were most consistently associated with diabetic peripheral neuropathy, high MNSI (Michigan Neuropathy Screening Instrument) score, and specific neuropathic deficits, whereas some inverse associations were seen for IL-18.

The population-based design of their study included patients with type II DM only and did not mention the reasons behind excluding patients with type I DM. In
addition, since they did not exclude other reasons for neuropathy in the sample included, in some cases neuropathy may not have been due to diabetes mellitus. Their justification for this was that an exclusion of other potential causes of neuropathy for this relatively large study sample was not feasible. Thirdly, the study lacked a non-diabetic control group, which would have been necessary to demonstrate unequivocally that the diabetic study participants had increased levels of pro-inflammatory markers compared with those in healthy individuals without DM.

2.5.4. Autoimmunity and DPN

Waksman and Adams (1955) suggested an autoimmune aetiology of peripheral neuropathy when they injected rabbits with neuronal components to produce what they called “allergic neuritis” (Vinik et al. 2005). A number of studies suggesting different mechanisms of autoimmune activation as a pathogenetic factor in the development of autonomic neuropathy in both type I and type II DM have been discussed by Vinik et al. (2005). Post-mortem studies of type I DM patients with severe autonomic neuropathy have shown lymphocytic infiltration and small nerve fibre damage in autonomic ganglia indicating vigorous immune response (Duchen et al. 1980). Circulating auto-antibodies in sera of type I DM patients have been noted to react with autonomic tissues, most notably sympathetic ganglia and the vagus nerve, which might be associated with future development of autonomic neuropathy (Granberg et al. 2005).

Regarding DPN, Granberg et al. (2005) noted that it is not clear whether autoimmunity plays a primary role in the pathogenesis or it accelerates DPN initiated by metabolic or vascular injury. Peripheral nerves are normally protected against the immune system by tight capillary endothelial junctions and the perineurium. Nerves are also a rich source of glycoproteins, lipopolysaccharides, and other lipoproteins that can potentially form active antigenic material (Vinik et al. 2005).
In the autoimmune onslaught against nerves, Boulton et al. (2004) stated that there is first damage to the protective sheath and then to the inner components. These can be brought on by viral or bacterial infections (e.g., polio, leprosy, and Lyme’s disease), neoplasm, or connective tissue disorders, and often there is strong genetic predisposition, such as HLA DR-3 and HLA DR-4, in type I DM (Vinik et al. 2005).

Type 1 diabetes mellitus results from an autoimmune destruction of pancreatic β-cells, and there may be a direct destruction of neurons by the same autoimmune process in DM (Vinik et al. 2005). The pancreatic islets of Langerhans are surrounded by a Schwann cell sheath. These cells form a tight cellular mantle that envelops the endocrine islet tissue. Components of the peri-islet Schwann cells include glutamate decarboxylase (GAD); an enzyme that catalyses the decarboxylation of glutamate to GABA and CO₂ (Vinik et al. 2005). GAD exists in two isoforms encoded by two different genes (GAD1 and GAD2). These isoforms are GAD65 and GAD67 (Erlander et al. 1991). There is an early appearance of anti-GAD65–specific T-cells in type 1 DM (Winer et al. 2003). Anti-GAD65 antibody is a strong predictive marker for the onset of type 1 DM (Winer et al. 2003). Presence of this antibody in patients with recent-onset type 1 DM is associated with worse glycaemic control and worse peripheral nerve function, suggesting a common mechanism for β-cell and neuronal damage (Hoeldtke et al. 2000). Patients with high GAD65 antibodies were shown to have reduced motor nerve conduction velocities in the median, ulnar and peroneal nerves, prolonged F wave latencies, high thermal threshold detection for hot and cold, and decreased cardiovascular autonomic function (decreased heart rate variability and lower post-Valsalva R-R intervals) (Hoeldtke et al. 2000).

Serum collected from type 1 DM patients has also demonstrated toxicity to neuroblastoma cells (Pittenger et al. 1995). About two-thirds of the toxicity was due to autoimmune serum factors. One of the components of this serum that mediates immune destruction of neuroblastoma cells in cultures was found to be Fas-specific IgG antibodies (Pittenger et al. 1997). These antibodies bind to Fas-ligand on the surface of neuroblastoma cells and induce apoptosis. Pittenger et al. (1997) found
that serum from patients with diabetic neuropathy contains an activator of Fas-regulated apoptosis that may contribute to the pathogenesis of diabetic neuropathy.

Autoimmune immunoglobulin was thought to induce apoptosis of the neuronal cells, and was further related to the severity of DPN (Pitteneger et al. 1999). This suggests that autoimmune mechanisms may act together with hyperglycaemia to damage sensory and autonomic neurons (Boulton et al. 2004; Vinik et al. 2005). Vinik et al. (2005) concluded that there is no doubt that a variety of antibodies are present in the sera of patients with DPN and that the sera exert apoptotic effects on neurons grown in culture, but the missing link is the relationship with clinical neuropathy and the potential for reversibility with immune therapy.

A number of articles described a significant link between autoimmunity and diabetic neuropathy such as proximal neuropathy, and autonomic neuropathies associated with DM (Vinik et al. 2005). However, studies investigating the relationship between clinical DPN and autoimmunity are almost nonexistent.

A study by Srinivasan et al. (1998) demonstrated that sera from type II DM patients with neuropathy contain an autoimmune immunoglobulin that induces complement-independent and calcium-dependent apoptosis in neuronal cells. Neuronal cells were cultured in the presence of complement-inactivated sera obtained from patients with type II DM with and without neuropathy and healthy adult control patients. Serum from DM patients with neuropathy in their study was associated with a significantly greater induction of apoptosis, compared to serum from DM patients without neuropathy and controls. In the presence of calcium channel antagonists, induction of apoptosis was reduced by approximately 50%. Furthermore, pre-treatment of neuronal cells with serum from DM patients with neuropathy was associated with a significant increase in elevated $K^+$ evoked cytosolic calcium concentration (Srinivasan et al. 1998). Similarly, treatment with an anti-human IgG antibody was associated with intense fluorescence on the surface of neuronal cells exposed to sera from patients with type II DM with neuropathy (Srinivasan et al. 1998).
Although this study by Srinivasan et al. (1998) suggests an autoimmune involvement in the pathogenesis of diabetic neuropathy, their study did not document the type of neuropathy witnessed in the sample investigated. Hence, the diabetic neuropathies they are referring to might have included autonomic or inflammatory neuropathies associated with DM as described shortly.

Based on this concept, a number of new treatment agents have been introduced and tried on a number of DM patients with peripheral neuropathies worldwide and some of the cases in fact presented significant reduction in pain levels and improvements in neuropathy symptoms (Veves and King 2001). Intravenous Immunoglobulin Injection for example is one of the new treatment plans suggested for patients with central and peripheral neuropathy (Elovaara et al. 2010). Intravenous immunoglobulin (IVIg) is increasingly used in neurological manifestations of autoimmune diseases as well as inflammatory diabetic neuropathies (Gullain-Barre syndrome, CIDP, Multifocal Motor Neuropathy for example) (Said et al. 2003). However, no randomised controlled trials have been published to date supporting the efficacy of using such agents to reduce the inflammation in patients with DPN, or how such agents which have been proven to be effective in conditions where inflammation or autoimmunity are present such as autoimmune neuropathies would actually work in reducing the symptoms of DPN.

Perhaps the clearest link between autoimmunity and neuropathy has been the demonstration of an increased likelihood of chronic inflammatory demyelinating polyneuropathy, multiple motor polyneuropathy, vasculitis, and monoclonal gammopathies in diabetes mellitus (Sharma et al. 2002). These are proximal neuropathies presenting with pain in the buttocks and thighs, fasciculation, and weakness with inability to rise from the sitting position or when kneeling on the floor. They may be the presenting symptom in many autoimmune vasculitides and celiac disease, a multigenetic, T-cell–mediated autoimmune disorder that results from a loss of tolerance to gluten (Chin and Latov 2005).
In support of an autoimmune mechanism for proximal neuropathies is the salutary response to intravenous Ig and immunotherapy (Sharma et al. 2002). Intravenous immunoglobulin (IVIG) has been successfully used to treat a number of immune-mediated disease of the central and peripheral nervous system (Levy et al. 1999; Sharma et al. 2002; Levy et al. 2003). Although underlying mechanisms of action of IVIG have not been fully explained, it is known that IVIG can interfere with the immune system at several levels (Elovaara et al. 2008). It has been demonstrated to inactivate auto-reactive T cells by competing for and interrupting their interaction with antigen presenting cells (Kaiseier et al. 2008). Numerous targets for IVIG include: T-cells, cytokines, immune cell trafficking, β-cells, complement and Fc-receptors.

The situation with sensory neuropathies is less clear. Several different autoimmune antibodies in human sera have been reported that may react with epitopes in neuronal cells (Vinik et al. 2005). Prominent among them are the gangliosides, and antibodies to GD1a, GD1b, GM1, GM2, and GalNAc-GD1a are not uncommon. Other antibodies include anti-sulfatide, anti–myelin-associated glycoprotein, anti-Hu (associated with neuropathy in paraneoplastic syndromes), perinuclear anti-neutrophilic cytoplasmic antibodies, and cytoplasmic anti-neutrophilic cytoplasmic antibodies (Vinik et al. 2005).

Pittenger et al. (1997) reported neurotoxicity of sera from 39 patients with diabetic neuropathy. Neurotoxicity was assessed using neuronal cell line (adrenal medulla and ventral spinal cord 4.1, and a motor cell line). Neurotoxicity correlated with vibration detection thresholds, and sera from patients with motor neuropathy were highly toxic to the VSC 4.1 line, indicating that there was a relationship between the specific nerve fibre function and the type of neuronal cell destroyed by the serum factors (Pittenger et al. 1997). Unfortunately, there have been no trials on immunotherapy for somatic neuropathies to confirm or refute the importance of these findings.
2.6. Treatment of DPN

The Diabetes Control and Complication Trial strongly suggest that strict control of blood glucose prevents long-term complications of DM, including DPN (DCACT 1995). Modification of vascular risk factors (for example, hypertension, hyperlipidaemia, weight gain, and smoking) is also recommended to slow the progression of neuropathy (Tesfaye et al. 2005).

Based on the different pathogenetic mechanisms of DPN, several therapeutic approaches have been implemented. Painful diabetic peripheral neuropathy management consists of excluding other causes of painful peripheral neuropathy, maximizing diabetic control and using medications to alleviate pain (Chong and Hester 2007). Pharmacological treatment for neuropathic pain in DM includes treatment with tricyclic antidepressants (TCAs), other types of antidepressants, anticonvulsants, and opioids and opioid-like drugs. Gabapentin is the most widely used drug for treatment of neuropathic pain (Betul and Gundogdu 2006). Duloxetine and pregabalin became available recently and are specifically licensed for the management of painful DPN (Rosenstock et al., 2002).

Based on the pathogenetic mechanisms of DPN, potential disease-modifying therapeutic approaches have been developed including antioxidants such as α-lipoic acid (ALA) (Ametov et al. 2003; Ziegler et al. 2006) to diminish increased oxidative stress (Ziegler et al. 2004). Other potential modalities include the aldose reductase inhibitors (Bril et al. 2009), growth factors (Ropper et al. 2009), and the protein kinase C-b inhibitor ruboxistaurin (Vinik et al. 2005). These drugs have been designed to favourably influence the underlying pathophysiology of the disorder rather than for symptomatic pain relief.

Alpha-lipoic acid has been licensed and used in Europe for treatment of symptomatic diabetic neuropathy for more than 20 years (Shakher and Stevens 2011). Reduction of neuropathic symptoms such as pain and paresthesia occurred with short-term use of the intravenous form of ALA (Ziegler et al. 2004). A large multicentre trial
(NATHAN 1 study) conducted in North America and Europe to evaluate the effects of long-term (4 years) treatment of α-lipoic acid on the progression of DPN stated that four year treatment with ALA in mild to moderate DPN did not influence the primary composite end point but resulted in a clinically meaningful improvement and prevention of progression of neuropathic impairments and was well tolerated (Zeigler et al. 2011).

Reversal of experimental DPN and clinical improvement in chronic ischaemic neuropathy has been demonstrated with vascular endothelial growth factor gene transfer, an inducer of angiogenesis (Schratzberger et al. 2001; Simovic et al. 2001). In one trial, several indices of nerve conduction studies, but not neuropathic symptoms and deficits, were improved after 1 year of treatment with the angiotension-converting enzyme inhibitor trandolapril (Malik et al. 1998).

Restoration of neurotrophic growth factors, recombinant human NGF, and brain-derived neurotrophic factor have not yet been shown to be of value in human clinical trials, but there is hope that future clinical trials will show them to be effective in the treatment of DPN (Apfel et al. 2000; Wellmer et al. 2001). Treatment with the PKC β-selective inhibitor ruboxistaurin restored several neuropathic deficits in a phase II clinical trial (Bastyr et al. 2003).

A comprehensive systematic review and meta-analytic comparison of all available randomized controlled clinical trials evaluating available pharmacologic therapies for the treatment of painful DPN was conducted by Snedecore et al. (2013) with the goal of comparing efficacy (pain reduction) and harms (discontinuations and adverse events).

Data from 58 studies including 29 interventions and 11,883 patients were analyzed. Pregabalin (300 mg/day) was the most effective on the 100-point visual analogue scale, while topiramate was the least. Relative risks (RRs) of 30% pain reduction ranged from 0.78 (Sativex®) to 1.84 (lidocaine™ 5% plaster). Analysis of the RR ratio of these two treatments revealed marginal significance for Sativex®, indicating
the best treatment is only slightly better than the worst. Relative risks of 50% pain reduction ranged from 0.98 (amitriptyline) to 2.25 (alpha-lipoic acid). Fluoxetine had the lowest risk of adverse events while oxycodone had the highest.

Clearly, selecting an appropriate DPN therapy remains challenging due to important differences in effectiveness and harms associated with a large number of available therapeutic options.

2.7. Summary of literature review

Diabetic Peripheral neuropathy presents with considerable morbidity and can result in significant decreases in quality of life worldwide. Diabetes mellitus and its complications within the MER region are rising. Previous clinic-based studies on Bahraini patients with DM demonstrated that a large proportion of the DM patients have neuropathic complications and are therefore, at substantial risk of developing foot complication that will affect their quality of life.

Although recent experimental studies suggest a multifactorial pathogenesis of DPN limited translational work in DM patients continues to generate much debate and controversy over the causes of human DPN, and to date we have little effective long-term treatments. While conventional medicine can offer some relief, the potential side effects or addictive nature of many of the medications render long term use undesirable. Such treatments, furthermore, simply mask the symptoms and do not address the underlying pathologies.

Available treatments to date consist of improved metabolic control and a focus on symptoms but do not concentrate on fundamental mechanisms in the pathogenesis of neuropathy. Despite efforts to make an early diagnosis and to halt the progression of DPN, currently there is no effective treatment at a global level, except for tight control of blood glucose. This might be as a result, at least in part, of insufficient
clarification of the pathogenesis of DPN, complicated clinical pictures that do not necessarily reflect progression of the disease, or inadequate design of clinical trials.

The pathogenetic vision of diabetes mellitus has changed in the last few years, with inflammatory and autoimmunity pathways playing roles in the development and progression of diabetic complications. These new pathogenic factors lead to a consideration of new therapeutic approaches. This study was therefore conducted to investigate whether inflammation and/or autoimmunity are associated with the pathogenesis of human DPN.

2.8. Research Aims and Objectives

2.8.1. The aims of the study are

1) to estimate the percentages of DM complications in the Kingdom of Bahrain through studying the demographics and the presence of aspects such as neuropathy and peripheral vascular disease within the population of patients with diabetes mellitus in the Royal Medical Services Bahrain Defence Force Hospital (RMS-BDF);

2) to compare the levels of pro-inflammatory markers and autoimmune markers between DM patients with peripheral neuropathy and DM control patients without peripheral neuropathy and the healthy control group;

3) to explore possible correlations between the levels of pro-inflammatory markers and autoimmune markers and other demographics or clinical variables, such as, types of DM, duration of DM, and neurological manifestations.
2.8.2. The Research Objectives are:

2.8.2.1. Primary Objectives

1) to explore the characteristics of the DM patient population attending the RMS-BDF Hospital through investigating their demographic profiles;

2) to compare the levels of pro-inflammatory cytokines in the blood serum between three groups; DM patients with peripheral neuropathy attending Diabetic Clinic at the RMS-BDF Hospital, DM patients with no neuropathy, and healthy subjects (with similar demographics);

3) to compare the percentages of patients who have positive autoimmune antibodies in the blood serum between three groups; DM patients with peripheral neuropathy attending Diabetic Clinic at the RMS-BDF Hospital, DM patients with no neuropathy, and healthy subjects (with similar demographics).

2.8.2.2. Secondary Objectives

1) to explore the possible correlations between the levels of pro-inflammatory markers and sample demographics (age, gender, type of DM, duration of DM, body mass index (BMI), random blood glucose (RBG) and systolic and diastolic BP) and clinical manifestations of peripheral neuropathy (Vibration perception threshold (VPT), Neuropathy symptom score (NSS) and Neuropathy disability score (NDS));

2) to explore the potential correlations between the presence of autoimmunity markers and sample demographics (age, gender, type of DM, duration of DM, BMI, RBG and systolic and diastolic BP), and the clinical manifestations of peripheral neuropathy (VPT, NSS and NDS);
3) to explore the potential correlations between sample demographics (age, gender, type of DM, duration of DM, BMI, RBG and systolic and diastolic BP), and the clinical manifestations of peripheral neuropathy (VPT, NSS and NDS).

2.9. Research Hypotheses

2.9.1. Hypothesis one:

There are significant differences in the levels of the pro-inflammatory markers (CRP, TNF-α, IFN-ɤ, IL-6, IL-8, and IL-1β) in the blood serum of patients with DM with peripheral neuropathy when compared with the levels of pro-inflammatory markers in the two control groups; DM control and healthy control.

Null Hypothesis one:

There are no significant differences in the levels of the pro-inflammatory markers (CRP, TNF-α, IFN-ɤ, IL-6, IL-8, and IL-1β) in the blood serum of patients with DM with peripheral neuropathy when compared with the levels of pro-inflammatory markers in the two control groups; DM control and healthy control.

2.9.2. Hypothesis two:

Autoimmune antibodies are present in the blood serum of patients with diabetic peripheral neuropathy.

Null Hypothesis two:

Autoimmune antibodies are not present in the blood serum of patients with diabetic peripheral neuropathy.
Chapter Three: Methodology

The research design of the study has a specific methodological direction based on its objectives and research question. This chapter provides detailed description and justifications of the methodological approach used in the study. The proposed framework looks at scientific investigations to quantify the relationships between inflammation and peripheral neuropathies, and autoimmunity and peripheral neuropathies in patients with diabetes mellitus, as well as exploring additional factors that might influence this relationship.

The goals of this chapter are to:

- Justify the quantitative methods used within this project and explore the strengths and limitations of the approach;
- Discuss the process of obtaining ethical approval;
- Explore the sampling technique and the study population;
- Provide a thorough explanation of the quantitative research method conducted to test the hypotheses;
- Detail the quantitative measures undertaken to test the null hypothesis which suggests no significant differences in the levels of pro-inflammatory markers between patients with DPN when compared to the control groups;
- Detail the quantitative measures undertaken to test the null hypothesis which suggests no presence of autoimmune antibodies in the blood serum of patients with DPN when compared to the control groups;
- Detail the statistical analysis undertaken to explore the methodological approach.
3.1. Methodological Approach

A quantitative retrospective analytical approach was employed to answer the research question which is investigating the relationship between pro-inflammatory markers and autoimmune markers and the pathogenesis of diabetic peripheral neuropathy. A cross-sectional case control study design was used where subjects were initially sampled from a population (patients with DM) without respect to specific disease status. The sample was then identified for a case-control analysis in which individuals with specific characteristics (cases) were identified, suitable comparison participants (controls) were identified, and the groups were compared.

As previously discussed in Chapters one and two, the research proposed an empirical setting to investigate the theoretical path drawn from the literature, which suggests an association between inflammation and/or autoimmunity and diabetic peripheral neuropathy, and test this through hypotheses. This research approach provides a possible answer to the research question scientifically which is defined in an objective way and measured through statistical tools and techniques.

The conceptual framework used in this investigation seeks to quantify the data for the purpose of explaining the casual relationships. The approach for this investigation is thus explanatory. It comprises quantitative research tools and techniques and incorporates quantitative collection of data where findings show the level of significance of the relationship and further explores the interoperation of the results.

The quality of research designs can range from meta-analyses and systematic reviews of double-blind, placebo-controlled clinical trials at the top end of a hierarchy, descending towards expert opinion or personal experience at the bottom. The NHS and the Oxford Centre for Evidence-based Medicine uses a system with categories labelled A, B, C, and D which clearly suggests the levels of evidence according to the study designs and critical appraisal of prevention, diagnosis, prognosis, therapy, and harm studies (OCEBM 2011):
Level A: Consistent Randomised Controlled Clinical Trial, cohort study;

Level B: Consistent Retrospective Cohort, Exploratory Cohort, Ecological Study, Outcomes Research, case-control study; or extrapolations from level A studies;

Level C: Case-series study or extrapolations from level B studies;

Level D: Expert opinion without explicit critical appraisal, or based on physiology, bench research or first principles.

It can be argued that it is widely recognised that the most powerful type of experimental study is the randomized controlled trial (Altman 1996; Chan 2003; Stolberg et al. 2004; OCEBM 2011). However, conducting an RCT in this study was not possible due to the precise inclusion and exclusion criteria required for this investigation.

### 3.2. Ethical consideration and approval

The Royal Medical Services - Bahrain Defence Force Hospital (RMS-BDF) and Queen Margaret University (QMU) Ethics and Research Committees granted favourable ethical opinions for the study titled “The role of pro-inflammatory cytokines and autoimmune antibodies in diabetic peripheral neuropathy” (Appendix 2 and Appendix 3) on the 24th of January 2012 and the 8th of May 2012, respectively.

The ethical consideration in the methodology adapted in this research was in accordance with the principles of the Declaration of Helsinki (WMA 2002). Efforts were made to protect the rights and dignity of study participants, including their rights to confidentiality, anonymity, informed consent and the right to withdraw from the study. This information is further illustrated in the information sheet and consent forms (Appendix 4 and Appendix 5).
All data were as anonymous as possible. All the record cards used for the purpose of investigating the demographics were recorded by the patient’s hospital number, and patients' details were kept confidential at all times. Patients' records were only accessed by the researcher involved in the study. Permission to access the data was also obtained from the Research Ethical Committee (REC) at RMS-BDF Hospital (Appendix 2) following the submission of a research proposal explaining the methodology in detail. The sample selected for further investigations were verbally informed about the aims, objectives, and the nature of the study. The invitation to participate in the study was made by the researcher and only those willing to participate identified themselves to the study facilitators. This process was aimed at further ensuring participants’ confidentiality and autonomy.

Interested participants were then given a detailed patient information sheet explaining the nature of research and the probable consequences of the investigation in lay terms, along with details of the investigator and how they might be contacted, the confidentiality of the data, any risks involved, and any other information which the participant might be required to know before giving informed consent. All participants were asked to make an independent decision without the influence of the researcher. Participants were given a period of one week to fully read and understand the research methodology. Researcher’s contacts were available for all participants should they have had any further enquiries.

For easy understanding, the consent forms and information sheet was made available in English and Arabic (being the most spoken languages in the province). Participants were requested to choose the language of preference for the consent forms and information sheets. Informed consent forms were signed in duplicate; a copy was kept by the researcher in a secure storage, while the second copy was kept by the respective participant.

Risk assessment was conducted by the researcher and assessed by the ethics committees (Appendix 6). The study method was classified as incurring major invasive risk due to the involvement of invasive investigations through blood sample
analysis. Although the research raised ethical concerns, the ethical board committees were provided with justifiable means to support the investigation.

As previously discussed in Chapter one, the research undertaken is considered to be essential for the advancement of knowledge that would benefit patients with diabetic peripheral neuropathy, and improves health professionals’ competencies in managing such patients. The study has the aim of contributing, through significant improvement in the scientific understanding of the individual's condition, to the ultimate attainment of results capable of conferring benefit to the person concerned or to other persons afflicted with the same condition.

To ensure risk minimization, blood investigations were carried out at hospital setting with an expert phlebotomist. Help was available at all times in case of emergencies (such as, needle breakage, needle stick injury, and emotional fear). All subjects were advised that they could withdraw from study at any time if they felt uncomfortable with the blood sample procedure.

Furthermore, the results of the blood investigations may show an increase or decrease in pro-inflammatory or autoimmune markers which the subjects may not be aware of. These results were however not relevant to the patients as the number of pro-inflammatory or autoimmune markers were not suggestive of any underlying diseases. This study was simply testing whether their presence was associated with DPN. Participants’ blood results were not reported back to all subjects, and were not saved in the hospitals computer system, unless the participants were interested in identifying the level of markers in their blood.

The participants’ study records (such as demographics and blood investigation results) will be kept by the researcher for a maximum of three years following the completion of the study. In line with the agreements with the participants as detailed in the consent forms, the records were kept by the researcher in a safe, suitable, sizeable, durable and locked container. The records were protected from any form of damage, unauthorized use, and removal or disclosure of the information they contain.
At the end of the three years storage period, the record will be destroyed by the researcher in a manner that continues to protect the anonymity and dignity of the participants. Signed consent forms were kept separately from the data and destroyed within 12 months of the end of the data collection period.

3.3. Population, sample size, and sample characteristics

3.3.1. Population and sample selection

A convenience random sample of 500 record cards of patients diagnosed with type I and type II DM who have attended the RMS-BDF Hospital Diabetic Clinic for their routine follow up appointment, between April 2012 and June 2012, were initially selected in order to study the general demographics and clinical characteristics of DM patients in Bahrain. The random sampling method was achieved by utilizing the hospital computer system to obtain a list of all the patients who have attended the DM clinic in the last three months, and assigning a number to all patients. This was followed by drawing a set of 500 random numbers (the odd numbers) which represented the total members of the population to be sampled (n=500).

Patients’ record cards were screened thoroughly by the researcher, and the following information was documented in separate charts:

- Age;
- Gender;
- Race;
- Type of DM;
- Duration of DM;
- Complete Medical History;
- Random Blood Glucose (mmol/l)
- Medications;
- Height (cm), weight (kg), and BMI (kg/m²);
- Blood Pressure measurement recorded in their last visit (systolic and diastolic BP in mmHg);
- Previous laboratory investigations (if available). This included HbA1c, CBC, LFT, LDL and LDH;
- Previous notes on diabetic foot assessment (neurological, vascular, and general foot condition, if available).

Once all the demographics and clinical characteristics of the patients had been obtained from the patient’s record cards, the sample was then divided into two groups for the variable of peripheral neuropathy. The groups were simultaneously screened to identify the samples required for the case control study based on the inclusion and exclusion criteria described shortly. The two groups were further divided into four groups (DM patients with neuropathy and no other medical conditions, DM patients with neuropathy and other medical conditions, DM patients with no neuropathy and no other medical conditions and DM patients with no neuropathy but diagnosed with other medical conditions) as illustrated in Figure 3.3.1.

All patients referred to the RMS-BDF Hospital diabetic clinic had established diabetes mellitus according to WHO criteria (1999). Hypertension was defined as previous treatment for hypertension or blood pressure measurement in the clinic equal to 160/95 mm Hg or higher on at least two successive visits (Chobanian et al. 2003). Peripheral neuropathy was defined as nerve damage characterized by sensory loss, pain, muscle weakness and wasting of muscle in the legs and feet (Claus et al. 1993). Peripheral arterial disease was defined as arteriosclerosis of the extremities; a disease of the blood vessels characterized by narrowing and hardening of the arteries that supply the lower limbs (Mills 2010).
The sample required for the case control analysis was then randomly selected by collecting 30 record cards from the DM patients with neuropathy and no other medical conditions group, and another 30 record cards from the DM patients with no neuropathy and no other medical conditions group. The method of randomisation
was similar to the one applied for the cross sectional sample collection previously mentioned.

Selecting the control groups followed the decision made on case selection to ensure homogeneity in all respects other than having the disease. Generalisability was obtained by including two types of controls for the cases studied (Paneth et al. 2002). Control selection was obtained through frequency matching (matching variables and matching criteria). This strategy was adapted to ensure balanced distribution across strata, stable odd ratio estimation and smaller standard errors (Rothman and Greenland 1998). This was completed by inviting subjects from the population to volunteer in the study through circulating an email to all staff working at the RMS-BDF Hospital (Approximately 5,000 physicians, health professionals, and other employees). The final samples used for investigating the hypothesis included 90 individuals; 60 patients with diabetes mellitus selected from the 500 sample population, and 30 healthy volunteers recruited from hospital staff population as illustrated in Figure 3.3.1. The final groups selected for the case control analysis were the following:

**Group (1):** DM patients diagnosed with peripheral neuropathy with no other medical conditions;

**Group (2):** Control DM patients with no history of peripheral neuropathy and no other medical conditions (control 1);

**Group (3):** Healthy individuals with no DM and no neuropathy with similar demographics as the cases (control 2).
3.3.2 Inclusion and Exclusion criteria

The inclusion criteria included:

- Patients with type I and type II DM with and without peripheral neuropathy attending the Diabetic Clinic at the RMS-BDF Hospital who agreed to participate in the study and signed the consent form;
- Healthy subjects with no known underlying disease who agreed to participate in the study and signed the consent form (Control Group).

The exclusion criteria included:

- Symptomatic peripheral arterial disease (ankle brachial index < 0.65 and/or symptoms of claudication);
- Congestive heart failure, cardiac arrhythmias, stroke or transient ischaemic attack;
- End stage renal failure;
- Uncontrolled hypertension;
- Severe dyslipidaemia;
- Chronic liver disease;
- DM patients with inflammatory neuropathies including chronic inflammatory demyelinating polyneuropathy (CIDP), proximal diabetes neuropathy, and autonomic neuropathies;
- Patients with other types of neuropathies not associated with DM such as B12 deficiency, hypothyroidism, and uraemia;
- Other severe chronic medical condition requiring active treatment;
- Subjects older than 70 years, or younger than 20 years;
- Subjects that currently smoke, or have been smoking in the last 5 years;
- Morbidly Obese patients;
- Pregnancy;
- Subjects reporting any active infection or inflammation;
- Subjects on hormone replacement therapy, statins, angiotension converting enzyme inhibitors or thiazolidiones;
- Subjects with abnormal values in their past blood investigation analysis.

The exclusion criteria for the study were established following a thorough literature search conducted to explore the possible factors that may affect the levels of pro-inflammatory markers and the presence of autoimmune antibodies in the blood serum, and thus bias the outcomes. For example, patients with other types of neuropathies associated with DM, such as chronic inflammatory demyelinating polyneuropathy, proximal diabetes neuropathy, and autonomic neuropathies, would bias the study results since such conditions are positively associated with inflammation and autoimmunity. Similarly, chronic smoking has been proven to be associated with increased pro-inflammatory cytokines (Barbosa et al. 2001).

However, the collection of data in a fairly homogeneous environment representing a single country and from a single hospital within that country might question the generalisability of the sample, but it eliminates the potential risk of interacting external variables (Amine and Cavusgil 1986). The rationale for selecting Bahrain as a context was: the researcher is a native of Bahrain and the only podiatrist in the country, which has facilitated easy access to Bahraini data sources. In addition, the study provides data from a developing country for increasing generalisability among other developing countries.

3.3.3. Sample size

The total population in Bahrain is approximately 1,281,332, according to the Bahrain demographics profile for the year 2013. The prevalence of DM in Bahrain is 16.3% of the total population (IDF 2013), suggesting that approximately 208,985 people in Bahrain are diagnosed with DM. The percentage of patients with DPN is suspected to be around 50% of DM patients (Sima 2003). The estimated population of DPN patients in Bahrain is therefore approximately 104,428 patients.
The ultimate sample size for the case control analysis was 90 (30 in each group). The size of the sample in similar extant review studies ranged from a low 45 in each group (Doupis et al. 2009) to a high 227 (Herder et al. 2009). Detecting a true effect size in order to estimate the sample size required for this study was not possible since median and interquartile ranges were used in the previous studies, which indicates a non-normally distributed data. Cohen (1992) stated that when the outcomes distribution is skewed, it is not possible to estimate a standard deviation from an interquartile range.

The standard and sophisticated statistical analysis including structural equation modelling introduced by Cohen (1992) recommends a sample size of 783 participants to detect a small effect size \((r = 0.1)\), 85 participants to detect a medium effect size \((r = 0.3)\) and 28 participants to detect a large effect size \((r = 0.5)\) when the standard \(\alpha\)-level of 0.05 and a power of 0.8 are considered.

Taking into consideration the limited financial and time resources available for undertaking this research, a total sample of approximately 100 was believed to be convenient to answer the research question for this study which will be considered as a pilot/exploratory study leaving the door open for further research to take place in the future.

### 3.3.4. Sample assessment

The selected sample including cases and control (total 90 participants) were invited to attend the diabetic clinic between July 1\(^{st}\) and July 10\(^{th}\) of 2012 for clinical assessment and one to one discussion with the researcher. This involved detailed verbal explanation of the study contribution and course of action and the completion of the consent form. A maximum number of 10 participants were scheduled per day to ensure adequate time for each subject. The assessment included past and current medical history and medication. Participants were requested to report any issues that might influence the results of the quantitative blood investigations. A thorough
neuropathy test, described shortly, was then conducted on all subjects within the three groups to ensure the exclusion of any undiagnosed neurological manifestations. Peripheral neuropathy was assessed according to the guidelines of the San Antonio Consensus Statement and the American Diabetes Association statement on diabetic neuropathy (ADA 1988). The neuropathic symptoms were evaluated using a Neuropathy Symptom Score (NSS) based on the original system proposed by Dyck (Dyck et al. 1999a), and the clinical signs by using a Neuropathy Disability Score (NDS) as described elsewhere (Veves et al. 1994, Pham et al. 2000).

More specifically, for the evaluation of the NSS, the participants were asked about the following symptoms in their feet or legs: 1) pins and needles; 2) abnormal cold or hot sensations; 3) lancinating or stabbing pain; 4) deep aching pain; 5) burning pain; and 6) irritation of the feet or legs by the bedclothes at night (hyperesthesia). Each symptom was scored with one point if it was present and two points if nocturnal exacerbation was also present. A score of four or more points was considered to be abnormal (Pham et al. 2000). Neuropathy symptoms score (NSS) is a widely studied scoring symptom, and it is used extensively in clinical practice. It has proven to be a valid and sensitive system by many authors (Dyck et al. 1985; Dyck 1988).

The Neuropathy Disability Score (NDS) was used to quantify the severity of diabetic neuropathy on clinical examination. The NDS is the most widely used and widely accepted scoring system for diabetic neuropathy; it has also been recommended in consensus reports (Dyck et al. 1980; ADA 1988). The sensations of pain, touch, cold, and vibration were tested in both legs and were scored according to the level up to which the sensation was impaired.

Sensory tests included a pinprick with a NeuroTip™ (NTS405, manufactured by Owen Mumford Ltd) using a NeuroPen® (NT0100, Owen Mumford Ltd), light touch with a strip of cotton ball, and temperature perception with a Bailey’s Tip-Therm temperature sensitivity device (CH536, Bailey Instruments Ltd.). Pain sensation was assessed with both a sterile pinprick and a blunt tip over the bottom of each foot (Smieja et al. 1999). Patients were asked after each application of the pinprick or
blunt tip application whether the sensation witnessed was sharp, blunt, or absent. Any differing response was deemed as an abnormal response to the stimuli, thereby indicating the presence of damage to the nerve fibres (Hoitsma 2004).

The NeuroTip™ is a short-round tipped blunt needle mounted in a plastic body used to assess small nerve fibres for pain perception. The device can be used in two ways; momentarily pressed against the test site whilst held in the examiner’s hand, or pressed against the test site whilst positioned in the spring-loaded compartment of a pen-like device known as a NeuroPen®. When used simply in the examiner’s hand, the force at which the NeuroTip™ is pressed against the test site is unknown and very difficult to repeat consistently (Hoitsma 2004). However, when the NeuroTip™ is pressed against the test site in the spring-loaded compartment of a NeuroPen® it is relatively simple for the examiner to repeatedly exert a force of approximately 40g due to a spring mechanism. However, a study comparing the use of a Neurotip™ by hand and a NeuroPen® showed no significant difference in performance (Soundararajan and Russel 2007). Although their performance may not differ significantly, NeuroPen® does limit the force exerted via the sharp point of NeuroTip™, thus providing a critical safety barrier to ensure the skin is not damaged during examination which could possibly result in foot ulceration.

It is common for thermal perception to be one of the first nerve functions to deteriorate as a result of DPN (Spencer and Schaumburg 1976). The screening of both hot and cold thermal perception in a diabetic foot assesses the presence of small nerve fibre neuropathy in the A-delta and C-nerve fibres respectively. The device known as Tip-Therm is a popular thermal assessment tool, a pen-like instrument used to assess patients’ thermal perception by requesting them to differentiate between a cold and not cold sensation. The device has two flat sides, one comprising of a metal tip and the other being the plastic casing that holds the device together. Due to the skin transferring energy to the metal tip upon contact due to its high heat capacity, the patient should detect a cold sensation if thermal perception is still present. The plastic casing end does not absorb as much energy from contact with the skin and therefore feels warmer than the metal end.
Tip-Therm has identified that by assessing cold perception, the deterioration of cold sensation can identify a significant correlation to the onset of neuropathy, demonstrating 92.1% sensitivity and 98.3% specificity when compared to a monofilament (Viswanathan et al. 2002). However, in normal clinical use the device is very dependent on the environmental climate in which it is used as the temperature of the metal is not controlled, and therefore the results of screening can be dramatically affected (Viswanathan et al. 2002). From the review of the current thermal assessment devices, there seems to be a lack of assessment to heat perception, which alongside cold perception screening would provide a much more thorough assessment for small nerve fibre neuropathy (Viswanathan et al. 2002).

A score was given according to the anatomical location in which the patient could not identify the stimuli introduced. If the patient perceived the stimulus at all levels, then a score of 0 was given. A score of 1 was given if the patient failed to perceive the stimulus at the base of the toe, a score of 2 was given if the patient failed to perceive the sensory at the midfoot, a score of 3 was given if the patient failed to perceive the stimulus at the heel, a score of 4 was given if the patient failed to perceive the stimulus at the lower leg and a score of 5 was given if the patient failed to perceive the stimulus at the knee. The average score of both feet was entered as the sensory score. Reflexes were scored in every leg as normal (0), present with reinforcement (1), and absent (2). The total sum represented the reflex score. If the NDS was greater than 5 (maximum, 28) it was considered abnormal (WHO 1999). The summation of reflex and sensory scores for each modality was entered as the NDS. An NDS of ≥ 5 was indicative of the existence of neuropathy.

Semmes-Weinstein 5.07 SWF (CH537, Bailey Duraban Retractable Monogilament 10 gram of pressure, Bailey Instruments Ltd) monofilaments were tested on the plantar surface of the great toe and centrally at the heel. This method was standardized according to generally accepted guidelines (Birke and Sims 1986; Kumar et al. 1991; Mueller 1996). The “yes/no” method was used, which means that the patient says “yes” each time he or she senses the application of a monofilament.
Inability to feel a 5.07 SWF (10 g of pressure) was considered to be indicative of peripheral neuropathy (Mcneely et al. 1995).

A key issue with monofilaments during use is how easily they can be damaged. A damaged monofilament can significantly affect the accuracy and reliability of an assessment (Kumar et al. 1991). Intensive studies have shown that out of the four major monofilaments manufacturers only 2 produced a monofilament that exerted a linear force within 10% of the specified 10g. The study further highlighted that only 50% maintained less than 10% deviation after 200 compressions (Booth 2000). There is also currently no universally accepted guideline on how to use the device during assessments. Differencing opinions on the number of test sites and the number of tests per test site all contribute to the misuse of monofilaments during an assessment.

Vibration perception is often one of the first sensory modalities to deteriorate upon the onset of neuropathy (Malik 2005). There are a number of instruments available to measure vibration perception; all the available tools apply a vibration to the test site of the foot to observe whether or not the patient can feel it. A lack of sensation identifies the presence of large nerve fibre neuropathy. The vibration perception threshold (VPT) was measured at the great toe of the dominant side using a Neurothesiometer (NEU1, Manufactured under BS 5750 Part 2 QAS34/51 (equivalent to ISO 90002 and EN29009), Horwell Ltd).The mean values after three readings were recorded according to the methods of Bloom et al. (1984).

In a study by Meijer et al. (2000) the sensitivity and specificity of the abnormal monofilament tests at a cut off level of 3 to 4 were 0.96 and 0.51 for abnormal monofilament scores, respectively. For abnormal vibration perception threshold scores, these values were 0.97 and 0.59, respectively (Meijer et al. 2000). A systematic review by Dros et al. (2009) noted sensitivity of the monofilament test ranged from 0.41 to 0.93, and specificity ranged from 0.68 to 1.00. All studies showed methodological limitations that could have inflated sensitivity or specificity (Dros et al. 2009).
Peripheral neuropathy was confirmed when at least two of the three quantitative measurements (NSS, NDS and VPT) were abnormal. Combinations of more than one test have ≥ 87% sensitivity in detecting DPN (Boulton et al. 2005).

Peripheral arterial assessment was subsequently carried out on all participants to exclude vascular pathologies that may bias the outcomes. Foot pulses were recorded as either present or absent by clinical palpation. The absence of one or more pulses, the presence of claudication, and an ABI < 0.65, and/or a history of previous revascularization was regarded as diagnostic for peripheral arterial disease (PAD) (Pham et al. 2000). Information on the existence of a foot ulcer or history of foot ulceration was obtained from the patients. Patients showing any clinical signs and symptoms of PAD, and/or with any present open wounds or signs of inflammation or cellulites were excluded from the study.

3.4. Blood investigations for the final groups

The total number of participants selected were then invited to attend the blood collection laboratory on specified days between the 15th and 30th of July 2012 for blood sample collection. A total number of 10 participants were scheduled on the specified collection days to minimise the risk of delaying blood processing. All tests were conducted by an experienced phlebotomist, who was exclusively assigned to this task on the days selected. All clinical examinations and evaluations were conducted under fasting conditions. Blood samples were taken from subjects in all three groups to measure the following:

- Pro-inflammatory marker: TNF-α/IFN-γ/IL-1β/ IL-6/ IL-8 and CRP;
- Autoimmune antibodies: ANA screen.
The five cytokines (TNF-α/IFN-γ/IL-1β/ IL-6/ IL-8) were chosen due to their known pro-inflammatory actions, and links to autoimmune disease. TNF-α is produced by activated macrophages in response to microbes, especially the lipopolysaccharide of gram negative bacteria. It is an important mediator of acute inflammation (Lees et al. 2013). It mediates the recruitment of neutrophils and macrophages to sites of infection by stimulating endothelial cells to produce adhesion molecules and by producing chemokines which are chemotactic cytokines. TNF-α is a mediator of both natural and acquired immunity (Akdis et al. 2011). Local increasing concentrations of TNF-α cause heat, swelling, redness and pain, which may be evident in DPN.

Interferon-γ is an important cytokine produced primarily by Th1 cells. It has numerous functions in both the innate and adaptive immune systems (Akdis et al. 2011). IFN-γ is a key regulatory molecule in cellular immune responses and may also have pleiotropic effects on the nervous system. The role of IFN-γ in immunomodulation includes activation of expression of major histocompatibility complex antigens and effects on cell proliferation and differentiation (Robertson et al. 1997). It is not known whether or not effects of IFN-γ are related to direct receptor-mediated effects on populations of neurones. However, Meller et al. (1994) reported that administration of IFN-γ induced thermal hyperalgesia, and this may be related to induction of inducible NOS-2 in macrophage cells.

Interleukin-1 was originally discovered as a factor that induced fever, caused damage to joints and regulated bone marrow cells and lymphocytes (Wills-Karp and Finkelman, 2008). IL-1 plays an important role in both innate and adaptive immunity, and is a crucial mediator of the host inflammatory response in natural immunity (Akdis et al. 2011). The major cell source of IL-1 is the activated mononuclear phagocyte. Other sources include dendritic cells, epithelial cells, endothelial cells, B cells, astrocytes, fibroblasts and large granular lymphocytes. IL-1 induces fever as a result of bacterial and viral infections. It suppresses the appetite and induces muscle proteolysis, which may cause severe muscle “wasting” in patients with chronic infection or inflammation. IL-1β causes the destruction of β cells leading to type 1 diabetes mellitus (Akdis et al. 2011). It inhibits the function
and promotes the apoptosis of pancreatic cells. Autoimmune diseases exhibit increased IL-1 concentrations. It suppresses further IL-1 production via an increase in the synthesis of PGE2.

IL-6 is a multifunctional, pleiotropic cytokine involved in regulation of immune responses, acute-phase responses, haematopoiesis, and inflammation (Akdis et al. 2011). It is produced by endothelial cells, fibroblasts, monocytes, and macrophages in response to different stimuli (IL-1, IL-17, and TNF-α) during systemic inflammation. In innate immunity, IL-6 directs leukocyte trafficking and activation and induces production of acute-phase proteins by hepatocytes (Saito et al. 1992). IL-6 promotes T-cell proliferation, β-cell differentiation and survival, and plasma-cell production of IgG, IgA, and IgM (Gerhartz et al. 1996). IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts and other cells in response to trauma, burns, tissue damage, inflammation, IL-1 and, to a lesser extent, TNF-α. It is elevated in patients with retroviral infection, autoimmune diseases and certain types of benign or malignant tumours. It stimulates energy mobilization in the muscle and fatty tissue, resulting in an increase in body temperature. A study by Araki et al. (2013) suggested the potential neuroprotective effect of IL-6 therapy in DPN.

IL-8 was identified as a neutrophil-specific chemotactic factor and later classified as a member of the chemokine family (Akdis et al. 2011). IL-8 production has been observed in vitro in a wide variety of cells including monocytes, T lymphocytes, neutrophils, vascular endothelial cells, dermal fibroblasts, keratinocytes, hepatocytes, and human gastric cancer cells (Akdis et al. 2011). The major effector functions of IL-8 are activation of neutrophils to the site of infection or injury. Intravenous injection of IL-8 in rabbits caused rapid neutrophilia (Holck et al. 2003). Increased concentrations of IL-8 were found in inflammatory sites in patients with diseases, such as, psoriasis, arthritis, and autoimmune disease (Holck et al. 2003). However, definitive evident is lacking on whether IL-8 was essentially involved in these inflammatory reactions.
Blood collection was obtained through venipuncture, which is one of the most routinely performed invasive procedures used to obtain blood for diagnostic purposes (Lavery & Ingram 2005). It is important to note that the methods of sampling and storage of blood serum are critical for accurate measurements (Bowen et al. 2010). Each participant was asked to sit in an upright position with their chosen arm outstretched and supported by a pillow. A 21G (Greiner Bio-One, Brunel Way, Stroudwater Business Park, Stonehouse, GL10 3SX) hypodermic needle was inserted into a suitable vein in the antecubital fossa. Blood (15 ml) was withdrawn into an EDTA vacuette (Greiner Bio-One) and 5ml of blood withdrawn into each of the serum clot activator vacuettes (Greiner Bio-One). This was conducted in accordance with the blood collection guidelines adapted at the RMS-BDF Hospital.

Serum collection tubes containing clot activators and gel for serum separations were used for each subject. A total number of 3 tubes were collected (5.0 ml BD Vacutainer SST™ 11 advance tube with clot activator and gel). All gel barrier and additive tubes were mixed by gentle inversion 5 to 10 times immediately after the blood was drawn. This assists in the clotting process, and assures homogeneous mixing of the additives with the blood (Augello et al. 2004). Tubes were filled to the marked level to achieve the right proportion. Otherwise, the anticoagulation will be high and falsify the results (Bowen et al. 2010). Special silicon gels are used in these tubes to achieve a more accurate and faster separation of serum (Bowen et al. 2010). These gels exhibit a special density that is intermediate between the density of cells and the serum. The semi fluid gel changes its stiffness during centrifugation. The cell-clot accumulates at the bottom while the serum stays at the top of the tube. Serum separator tubes were left to clot for a full 30 minutes in a vertical position prior to centrifugation. Short clotting times can result in fibrin formation, which may interfere with complete gel barrier formation (Bowen et al. 2010).

The tubes collected were centrifuged within one hour of blood collection for a spin time of 15 minutes at 1000 × g to separate cellular components and serum. The best temperature for centrifugation is at 20-25°C as cooling may impair the flow characteristics while high temperatures may cause a breakdown of the gel (Bowen et
al. 2010). Following centrifugation, the serum was transferred from each tube to seven transport containers using a transfer pipette. All containers were marked with the subject’s given number and placed in a storage box. The marked containers for CRP and ANA analysis were transferred directly to the RMS-BDF laboratory for immediate processing as explained shortly. The five other containers for each subject were kept in the storage boxes for cytokines analysis at a later stage. Storage boxes containing the mini transport containers were then cryopreserved at -80°C.

3.5. Measurements of immune mediators

3.5.1. C-reactive protein analysis

Immunological variables were measured from serum sample described earlier. For CRP analysis, serum concentrations of C-reactive protein were tested immediately following blood collection and serum preparation at the RMS-BDF laboratory by a CRP (Latex) High Sensitive Immunoturbidimetric assay on COBAS integra 700 analyzer (details described in Appendix 7).

The Immunoturbidimetric methodology used in this assay is well-established and is the basis for the already cleared Integra CRP assay (K981897). During the reaction, anti-CRP antibodies coupled with latex microparticles react with CRP in the sample to form an antigen-antibody agglutinate, which is measured turbidimetrically. The calibrator is the Calibrator for automated systems (C.f.a.s). Proteins; and the recommended control materials are CRP T Control N and Precinorm Protein. The reagents are for use on the Integra 400, 400 plus, 700 and 800 analyzers.

This method has been standardised against the reference preparation of IRMM (Institute for Reference Materials and Measurements) and RPPHS (Reference preparation for proteins in human serum) (Baudner et al., 1993), and is currently being conducted for CRP analysis for patients attending the RMS-BDF Hospital.
The CRP (Latex) High Sensitive Immunoturbidimetric assay is for the in vitro quantitative determination of C-reactive protein (CRP) in human serum and plasma on Roche automated clinical chemistry analysers, with a functional sensitivity of 0.3 mg/L. Measurement of CRP is of use for the detection and evaluation of inflammatory disorders and associated diseases, infection and tissue injury. Highly sensitive measurement of CRP may also be used as an aid in the assessment of the risk of future coronary heart disease (Cook et al. 2006). When used as an adjunct to other laboratory evaluation methods of acute coronary syndromes, it may also be an additional independent indicator of recurrent event prognosis in patients with stable coronary disease or acute coronary syndrome (Cook et al. 2006).

The conventional method for CRP measurement is by an immunoturbidimetric assay (imCRP). This method is available in routine testing laboratories and is suitable for measurement of CRP concentrations during infection. However, it is regarded as a relatively insensitive method for determination of changes in concentrations within the normal range (<10 mg/l) (Ledue and Rifai 2003). Therefore, hsCRP assays have been developed which can measure CRP at concentrations as low as 0.1 mg/l. Measurement of hsCRP can demonstrate subclinical inflammatory states, which may reflect inflammation (Wilson et al. 2006).

3.5.2. Pro-inflammatory Cytokines analysis

The pro-inflammatory cytokines were analysed from the sample blood serum stored at -80°C. The cytokines TNF-α, IFN-ɤ, Interleukin IL-1β, Interleukin IL-6, and Interleukin IL-8 were measured at Princess Al-Jawhara Research Centre laboratory using Quantikine ELISA kits from R&D Systems (Weisbaden, Germany). This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for the markers mentioned above has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any marker present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for the markers is added
to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is then added to the wells and colour develops in proportion to the amount of marker bound in the initial step. The colour development is stopped and the intensity of the colour is measured. For calculating the results, the readings for each standard, control, and sample was subtracted by the average zero standard optical density as described in the kit manual. A standard curve was then created by reducing the data using computer software capable of generating a four parametric logistic (4-PL) curve fit.

Enzyme-Linked ImmunoSorbent Assay (ELISA) was introduced in the 1970s (Crowther 2001). In the typical double antibody sandwich ELISA, antibody attached to the bottom of a well provides both antigen capture and immune specificity, while another antibody linked to an enzyme provides detection and an amplification factor. This approach enables accurate and sensitive detection of the antigen, the cytokine of interest. Because of these desirable features, ELISA has been considered the standard cytokine measurement method and is widely utilized in clinical laboratories and biomedical research. Additional advantages of ELISA include the fact that results are highly quantitative and generally reproducible (Leng et al. 2008). ELISAs offer excellent specificity and, once fully optimized, sensitivity that rivals that of bioassays (Stefura et al. 2008).

### 3.5.2.1. R&D Systems Human Quantikine® ELISA TNF-α

The Quantikine Human TNF-α Immunoassay is a 3.5 or 4.5 hour solid phase ELISA designed to measure human TNF-α in cell culture supernates, serum and plasma. It contains E. coli-derived recombinant human TNF-α and antibodies raised against this protein. It has been shown to accurately quantitate the recombinant factor. Results obtained with naturally occurring TNF-α samples showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards (Appendix 8). These results indicate that this kit can be used to determine relative mass values for natural human TNF-α. Sensitivity, determined by assaying multiple replicates of
the zero standards is 5.5pg/mL. Less the 0.5% cross-reactivity with available related molecules is observed with this ELISA kit (Stefura et al. 2008). Intra-assay precision (precision within an assay) and inter-assay precision (precision between assays) are presented in Appendix 8.

3.5.2.2. R&D Systems Human Quantikine® ELISA IFN-ɤ

The Quantikine Human IFN-ɤ Immunoassay is a 4.5 hour solid phase ELISA designed to measure IFN-ɤ levels in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human IFN-ɤ and antibodies raised against the recombinant factor. Results obtained for naturally occurring human IFN-ɤ samples showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IFN-ɤ. Sensitivity, determined by assaying multiple replicates of the zero standards is 8pg/mL. Less the 0.5% cross-reactivity with available related molecules is observed with this ELISA kit (Stefura et al. 2008). Intra-assay precision (precision within an assay) and inter-assay precision (precision between assays) are presented in Appendix 8.

3.5.2.3. R&D Systems Human Quantikine® ELISA IL-6

The Quantikine Human IL-6 Immunoassay is a 4.5 hour solid phase ELISA designed to measure IL-6 in cell culture supernates, serum, and plasma. It contains recombinant human IL-6 and antibodies raised against recombinant human IL-6 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural IL-6 showed linear curves that were parallel to the standard curves obtained using the *E. coli*-expressed Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IL-6. Sensitivity, determined by assaying multiple replicates of the zero standards is 0.7pg/mL. Less the 0.5% cross-reactivity with available related molecules is observed with this ELISA kit (Stefura et al. 2008). Intra-assay precision (precision
within an assay) and inter-assay precision (precision between assays) are presented in Appendix 8.

3.5.2.4. R&D Systems Human Quantikine® ELISA IL-8

The Quantikine Human IL-8 Immunoassay is a 3.5 hour solid phase ELISA designed to measure human IL-8 in cell culture supernates, serum, and plasma. It is based on antibodies raised against the 72 aa variant of human IL-8 derived from *E. coli*. It is calibrated with the same recombinant factor. This immunoassay accurately quantitates recombinant human IL-8. Measurement of natural human IL-8 or the 77 aa variant of human IL-8 gave results parallel to the standard curves obtained using the *E. coli*-expressed Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IL-8. Sensitivity, determined by assaying multiple replicates of the zero standards is 7.5pg/mL. Less than 0.5% cross-reactivity with available related molecules is observed with this ELISA kit (Stefura et al. 2008). Intra-assay precision (precision within an assay) and inter-assay precision (precision between assays) are presented in Appendix 8.

3.5.2.5. R&D Systems Human Quantikine® ELISA IL-1β

The Quantikine Human IL-1β Immunoassay is a 3.5 or 4.5 hour solid phase ELISA designed to measure IL-1β in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant human IL-1β and antibodies raised against the recombinant factor. It has been shown to quantitate recombinant human IL-1β accurately. Results obtained using natural IL-1β showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural IL-1β. Reports indicate that this and other ELISA kits calibrated using mature IL-1β as a standard will detect, but considerably underestimate, the unprocessed IL-1β precursor present in samples. In biological samples other than cell lysates, the precursor form of IL-1β is usually not the predominant form of IL-1β present and,
additionally, is not biologically active. Therefore, results obtained using this kit should provide a useful measure of the levels of active IL-1β present in biological fluids. Sensitivity, determined by assaying multiple replicates of the zero standards is 1pg/mL. Less the 0.5% cross-reactivity with available related molecules is observed with this ELISA kit (Stefura et al. 2008). Intra-assay precision (precision within an assay) and inter-assay precision (precision between assays) are presented in Appendix 8.

3.6. Serology measurements

Anti-nuclear antibody was detected at the RMS-BDF laboratory using the Varelisa® ReCombi ANA screen, manufactured by Pharmacia & Upjohn Diagnostics GmbH Co. KG, which is a qualitative method designed for the determination of eight antinuclear antibodies in human serum. The Varelisa® Recombi ANA screen detects anti-bodies against dsDNA, RNP, Sm, SS-A/Ro, SS-B/La, Scl-70, Centromere and Jo-1 in a single microwell.

In the Varelisa® Recombi ANA screen the wells of a microwell are coated with human recombinant and native purified nuclear antigens and dsDNA. Antibodies, specific for the nuclear antigens present in a patient sample bind to these nuclear antigens. Once ANA test is positive the ANA profile can then be used using the Varelisa® Recombi ANA Profile to individually detect anti-bodies mentioned above. This, however, was considered beyond the objectives of this study.

Varelisa® ReCombi ANA Screen is a well-established and reliable system which was initially introduced in 1994 (Pharmacia Diagnostica 2010), and was cited in many independent publications since. The use of recombinantly produced human antigens of very high purity ensures the Varelisa® ReCombi ANA Screen has an outstanding specificity decreasing the amount of false positive results (Phadia 2010). The higher antigen concentration and the use of human recombinant antigens guarantee a
noticeably higher sensitivity of the Varelisa® in comparison with the Hep-2 cell Immunoflurescence test. High positive productive values of the Varelisa® ReCombi ANA Screen were also proved in research (Phadia 2010).

3.7. Reliability and validity of data collection tools

Reliability and validity of a research study helps in providing its authenticity; it also helps in understanding the accuracy of data collected and analysis done (Carlson and Morrison 2008). The reliability and validity of the collected data of the research is largely dependent on the research instrument used for the purpose of answering the hypothesis (Bhattacherjee 2012). This includes study design, sample used, and instrumentation used for data collection and analysis.

The investigation and refinement of the case-control design used in this study, a process which began in about the middle of the 20th century and described by Cornfield (1951) and Mantel and Haenszel (1959) constitutes a significant innovation in population-based research (Schoenbach 1999). By comparison with other study types, case-control studies can yield important findings in a relatively short time, and with relatively little money and effort. However, case-control studies tend to be more susceptible to biases than other analytical, epidemiological designs (Schulz and Grimes 2002) since choosing a control group and obtaining exposure history can greatly affect a study’s vulnerability to bias (Kelsey et al. 1996). Nevertheless, ensuring a greater balance between the numbers of cases and non-cases (control), the case-control design generally offers much greater statistical efficiency than other designs, giving it a crucial advantage for studying rare diseases (Schoenbach 1999).

Random variations that could affect the study’s precision were minimised with precise sample selection in this study (cases and control) as described earlier. The use of reliable methods for measuring variables (population demographics, sample
assessment, blood collection and analysis of immunological and serological markers) also improved precision. Potential confounding variables which might compromise the internal validity of the study results were controlled by gathering the demographic variables and neuropathy symptom variables in order to investigate the correlations between confounding variables and study’s primary results. Internal validity was also improved by including two control groups in order to minimise the risk of results being influenced by other possible causes. The rationale behind including a group of subjects who were diagnosed with only diabetes mellitus without signs of neuropathy was to exclude the possibility of marker levels being linked to DM itself rather than the neuropathy. External validity of the study however raises questions from the aspect of generalisation of study results to a more universal population. The most common loss of external validity in observational research comes from the fact that studies often employ small samples obtained from a single geographic location or facility. Because of this, one cannot confirm that the conclusions drawn about cause-effect relationships apply to people in other geographic locations or at other facilities.

As mentioned earlier, using multiple geographical locations and a large sample size was unfortunately unattainable for this particular research due to the limited time frame and budget available. The preeminent way to improve the external validity of the research is therefore to replicate this methodology in different populations, places, and time periods at a later stage, and to consider this research as a pilot which will set the foundation that supports the commencement of larger studies in the near future.

3.8. Analysis of primary and secondary data

Data entry and statistical analysis was performed using Statistical Package for Social Sciences (SPSS), officially named “IBM® SPSS®”, for windows (version 18.0) also called PASW statistics (18.0). SPSS is one of the most used programmes for
statistical analysis in social science (Levesque 2007). SPSS Statistics leverages a powerful set of statistical capabilities, enabling researchers to produce valuable information from the data provided. Complex analysis allows discovering of information that improves decision-making, research outcomes and ultimately expanding practice (Levesque 2007).

Expert statistical advice was consulted from the biostatistician at Queen Margaret University in Edinburgh. Independent and dependent variables were all entered into the SPSS data editor in preparation for analysis. Unless stated otherwise, significance of test results is usually reported in the three ways suggested by Coolican (1990), based on $p$ the probability level:

- 'significant': $0.05 > p < 0.01$;
- 'highly significant': $0.01 > p < 0.001$; and
- 'very highly significant': $0.001 > p$.

In this study the statistical analysis $p$ value was accepted at 0.05; this value is indicative that there is less than 5% chance of achieving results by chance assuming that the null hypothesis is true. It appears that 5% is an arbitrary significance level that is used to avoid Type I errors (assuming an effect where there is not one), but equally not too low to exclude too many Type II errors (assuming there is not an effect where there is one) (Field 2009).

The analysis of primary data was performed by multivariate techniques and modelling the data through descriptive statistics using frequency distributions, box-plots, means and standard deviations (described in details in Chapter four).

The data analysis proceeded with the examination of data entry. This was significantly relevant to gain some critical insights into the data characteristics and analysis (Hair et al. 1998). Prior to running the inferential analysis for the independent and dependent variables, the normality distribution per variable was explored for each group separately. Normality in the data is often a conventional
assumption in the estimation process (Bai and Ng 2005). Outliers may affect the normality of the data. As Tabachnick and Fidell (2001) argue, outlier is a sample with such an extreme value on one variable (a univariate outlier) or such a strange combination of scores on two or more variables (multivariate outlier) that they distort statistics. Therefore an attempt was made to assess the normality of the data in order to explore whether the data were parametric or non-parametric for the statistical tests using frequency distributions, box-plots, histograms, and Kolmogorov-Smirnov and Shapiro-Wilk quantitative normality tests (Appendix 9).

Most of the data were not parametric therefore data did not fit a normal distribution curve. If results were parametric, it can be argued that results may have been more robust, since parametric methods make more assumptions than non-parametric methods, such as normality (Corder and Foreman 2009). However, the non-parametric data obtained in this research do not rely on assumptions that the data are drawn from a given probability distribution. The non-parametric test of homogeneity of variance (Modified levene’s test for non-parametric distribution) was subsequently conducted. The assumption of homogeneity of variances is essential when comparing groups, because if variances are unequal, the validity of the results are jeopardized (i.e., increased Type I error rates leading to invalid inferences) (Glass et al. 1972). To further investigate the homogeneity between the demographic variables, a non-parametric Kruskal-Wallis test was conducted between the three groups for each demographic variable.

Quantitative testing for hypothesis one (rejecting null hypothesis) was achieved using Kruskal-Wallis, a non-parametric test of whether more than two groups differ followed by the Mann-Whitney U test, a non-parametric test that looks for differences between two independent groups.

The Kruskal-Wallis test is the non-parametric counterpart to the one-way independent ANOVA designed to compare variables by ranks between more than two independent groups when the data is not normally distributed (Field 2009). A \( p < 0.05 \) implies that a significant difference exists between at least two of the
samples. Therefore, a sample contrast between individual sample pairs, or post hoc tests, are recommended to determine which of the sample pairs are significantly different (Meyer and Seaman, 2006).

Mann-Whitney U test, a non-parametric test of null hypothesis comparing two independent groups, was used to follow up this finding. The efficiency of the Mann-Whitney U test is about 0.95 when compared to the $t$ test in non-normally distributed samples (Lehamnn 1999). Given that it is being conducted as a post hoc test to the Kruskal-Wallis test, a Bonferroni correction was applied and all effects were reported at a $p < 0.0167$ level of significance.

Chi-square or Fisher’s Exact tests were used to compare the presence of autoimmune antibodies in the three groups by testing the relationship between the percentages of positive and negative values (categorical variables) in the blood serum (testing hypothesis two). Chi-square test was used when the expected frequencies in each cell were greater than 5 (not more than 20% of the cells have expected frequency less than 5). Chi-Square test was also used to demonstrate the relationship in the percentages of positive and negative ANAs between group 1 and group 2 and between group 1 and group 3.

Spearman’s correlation coefficient (a non-parametric test for non-normally distributed data) was used to explore the possible correlation between the quantitative values of the inflammatory markers and the demographic variables for each group separately. Associations between serum concentrations of immunological variables and measures of neuropathy were also analyzed using Spearman’s correlation coefficient. Binary logistic regression was used to explore the correlation between the presence of ANA and the demographical variables and clinical manifestations of peripheral neuropathy in the total sample. The relationship between the demographic variables and the neurological manifestations within the three groups were also explored using Spearman’s correlation coefficient. Scatter plots were used to illustrate graphical correlations between the variables tested.
To further analyze the results, multiple linear regressions were conducted to explore whether neuropathy was independently associated with an increase in the level of inflammatory markers and the presence of antibodies.
Chapter Four: Results and Findings of study

The present study as described in Chapter three was divided into two main sections; section one (Primary data analysis) and section two (Secondary data analysis). Primary data analysis was further divided into two sections; cross-sectional analysis and a case control analysis.

The cross-sectional analysis studied the demographics and the presence of aspects such as neuropathy and peripheral vascular disease within the population of patients with diabetes mellitus in the RMS-BDF Hospital. The case-control analysis investigated the differences in the levels of pro-inflammatory markers and autoimmune antibodies between patients with diabetic peripheral neuropathy, diabetic controls and healthy controls (hypothesis one and hypothesis two testing). Section two (secondary analysis) further authenticated section one by exploring the relationship between pro-inflammatory markers and autoimmune markers and the sample demographics and clinical characteristics.

The goals of this chapter are to:

- Describe the population from which the sample has been selected, and explore the demographics and clinical characteristics of patients with DM attending the RMS-BDF Hospital;
- Present the primary research findings, which are set to test the research hypotheses;
- Present the secondary research findings, which are set to test the correlations between different variables.
4.1. Primary data analysis

4.1.1. Part 1: Cross-sectional study - Population demographics and clinical characteristics

The aim of the cross-sectional analysis was to gain an overall understanding of the population of patients with DM attending the RMS-BDF Hospital, and the percentages of DM complications among Bahrainis. With regards to demographics, all the patients randomly selected were Bahraini natives diagnosed with either type I or type II DM. From the 500 record cards used 48% (n=242) were male, and 52% (n=258) were female. Descriptive statistics were conducted to explore the demographic variables. Means and standard deviations were computed for quantitative variables (Table 4.1.1a), while frequencies and percentages were computed for categorical variables (Table 4.1.1b).

| Table 4.1.1a: Descriptive statistics on the demographics of patients with DM in the RMD-BDF Hospital (Age, BMI, systolic and diastolic BP, RBG and duration of DM) n=500 (SD: Standard deviation) |
|------------------------|--------|------|
| Age (years)            | 55     | 14   |
| BMI (kg/m²)            | 35     | 9    |
| Systolic BP Last Visit (mmHg)* | 147 | 21   |
| Diastolic BP Last Visit (mmHg)* | 73  | 15   |
| RBG (mmol/l)*          | 10     | 4    |
| Duration (years)       | 8      | 7    |

*Random Blood glucose taken from non-fasting sample (normal range in adults is between 4.4 and 7.8 mmol/l)

*Normal blood pressure for an adult age 20 or over should be less than 120 systolic and less than 80 diastolic. A value of less than 140 systolic and 90 diastolic is considered normal in people over 60 years (AHA 2012)
**Table 4.1.1b: Descriptive statistics on the demographics of patients with DM in the RMS-BDF Hospital (Gender, type of DM and OMC) n=500**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>242</td>
<td>48%</td>
</tr>
<tr>
<td>Female</td>
<td>258</td>
<td>52%</td>
</tr>
<tr>
<td><strong>Type of DM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>38</td>
<td>8%</td>
</tr>
<tr>
<td>Type II</td>
<td>462</td>
<td>92%</td>
</tr>
<tr>
<td><strong>Other Medical Conditions (OMC)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM with no other conditions</td>
<td>169</td>
<td>24%</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>186</td>
<td>26%</td>
</tr>
<tr>
<td>Vascular insufficiency</td>
<td>141</td>
<td>20%</td>
</tr>
<tr>
<td>Retinopathy</td>
<td>115</td>
<td>16%</td>
</tr>
<tr>
<td>Kidney problems</td>
<td>61</td>
<td>9%</td>
</tr>
<tr>
<td>Cardiac problems</td>
<td>45</td>
<td>6%</td>
</tr>
</tbody>
</table>
4.1.1.1. **Age**

Table 4.1.1a presents the demographic features of the population studied (n=500). The mean age of the total population was 55 years with a standard deviation of ±14 years. The histogram in Figure 4.1.1.1 shows the distribution of patient’s age for the 500 record cards. The X axis represents the age in years, while the Y axis represents the frequency distribution of the DM population according to the patient’s age. The distribution is symmetrical with a positive kurtosis suggesting that the highest numbers of patients were close to the mean age of the population.

![Histogram presenting age distribution of DM population at the RMS-BDF Hospital (Mean age = 55 years, Standard deviation = ±14 years)](image)

Figure 4.1.1.1: Histogram presenting age distribution of DM population at the RMS-BDF Hospital (Mean age = 55 years, Standard deviation = ±14 years)
4.1.1.2. Body Mass Index

The mean Body Mass Index (BMI) of the total population was 35 kg/m² with a standard deviation of ± 9 kg/m² (Table 4.1.1a). BMI values were transferred into ordinal data in accordance to the categories based on the US WHO (2004) standards: <18.5 underweight, 18.5-25.0 normal weight, 25.0-30.0 overweight, and > 30.0 obese.

Figure 4.1.1.2 demonstrates the differences in the percentages of DM population according to their BMI classification. The X axis represents the classifications of BMI according to the WHO (2013), while the Y axis demonstrates the percentages of patients within each category. Interestingly, 64.8% (n=324) of the population was classified as obese (BMI> 30), 22.0% (n=110) were overweight, 13.0% (n=65) were within the normal BMI range, and 0.2% (n=1) was underweight.

![Simple bar chart demonstrating the differences between DM population according to the WHO (2013) BMI classifications (<18.5 underweight, 18.5-25.0 normal weight, 25.0-30.0 overweight, and > 30.0 obese (n=500).](image)
4.1.1.3. Duration of DM

The mean known duration of DM was 8 years with a standard deviation of 7 years (Table 4.1.1a). Interestingly, the majority of patients within the 500 sample reported that they have been diagnosed with DM within the last 10 years.

Figure 4.1.1.3 displays a histogram, where the number of years representing the duration of DM since diagnosis is presented on the X axis, and the frequency distribution is presented on the Y axis. The positive skewness suggests that most of the DM patients attending the diabetic clinic at the RMS-BDF Hospital have been diagnosed with DM within the last 10 years.

Figure 4.1.1.3: Histogram presenting the distribution of DM duration (years) between the DM population at the RMS-BDF Hospital
4.1.1.4. **Random Blood Glucose**

Random Blood Glucose (RBG) displayed a mean of 10 mmol/L with a standard deviation of 4 mmol/L (Table 4.1.1a), which is above the normal range (RBG normal values are between 4-7 mmol/L according to the ADA guidelines (2013)).

4.1.1.5. **Systolic and diastolic BP**

The mean systolic blood pressure within this population was 147 mmHg with a standard deviation of 21 mmHg, and the mean diastolic blood pressure reading was 73 mmHg with a standard deviation of 15 mmHg (Table 4.1.1a). The mean of the systolic BP displayed a value slightly higher than the normal values of systolic BP according to the American Heart Association (2012). The mean of diastolic BP, however, was within the normal range (AHA, 2012).

4.1.1.6. **Type of DM**

The clinical characteristics of the patients with DM attending the diabetic clinic presented a noticeable difference in the percentages of type I and type II DM (Table 4.1.1b). From the 500 record cards used, type I DM was only present in 8% (n=38) of the total population, while type II DM was present in 92% (n=462) of the total population.

Figure 4.1.1.6 describes the differences in percentages of type I and type II DM in both male and female patients. The X axis represents the gender (male, female), and the Y axis demonstrates the percentages of male and female patients diagnosed with type I and type II DM. Figure 4.1.1.6 suggests an obvious increase in the percentages of patients diagnosed with type II DM in both genders in comparison to patients diagnosed with type I DM. The percentages of female patients diagnosed
with type II DM was 47% (n=235) in comparison to 5% (n=23) female patients with type I DM, and 45% (n=227) male patients diagnosed with type II DM in comparison to, 3% (n=15) male patients with type I DM. No obvious gender differences in relation to type of DM were noted.

Figure 4.1.6: Bar chart demonstrating the differences in the percentages of male and female patients diagnosed with type I and type II DM in a the population of 500 patients with DM.

4.1.1.7. Percentages of DM complications

From the 500 sample, the prevalence of DM complications within the population of DM patients attending the RMS-BDF hospital was studied by calculating the percentages of the patients presenting with the most common DM complications
(DM patients without any DM complications, diabetic peripheral neuropathy (DPN), peripheral vascular disease (PVD), retinopathy, nephropathy, and cardiac complications (Table 4.1.1b).

Figure 4.1.1.7 represents a bar chart demonstrating the percentages of DM with associated DM complications. The X axis of the graph resembles the six categories mentioned earlier, while the Y axis represents the percentages of patients within each category. Out of the 500 record cards screened, 169 had no complications associated with DM (24%) (Figure 4.1.1.7). More than half of the sample demonstrated different complications associated with DM, the highest being peripheral neuropathy at 26% (n=186) followed by vascular insufficiency at 20% (n=141), and the lowest being cardiac complications associated with DM at 6% (n=45). This further confirms the importance of investigating the pathogenesis of DPN being it one of the highest complications of DM in the population of patients with DM in Bahrain.

Figure 4.1.1.7: Bar chart demonstrating the differences between the percentages of medical complications associated with diabetes mellitus
4.1.2. Part two- Case control study

4.1.2.1. Examination of data entry, assessment of normality, and homogeneity of variance

4.1.2.1.1. Examination of data entry and assessment of normality and outliers

As a first check, all entries were verified case by case, and at second check descriptive statistics including frequency distribution, mean and standard deviation were conducted and verified. The frequency distribution yielded no mistakes in the data entry process and ensured the accuracy of data entry and the absence of missing data.

The Shapiro-Wilk test (Table. 4.1.2.1.1a, Table 4.1.2.1.1b) compares the scores in the sample to a normally distributed set of scores with the same mean and standard deviation. The Shapiro-Wilk normality test does the same as the Kolmogorov-Smirnov test, however it has more power to detect differences from normality, hence, it is often being used with a sample of less than 50 in each group tested (Kirkpatrick & Feeney, 2010). Shapiro-Wilk test for all the dependant and independant variables presented significant values ($p<0.05$) suggesting that the distribution of the sample is not normal Table (4.1.2.1.1a, Table 4.1.2.1.1b).
Table 4.1.2.1a: Shapiro-Wilk Tests for Normality for the dependant variables, *means $p<0.05$, df: degree of freedom

<table>
<thead>
<tr>
<th>Group</th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-ɤ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1Beta (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistic</td>
<td>Df</td>
<td>P-value</td>
<td>Statistic</td>
<td>Df</td>
<td>P-value</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.764</td>
<td>30</td>
<td>0.000*</td>
<td>0.808</td>
<td>30</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.515</td>
<td>30</td>
<td>0.000*</td>
<td>0.865</td>
<td>30</td>
<td>0.001*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.466</td>
<td>30</td>
<td>0.000*</td>
<td>0.732</td>
<td>30</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.938</td>
<td>30</td>
<td>0.078</td>
<td>0.933</td>
<td>30</td>
<td>0.059</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.933</td>
<td>30</td>
<td>0.059</td>
<td>0.803</td>
<td>30</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.803</td>
<td>30</td>
<td>0.000*</td>
<td>0.803</td>
<td>30</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 1</td>
<td>0.938</td>
<td>30</td>
<td>0.078</td>
<td>0.933</td>
<td>30</td>
<td>0.059</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.933</td>
<td>30</td>
<td>0.059</td>
<td>0.803</td>
<td>30</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.803</td>
<td>30</td>
<td>0.000*</td>
<td>0.803</td>
<td>30</td>
<td>0.000*</td>
</tr>
</tbody>
</table>
Quantitative tests were used in conjunction with histograms, box-plots and P-P in order to make an informed decision regarding the extent of non-normality (Appendix 9). All the descriptive and quantitative tests for normality described in appendix 9 demonstrate that the majority of the variables in each group of the sample studied deviated from a normal distribution. Histograms presented positively skewed distribution in all the variables suggesting larger concentration were at lower end of scores. Box-plots were used to detect outliers which might influence the inferential tests described shortly. Nonparametric tests were therefore conducted for all the variables given since most of the variables are clearly not normally distributed ($p<0.05$) (Table 4.1.2.1.1a, Table 4.1.2.1.1b).
4.1.2.1.2. Test of homogeneity of variance

The modified Levene’s test described by Nordstokke et al. (2011) was used instead of the traditional Levene’s test due to the fact that it is considered to be more powerful for nonparametric data and reduces the likelihood of Type 1 and Type 2 errors (Nordstokke et al. 2011). According to the normality tests described in appendix 10, all the dependent variables deviated from a normal distribution; hence a non-parametric Levene’s test was used (Table 4.1.2.1.2).

Table 4.1.2.1.2: Non-parametric Levene’s Test for homogeneity of variances between the group characteristics, *means $p<0.05$, df: degrees of freedom

<table>
<thead>
<tr>
<th></th>
<th>Levene Statistic</th>
<th>df1</th>
<th>df2</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>age_difference_rank</td>
<td>.378</td>
<td>2</td>
<td>87</td>
<td>0.686</td>
</tr>
<tr>
<td>BMI_difference_Rank</td>
<td>4.281</td>
<td>2</td>
<td>87</td>
<td>0.017*</td>
</tr>
<tr>
<td>Syst_difference_rank</td>
<td>1.490</td>
<td>2</td>
<td>87</td>
<td>0.231</td>
</tr>
<tr>
<td>dia_difference_rank</td>
<td>1.149</td>
<td>2</td>
<td>87</td>
<td>0.322</td>
</tr>
<tr>
<td>RBG_difference_rank</td>
<td>8.545</td>
<td>2</td>
<td>87</td>
<td>0.000*</td>
</tr>
<tr>
<td>duration_difference_rank</td>
<td>1.855</td>
<td>1</td>
<td>58</td>
<td>0.178</td>
</tr>
</tbody>
</table>

Non-significant results were noted for the age, systolic BP, diastolic BP, and duration of DM ($p>0.05$) ($F(2, 87) = 0.378$, $F(2, 87) = 1.490$, $F(2, 87) = 1.149$, $F(2, 87) = 1.855$ respectively), indicating that the variance is not significantly different and the homogeneity of variance is tenable. However, for the BMI and RBG the homogeneity of variance assumption has been violated since the levene’s test results were significant ($F(2, 87) = 4.281$, $F(2, 87) = 8.545$, $p<0.05$ respectively) (table 4.1.2.1.2).
4.1.2.2. Describing the sample studied (Sample characteristics)

As described in Chapter three, the research sample included 90 individuals (all Bahraini natives); 60 patients with diabetes mellitus randomly selected from the 500 sample population described earlier, and 30 healthy volunteers recruited from hospital staff population. The comparison between the characteristics of the sample selected (Group 1, Group 2, and Group 3) were demonstrated by multivariate descriptive statistics using frequencies and percentages for categorical variables (Table 4.1.2.2a), and median and interquartile ranges for quantitative variables (Table 4.1.2.2b).

The percentages of male to female ratio in all the three groups studied were relatively similar (57:43, 53:47 and 47:53, respectively), with the number of females being slightly higher than number of males in the control group (53% (n=16) females: 47% (n=14) males) (Table 4.1.2.2a).

The number of patients with type 1 DM was higher in the neuropathy group (group 1) when compared to the non neuropathy group (group 2) (43% (n=13) in group 1, 23% (n=7) in group 2). Both groups however demonstrated higher percentages in type II DM when compared to the percentages of type I DM (57% (n= 17) in group 1, 77% (n=23) in group 2) (Table 4.1.2.2a).

Table 4.1.2.2a: Descriptive statistics displaying numbers (N) and frequencies (%) of demographics and clinical characteristics (Gender and type of DM) of groups 1, 2 and 3 (Group 1: Dm neuropathy, Group 2: DM control, Group 3: health control)

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>57%</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>43%</td>
<td>14</td>
</tr>
<tr>
<td>Type of DM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>13</td>
<td>43%</td>
<td>7</td>
</tr>
<tr>
<td>Type II</td>
<td>17</td>
<td>57%</td>
<td>23</td>
</tr>
</tbody>
</table>
Table 4.1.2.2: Descriptive statistics displaying median and interquartile ranges of demographics and clinical characteristics (Age, BMI, systolic and diastolic BP, RBG and duration of DM) for groups 1, 2 and 3. (Group 1: DM neuropathy, Group 2: DM control, Group 3: healthy control)

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>16</td>
<td>47</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>30</td>
<td>11</td>
<td>27</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>144</td>
<td>28</td>
<td>133</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>80</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td><strong>RBG (mmol/l)</strong></td>
<td>13</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td><strong>Duration (years)</strong></td>
<td>17</td>
<td>9</td>
<td>6</td>
</tr>
</tbody>
</table>

4.1.2.2.1. Age

The median age of the DM patients with neuropathy (group 1) and the DM patients with no neuropathy (group 2) was similar although slightly higher than the third group (healthy control) (49 (IQR= 42-57), 47 (IQR= 30-47), 37 (IQR= 32-43) years; respectively). The age of DM patients with neuropathy ranged from 26 to 66 years. The minimum age of patients in group 2 (DM control) was 20 years, while the maximum was 55 years. In group 3 (healthy control) the minimum age was 28 years and the maximum age was 57 years (Table 4.1.2.1). Despite the fact that the medians of group 1 and group 2 were almost similar, significant differences were observed when the three groups were compared together (p<0.05).

Table 4.1.2.1: Descriptive statistics for the variable (age in years) in group 1, group 2 and group 3

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>49</td>
<td>47</td>
<td>37</td>
<td>0.00*</td>
</tr>
<tr>
<td>Minimum</td>
<td>26</td>
<td>20</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>66</td>
<td>55</td>
<td>57</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal-Wallis significant (p<0.05)*
4.1.2.2.2. Body Mass Index

The BMI in group 1 (the neuropathy group) displayed a median of 30 kg/m² (IQR= 28-40), which is placed within the obese range according to the WHO (2013) for BMI classification. The median BMI for the other two groups is placed within the overweight range based on the same classification (27 kg/m² (IQR= 24-32) for the DM control, and 24 kg/m² (IQR= 23-25) for the healthy control).

In group 1 (neuropathy group), the BMI ranged between a minimum of 26 to a maximum of 51 kg/m². The minimum BMI in group 2 (DM control) was 22, and the maximum BMI was 42 kg/m². In group 3 (healthy control), the minimum BMI was 20 kg/m², and the maximum BMI was 33 kg/m². Significant differences were observed when the three groups were compared (p<0.05) (Table 4.1.2.2).

<p>| Table 4.1.2.2: Descriptive statistics for BMI in kg/m² in group 1, group 2 and group 3 |
|-----------------------------------|---------|---------|---------|-----------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>30</td>
<td>27</td>
<td>24</td>
<td>0.00*</td>
</tr>
<tr>
<td>Minimum</td>
<td>26</td>
<td>22</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>51</td>
<td>42</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

* Kruskal-Wallis test sig p<0.05

4.1.2.2.3. Random Blood glucose

Random Blood Glucose (RBG) was evidently raised in group 1 (the neuropathy group) and group 2 (the DM control group). The minimum RBG in group 1 was 3.2 mmol/L while the maximum was 20.6 mmol/L. In group 2, RBG ranged between 6 and 18.6 mmol/L. In the healthy control group RBG levels ranged between 4 and 8.4 mmol/L (Table 4.1.2.2.3). When the three groups were compared, significant differences between the groups in RBG were observed (p<0.05).
Table 4.1.2.2.3: Descriptive statistics for RBG (mmol/L) in group 1, group 2 and group 3

<table>
<thead>
<tr>
<th>RBG (mmol/L)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>13</td>
<td>9</td>
<td>6</td>
<td>0.00*</td>
</tr>
<tr>
<td>Minimum</td>
<td>3.2</td>
<td>6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>20.6</td>
<td>18.6</td>
<td>8.4</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal-Wallis test sig p<0.05

4.1.2.2.4 Systolic and diastolic BP

The median systolic and diastolic blood pressures demonstrated values approximately within the normal ranges according to the AHA (2012). Systolic BP in group 1 presented a median of 144 mmHg (IQR= 129-158), while groups 2 and 3 presented medians of 133 mmHg (IQR= 127-142), and 122 mmHg (IQR= 115-125). However, when the median of the three groups were compared, significant differences were observed in the values of systolic BP (p<0.05) (Table 4.1.2.2.4a).

The diastolic BP ranged between 52 and 123 mmHg in group 1 (Median= 80 mmHg (IQR= 65-88)), 62 and 104 mmHg in group 2 (Mdn= 80 (IQR= 74-83)), and 65 and 86 mmHg in group 3 (Mdn= 78 (IQR= 78-81)) (Table 4.1.2.2.4b). No significant differences between the three groups were observed in the values of diastolic BP (p>0.05).

Table 4.1.2.2.4a: Descriptive statistics for systolic BP in mmHg in group 1, group 2 and group 3

<table>
<thead>
<tr>
<th>Systolic BP (mmHg)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>144</td>
<td>133</td>
<td>122</td>
<td>0.00*</td>
</tr>
<tr>
<td>Minimum</td>
<td>106</td>
<td>108</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>187</td>
<td>167</td>
<td>151</td>
<td></td>
</tr>
</tbody>
</table>

*Kruskal-Wallis test sig p<0.05
Table 4.1.2.2.4b: Descriptive statistics for diastolic BP in mmHg in group 1, group 2 and group 3

<table>
<thead>
<tr>
<th>Diastolic BP (mmHg)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>80</td>
<td>80</td>
<td>78</td>
<td>0.9</td>
</tr>
<tr>
<td>Minimum</td>
<td>52</td>
<td>62</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>123</td>
<td>104</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>

4.1.2.2.5. Duration of DM

The median duration of DM was significantly higher in the neuropathy group (p<0.05) when compared to the no neuropathy group (17 years (IQR= 12-20) in group 1, and 6 years (IQR= 2-9) in group 2) (Table 4.1.2.2.5a). The minimum duration of DM in group 1 was 6 years, while the maximum duration was 45 years. In group 2, the duration of DM ranged between 1 year and 24 years.

Table 4.1.2.2.5a: Descriptive statistics for the duration of DM in years in group 1 and group 2

<table>
<thead>
<tr>
<th>Duration of DM (years)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>17</td>
<td>6</td>
<td>0.000*</td>
</tr>
<tr>
<td>Minimum</td>
<td>6</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>45</td>
<td>24</td>
<td></td>
</tr>
</tbody>
</table>

* Mann-Whitney (U) Test significant at p< 0.05
4.1.2.3. Case control analysis for hypotheses testing.

The aim of this analysis was to investigate whether immunological markers are associated with the pathogenesis of DPN by comparing the levels of pro-inflammatory markers and autoimmune markers between the three groups as discussed in Chapter three.

4.1.2.3.1. Hypothesis one: Rejecting the null hypothesis that proposed no significant differences in the levels of pro-inflammatory markers between the three groups.

Kruskal Wallis test (Table 4.1.2.3.1a) was used to compare the levels of the C Reactive Protein (CRP) and inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-8, and IL-1β) between the three groups. The results for all the variables demonstrated highly significant differences between group 1, group 2 and group 3 in the levels of IL-6, IL-8, and IL-1β ($H(2) = 21.06$, $H(2) = 15.62$, $H(2) = 21.08$ respectively) ($p<0.001$), highly significant differences in the levels of TNF-α and IFN-γ ($H(2) = 12.56$, $H(2) = 14.78$, respectively) ($p<0.01$), and a significant difference between the values of CRP ($H(2) = 8.75$, $p<0.05$) (Table 4.1.2.3.1a). The Null hypothesis 1 is thus rejected as the differences in the levels of pro-inflammatory markers between the neuropathy group and the other two control groups were significant.

Table 4.1.2.3.1a: Comparing the levels of pro-inflammatory markers between groups 1, 2 and 3 (Kruskal Wallis Test, $H$: Chi-Square, df: degrees of freedom, p value significant*: $0.05 > p < 0.01$, **: $0.01 > p < 0.001$; ***: $0.001 > p$)

<table>
<thead>
<tr>
<th></th>
<th>Group 1 Median</th>
<th>Group 2 Median</th>
<th>Group 3 Median</th>
<th>Chi-Square</th>
<th>df</th>
<th>P-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (g/l)</td>
<td>0.005</td>
<td>0.000</td>
<td>0.000</td>
<td>8.75</td>
<td>2</td>
<td>0.0127*</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>0.455</td>
<td>0.270</td>
<td>0.145</td>
<td>12.56</td>
<td>2</td>
<td>0.0020**</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>17.890</td>
<td>10.060</td>
<td>10.980</td>
<td>14.78</td>
<td>2</td>
<td>0.0014**</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.635</td>
<td>0.250</td>
<td>0.780</td>
<td>21.06</td>
<td>2</td>
<td>0.0005***</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>6.750</td>
<td>2.965</td>
<td>1.650</td>
<td>15.61</td>
<td>2</td>
<td>0.0004***</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>7.220</td>
<td>3.700</td>
<td>3.655</td>
<td>21.08</td>
<td>2</td>
<td>0.0005***</td>
</tr>
</tbody>
</table>
The median levels of C-reactive proteins in the three groups studied are demonstrated in Figure 4.1.2.3.1a. The X axis of the box-plot represents the groups 1, 2 and 3, while the Y axis represents the concentrations of CRP in the blood serum in gram per litre (g/l).

Figure 4.1.2.3.1a describes the highest concentration of CRP detected (0.20 g/l) which was evident in group 1 (the neuropathy group), and the median concentration of CRP within this group was 0.005 g/l. The concentration of CRP in group 2 (DM control) and group 3 (Healthy control) did not exceed a concentration of 0.00 g/l, except for some outliers detected in both groups.

![Figure 4.1.2.3.1a: Box-plot presenting a comparison between the median levels of CRP concentrations between groups 1, 2 and 3 (p<0.05)](image)
Figure 4.1.2.3.1b compared the median levels of TNF-α concentration in picograms per millilitre (pg/ml) in all three groups. The median level of TNF-α in group 1 (neuropathy group) (0.46 pg/ml) was higher than the other two control groups (0.27 pg/ml in group 2, and 0.14 in group 3). The highest concentration of TNF-α detected in the neuropathy group was 1.65 pg/ml (Figure 4.1.2.3.1b).
The comparison between the median levels of IFN-γ concentrations between the three groups is displayed in Figure 4.1.2.3.1c. The X axis presents the groups, while the Y axis displays the concentrations in picograms per millilitre. Similarly, the highest concentration was detected in group 1 (the neuropathy group) where the median was 17.9 pg/ml in comparison to 10.1 pg/ml and 10.9 pg/ml in groups 2 and 3, respectively. The highest concentration that was not considered an outlier detected was 31.8 pg/ml in group 1.

Figure 4.1.2.3.1c: Box-plot presenting a comparison between median levels of IFN-γ concentrations in the blood serum groups 1, 2 and 3 (p<0.01)
Figure 4.1.2.3.1d compared the median concentration of IL-6 between the three groups. The X axis represents the groups, while the Y axis displays the concentration in picograms per millilitre. The graph demonstrates the highest concentration in group 1 (the neuropathy group) at a concentration of 6.60 pg/ml. The median in group 1 (1.63 pg/ml) was higher than the two control groups. Interestingly, the median concentrations of IL-6 in group 3 (the healthy control) was slightly higher than the DM control group (group 2) (0.25 pg/ml in group 2, and 0.78 pg/ml in group 3). Some outliers were detected in all three groups (high concentration of IL-6) (Figure 4.1.2.3.1d).
The comparison between the median levels of IL-8 concentration between the three groups is displayed in Figure 4.1.2.3.1e. The X axis presents the groups, while the Y axis displays the concentrations in picograms per millilitre. Similarly, the highest concentration was detected in group 1 (the neuropathy group) where the median was 6.7 pg/ml in comparison to 2.9 pg/ml and 1.6 pg/ml in groups 2 and 3, respectively. The highest concentration detected, that was not considered an outlier, was 22.3 pg/ml in group 1.

Figure 4.1.2.3.1e: Box-plot presenting a comparison between median levels of IL-8 concentration in the blood serum in groups 1, 2 and 3 ($p<0.01$)
Figure 4.1.2.3.1f compared the median concentration of IL-1β between the three groups. The X axis presented the groups, while the Y axis displayed the concentration in picogram per millilitre. The box-plots demonstrate the highest concentration in group 1 (the neuropathy group) at a concentration of 24.7 pg/ml. The median in group 1 (7.2 pg/ml) was higher than the two control groups. Interestingly, the median concentrations of IL-1β in group 3 (the healthy control) was slightly higher than the DM control group (group 2) (3.7 pg/ml in group 2, and 3.6 pg/ml in group 3). Some outliers were detected in all three groups (high concentration of IL-1β).

Figure 4.1.2.3.1f: Box-plot presenting a comparison between median levels of IL-1β concentration in the blood serum in groups 1, 2 and 3 ($p < 0.001$)
Table 4.1.2.3.1b presents the Mann-Whitney U test results which further suggested that the levels of the pro-inflammatory cytokines between the neuropathy group (group 1) and the diabetic control group (group 2) were significantly different when the two groups were compared \( (U(\text{TNF-} \alpha) = 289.000, \ U(\text{INF} \gamma) = 229.500, \ U(\text{IL-}6) = 179.000, \ U(\text{IL-}8) = 283.500, \ U(\text{IL-}1\beta) = 134.500, \ p<0.0167) \). C-Reactive proteins levels, however, showed no significant differences when these two groups were compared \( (p>0.0167) \).

Likewise, significant differences were noted between group 1 (neuropathy group) and group 3 (healthy control) for the C Reactive Protein \( (U = 306.00) \) and the inflammatory cytokines \( (U(\text{TNF-} \alpha)= 221.00, \ U(\text{INF} \gamma)= 221.50, \ U(\text{IL-}6)= 220.00, \ U(\text{IL-}8)= 214.50, \ U(\text{IL-}1\beta)= 233.00, \ p <0.0167)(\text{table 4.1.2.3.1c}) \). The \( p \) value was not significant \( (p>0.0167) \) (table 4.1.2.3.1d) when the median values for the inflammatory markers were compared between groups 2 (DM control) and group 3 (healthy control).
Table 4.1.2.3.1c: Mann-Whitney U test demonstrating differences in the median concentrations of pro-inflammatory markers between groups 1 and 3. P value significant*: $p<0.0167$

<table>
<thead>
<tr>
<th></th>
<th>Mann-Whitney U</th>
<th>P-value $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (g/l)</td>
<td>306.000</td>
<td>0.010*</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>221.000</td>
<td>0.001*</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>221.500</td>
<td>0.001*</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>220.000</td>
<td>0.001*</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>214.500</td>
<td>0.000*</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>233.000</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

1. Mann-Whitney U Test significant at $p<0.0167$

Table 4.1.2.3.1d: Mann-Whitney U test demonstrating no differences in the median concentrations of pro-inflammatory markers between group 2 and group 3. P value significant*: $p<0.0167$

<table>
<thead>
<tr>
<th></th>
<th>Mann-Whitney U</th>
<th>P-value $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (g/l)</td>
<td>435.000</td>
<td>0.742</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>365.500</td>
<td>0.210</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>450.000</td>
<td>1.000</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>306.000</td>
<td>0.033</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>288.500</td>
<td>0.017</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>444.000</td>
<td>0.929</td>
</tr>
</tbody>
</table>

1. Mann-Whitney U Test significant at $p<0.0167$

The levels of inflammatory markers were considerably different when all the three groups were compared (rejecting null hypothesis). When every two groups were further evaluated, the levels of the inflammatory markers were significantly different between group 1 (neuropathy group) and group 2 (DM control), and between group 1 (neuropathy group) and group 3 (healthy control). The differences in the level of markers were not noticeable when groups 2 and 3 (the two control groups) were compared (Table 4.1.2.3.1d).
4.1.2.3.2. Hypothesis two: Rejecting the null hypothesis that proposed no significant differences in the presence of autoimmune antibodies (ANA) between the three groups.

Chi-square or Fisher’s Exact tests were conducted to compare the presence and absence of autoimmune antibodies (categorical variables) in the blood serum between all three groups. Chi-Square test was then performed compare the presence and absence of ANAs between group 1 and group 2, and between group 1 and group 3 as explained in Chapter three.

There were highly significant differences between the percentages of positive and negative autoimmune antibodies (ANA) when all the three groups were compared $\chi^2 = 33.91$, df = 2, $p<0.001$ (Table 4.1.2.3.2a). The Null hypothesis 2 was thus rejected as the differences between the autoimmune antibodies between the three groups were significant.

Table 4.1.2.3.2a: Chi-square test presenting a comparison between the percentages of positive and negative ANAs in group 1, group 2 and group 3 ($p<0.001$), df: degree of freedom.

<table>
<thead>
<tr>
<th>Group</th>
<th>ANA screen</th>
<th>Chi-Square</th>
<th>df</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Group 1</td>
<td>12</td>
<td>40.0%</td>
<td>18</td>
<td>60.0%</td>
</tr>
<tr>
<td>Group 2</td>
<td>29</td>
<td>96.7%</td>
<td>1</td>
<td>3.3%</td>
</tr>
<tr>
<td>Group 3</td>
<td>28</td>
<td>93.3%</td>
<td>2</td>
<td>6.7%</td>
</tr>
</tbody>
</table>

*Chi-Square test

Figure 4.1.2.3.2a presents a bar chart illustrating the comparison between the percentages of positive values of antinuclear antibodies presented in the blood serum in the three groups studied. The X axis of the graph presents the three groups divided by the positive and negative values of each group separately, while
the Y axis represents the number of cases of positive and negative antinuclear antibodies detected in the blood serum of all subjects. The number of positive ANA values in group 1 (the neuropathy group) is visibly higher (60%, (n= 18)) than the positive values detected in the other two groups (3.3% (n= 1)) in group 2, and 6.7% (n= 2) in group 3) (Figure 4.1.2.3.2a). Although some negative values were detected in group 1 (the neuropathy group), the percentages of positive values were higher (60% positive, 40% negative).

**Figure 4.1.2.3.2a:** Box-plot presenting a comparison between the percentages of positive and negative ANA screen between groups 1, 2 and 3
Chi-Square test was additionally used to demonstrate the relationship in the percentages of positive and negative ANAs between group 1 (neuropathy group) and group 2 (DM control) \((\text{Chi-square } (1) = 22.26, \text{ odds ratio} = 50, p<0.01)\) (Table 4.1.2.3.2b). The odds ratio was then calculated by the following equation.

\[
\text{Odds of +ve values in group 1} = \frac{\text{+ve values in group 1}}{\text{-ve values in group 1}} = \frac{18}{12} = 1.5
\]

\[
\text{Odds of +ve values in group 2} = \frac{\text{+ve values in group 2}}{\text{-ve values in group 2}} = \frac{1}{29} = 0.03
\]

\[
\text{Odds ratio} = \frac{\text{Odds of +ve values in group 1}}{\text{Odds of +ve values in group 2}} = 50
\]

Therefore, the odds of positive values of ANAs in the neuropathy group were 50 times higher than the diabetic control group.

**Table 4.1.2.3.2b:** Chi-square test demonstrating a comparison between the percentages of positive and negative ANA in group 1 and group 2 \((p<0.001)\)

<table>
<thead>
<tr>
<th>ANA screen</th>
<th>Negative</th>
<th>Positive</th>
<th>Chi-square</th>
<th>Df</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>12</td>
<td>40.0%</td>
<td>18</td>
<td>60.0%</td>
<td>22.259*</td>
</tr>
<tr>
<td>Group 2</td>
<td>29</td>
<td>96.7%</td>
<td>1</td>
<td>3.3%</td>
<td></td>
</tr>
</tbody>
</table>

*Chi-Square test

Table 4.1.2.3.2c demonstrates the relationship between group 1 (neuropathy group) and group 3 (healthy control) \((\text{Chi-square } (1) = 19.20, p<0.01)\). The odds ratio was calculated according to the formula described earlier = 21.4.
Table 4.1.2.3.c: Chi-square test demonstrating the relationship between the percentages of positive and negative ANA in group 1 and group 2 (p<0.001), df: degree of freedom.

<table>
<thead>
<tr>
<th>ANA screen</th>
<th></th>
<th></th>
<th></th>
<th>Chi-square</th>
<th>Df</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>N</td>
<td>%</td>
<td>Positive</td>
<td>N</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>12</td>
<td>40.0%</td>
<td>18</td>
<td>60.0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>28</td>
<td>93.3%</td>
<td>2</td>
<td>6.7%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Chi-Square test

Fisher’s Exact test was used to explore the relationship between positive and negative ANAs in groups 2 and 3 due to the fact that the expected frequencies in the cells were greater than 5. The comparison test produced insignificant differences between group 2 (DM control) and group 3 (healthy control) in the percentages of ANAs in blood serum (p>0.05) suggesting no obvious difference between the two control groups in this variable (Table 4.1.2.3.d).

Table 4.1.2.3.d: Fisher’s Exact Test presenting the relationship between positive and negative values of ANA in group 2 and group 3 (p>0.01)

<table>
<thead>
<tr>
<th>ANA screen</th>
<th></th>
<th></th>
<th>P-value1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>N</td>
<td>%</td>
<td>Positive</td>
</tr>
<tr>
<td>Group 2</td>
<td>29</td>
<td>96.7%</td>
<td>1</td>
</tr>
<tr>
<td>Group 3</td>
<td>28</td>
<td>93.3%</td>
<td>2</td>
</tr>
</tbody>
</table>

1. Fisher’s Exact test
4.2. Secondary analysis

4.2.1. Exploring the correlations between the pro-inflammatory markers and the sample demographics and clinical characteristics

4.2.1.1. Correlation Co-efficient between dependant variables and sample characteristics

Spearman’s correlation coefficient (a non-parametric test for non-normally distributed data) was used to explore the possible correlation between the quantitative values of the inflammatory markers and the demographic variables for each group separately as illustrated in tables 4.2.1.1a, 4.2.1.1b, and 4.2.1.1c. Within the sample group 1 (the neuropathy group), significant relationships between the systolic BP and the values of TNF-α and IL-6 were presented \((p<0.05)\) (Table 4.2.1.1a). Systolic blood pressures showed a weak positive correlation with the values of TNF-α \((r = 0.430, R^2 = 0.18)\), and a weak negative correlation with the values of IL-6 \((r = -0.382, R^2 = 0.15)\), following the exclusion of extreme outliers (Table 4.2.1.1a). This suggests that a rise in systolic BP may affect 18% of the raised variance in the values of TNF-α (positive relationship). A rise in systolic BP however may be responsible for 15% of the low variance in the values of IL-6 (negative relationship).

Similarly, type of DM showed a weak positive correlation with the values of CRP in the blood serum of group 1 \((r = 0.372, R^2= 0.13)\) (Table 4.2.1.1a). Binary logistic regression indicated that type II DM was significantly correlated with CRP values \((p= 0.038)\), while type I DM was insignificant \((p= 0.663)\).
Table 4.2.1.1a: Spearman’s Correlations between pro-inflammatory markers and demographic variables in group 1

<table>
<thead>
<tr>
<th></th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spearman Correlation</td>
<td>0.298</td>
<td>0.220</td>
<td>-0.154</td>
<td>0.086</td>
<td>0.255</td>
<td>0.154</td>
</tr>
<tr>
<td>P-value</td>
<td>0.110</td>
<td>0.907</td>
<td>0.415</td>
<td>0.651</td>
<td>0.174</td>
<td>0.417</td>
</tr>
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<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spearman Correlation</td>
<td>-0.087</td>
<td>-0.011</td>
<td>0.132</td>
<td>0.129</td>
<td>-0.290</td>
<td>0.026</td>
</tr>
<tr>
<td>P-value</td>
<td>0.648</td>
<td>0.955</td>
<td>0.488</td>
<td>0.498</td>
<td>0.120</td>
<td>0.891</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Systolic BP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Last Visit</td>
<td>Spearman Correlation</td>
<td>-0.201</td>
<td>0.430*</td>
<td>-0.157</td>
<td>-0.382*</td>
<td>-0.028</td>
</tr>
<tr>
<td>P-value</td>
<td>0.287</td>
<td>0.018</td>
<td>0.408</td>
<td>0.037</td>
<td>0.884</td>
<td>0.363</td>
</tr>
<tr>
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<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Diastolic BP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Last Visit</td>
<td>Spearman Correlation</td>
<td>-0.074</td>
<td>0.165</td>
<td>0.036</td>
<td>-0.127</td>
<td>0.077</td>
</tr>
<tr>
<td>P-value</td>
<td>0.697</td>
<td>0.382</td>
<td>0.852</td>
<td>0.503</td>
<td>0.684</td>
<td>0.703</td>
</tr>
<tr>
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<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>RBG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spearman Correlation</td>
<td>0.163</td>
<td>0.245</td>
<td>-0.003</td>
<td>0.035</td>
<td>0.274</td>
<td>0.115</td>
</tr>
<tr>
<td>P-value</td>
<td>0.390</td>
<td>0.192</td>
<td>0.986</td>
<td>0.854</td>
<td>0.143</td>
<td>0.544</td>
</tr>
<tr>
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<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spearman Correlation</td>
<td>0.259</td>
<td>0.172</td>
<td>-0.216</td>
<td>0.230</td>
<td>-0.221</td>
<td>0.354</td>
</tr>
<tr>
<td>P-value</td>
<td>0.167</td>
<td>0.362</td>
<td>0.251</td>
<td>0.221</td>
<td>0.240</td>
<td>0.055</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Type of DM</strong></td>
<td>Correlation</td>
<td>0.372*</td>
<td>-0.292</td>
<td>0.175</td>
<td>0.132</td>
<td>0.082</td>
</tr>
<tr>
<td>Coefficient</td>
<td>P value</td>
<td>0.043</td>
<td>0.595</td>
<td>0.118</td>
<td>0.355</td>
<td>0.486</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Correlation</td>
<td>0.269</td>
<td>0.074</td>
<td>0.148</td>
<td>0.210</td>
<td>0.288</td>
</tr>
<tr>
<td>Coefficient</td>
<td>P value</td>
<td>0.150</td>
<td>0.698</td>
<td>0.436</td>
<td>0.265</td>
<td>0.123</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level.
Table 4.2.1.1b: Spearman’s Correlations between pro-inflammatory markers and demographics variables in group 2

<table>
<thead>
<tr>
<th></th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td>Spearman Correlation</td>
<td>-0.183</td>
<td>0.345</td>
<td>-0.081</td>
<td>-0.066</td>
<td><strong>-0.374</strong>*</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.332</td>
<td>0.062</td>
<td>0.671</td>
<td>0.728</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>Spearman Correlation</td>
<td><strong>0.416</strong>*</td>
<td><strong>-0.369</strong>*</td>
<td>0.104</td>
<td>0.137</td>
<td>-0.135</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.022</td>
<td>0.045</td>
<td>0.583</td>
<td>0.472</td>
<td>0.476</td>
</tr>
<tr>
<td></td>
<td>N</td>
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<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Systolic BP</strong></td>
<td>Spearman Correlation</td>
<td><strong>0.439</strong>*</td>
<td>-0.085</td>
<td>-0.141</td>
<td>0.274</td>
<td>0.322</td>
</tr>
<tr>
<td>Last Visit</td>
<td>P-value</td>
<td>0.015</td>
<td>0.655</td>
<td>0.457</td>
<td>0.143</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Diastolic BP</strong></td>
<td>Spearman Correlation</td>
<td>0.044</td>
<td>-0.131</td>
<td>-0.208</td>
<td>0.285</td>
<td>0.068</td>
</tr>
<tr>
<td>Last Visit</td>
<td>P-value</td>
<td>0.817</td>
<td>0.490</td>
<td>0.269</td>
<td>0.127</td>
<td>0.723</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
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<td>30</td>
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<td>30</td>
</tr>
<tr>
<td><strong>RBG</strong></td>
<td>Spearman Correlation</td>
<td>0.210</td>
<td>-0.045</td>
<td>0.204</td>
<td>-0.005</td>
<td>-0.178</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.264</td>
<td>0.813</td>
<td>0.279</td>
<td>0.981</td>
<td>0.346</td>
</tr>
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<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Duration</strong></td>
<td>Spearman Correlation</td>
<td>-0.259</td>
<td>-0.162</td>
<td>0.260</td>
<td>-0.235</td>
<td>-0.109</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.167</td>
<td>0.391</td>
<td>0.166</td>
<td>0.210</td>
<td>0.565</td>
</tr>
<tr>
<td></td>
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<td>30</td>
<td>30</td>
<td>30</td>
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</tr>
<tr>
<td><strong>Gender</strong></td>
<td>Correlation</td>
<td>-0.155</td>
<td>0.252</td>
<td>0.104</td>
<td>-0.136</td>
<td>0.097</td>
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<tr>
<td></td>
<td>Coefficient</td>
<td>0.413</td>
<td>0.179</td>
<td>0.583</td>
<td>0.474</td>
<td>0.612</td>
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<td></td>
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<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Type of DM</strong></td>
<td>Correlation</td>
<td>0.059</td>
<td>-0.027</td>
<td>-0.319</td>
<td>-0.005</td>
<td>-0.196</td>
</tr>
<tr>
<td></td>
<td>Coefficient</td>
<td>0.758</td>
<td>0.886</td>
<td>0.086</td>
<td>0.981</td>
<td>.299</td>
</tr>
<tr>
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<td>30</td>
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<td>30</td>
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</tbody>
</table>

* Correlation is significant at the 0.05 level.
Table 4.2.1.1c: Spearman’s Correlations between pro-inflammatory markers and demographic variables in group 3

<table>
<thead>
<tr>
<th></th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Spearman Correlation</td>
<td>0.127</td>
<td>0.129</td>
<td>0.093</td>
<td>-0.001</td>
<td>-0.200</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.505</td>
<td>0.497</td>
<td>0.624</td>
<td>0.995</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
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<td>30</td>
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</tr>
<tr>
<td>BMI</td>
<td>Spearman Correlation</td>
<td>0.228</td>
<td>-0.093</td>
<td>0.035</td>
<td>0.048</td>
<td>0.057</td>
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<td>P-value</td>
<td>0.225</td>
<td>0.626</td>
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<td>0.763</td>
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</tr>
<tr>
<td>Systolic BP</td>
<td>Spearman Correlation</td>
<td>0.174</td>
<td>-0.026</td>
<td>0.261</td>
<td>0.099</td>
<td>0.204</td>
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<td>Last Visit</td>
<td>P-value</td>
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<td>0.890</td>
<td>0.164</td>
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</tr>
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<tr>
<td>Diastolic BP</td>
<td>Spearman Correlation</td>
<td>0.175</td>
<td>-0.089</td>
<td>0.140</td>
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<tr>
<td>Last Visit</td>
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<td>0.354</td>
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</tr>
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<td>RBG</td>
<td>Spearman Correlation</td>
<td>-0.258</td>
<td>-0.072</td>
<td>0.164</td>
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</tr>
<tr>
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<td>P-value</td>
<td>0.169</td>
<td>0.704</td>
<td>0.387</td>
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</tr>
<tr>
<td>Gender</td>
<td>Spearman Correlation</td>
<td>0.054</td>
<td>-0.322</td>
<td>-0.266</td>
<td>0.116</td>
<td>-0.430</td>
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<td>0.779</td>
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<td>Type of DM</td>
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</tbody>
</table>

This is further illustrated in the scatter plots displayed in Figure 4.2.1.1a and Figure 4.2.1.1b, which was used to demonstrate the correlations between the two variables. Figure 4.2.1.1a and Figure 4.2.1.1b describe the correlation between the systolic BP of patients in group 1 (the neuropathy group) and the levels of TNF-α and IL-6 in their blood serum. The systolic BP values are displayed in the X axis, while the Y axis represents the concentration of the cytokines; TNF-α and IL-6. In addition to the correlation, a linear relationship is also described by introducing the line of best fit.
A weak positive relationship is observed in Figure 4.2.1.1a suggesting that the TNF-α concentrations may possibly increase with a rise in systolic BP. Figure 4.2.1.1b, however, describes a weak negative correlation, where the values of IL-6 may possibly decrease with a rise in systolic BP.

![Figure 4.2.1.1a: Scatter plot demonstrating weak positive correlation between systolic BP (mmHg) and TNF-α (pg/ml) value in group 1 (Spearman’s correlation is 0.430, p<0.05)]
In Sample group 2 (DM control), a weak positive correlation was significant between the BMI of the patients and the level of CRP values ($r = 0.416, R^2 = 0.17, p<0.05$) (table 4.2.1.1b), and a weak negative correlation between the BMI and the level of TNF-α ($r = -0.369, R^2 = 0.14, p<0.05$) (table 4.2.1.1b). A weak positive correlation was also noted between the systolic BP and the value of CRP in the same group of patients ($r = 0.439, R^2 = 0.19, p<0.05$) (table 4.2.1.1b). A weak negative correlation between the age of the sample and IL-8 was also witnessed ($r = -0.374, R^2 = 0.14, p<0.05$). No significant correlations between the inflammatory markers and the demographics in group 3 were observed (Table 4.2.1.1c).
Figure 4.2.1.1c presents a scatter plot describing the correlation between the CRP levels (Y axis) and the BMI values (X axis) of the patients in group 2 (DM control). A weak positive correlation is detected between these two variables suggesting that an increase in patient’s BMI may be associated with an increase in the values of CRP (Figure 4.2.1.1c).
The scatter plot in Figure 4.2.1.1d demonstrates the observed and linear relationship between the levels of TNF-α and the values of BMI of the patients in group 2 (DM control). The X axis represents the BMI values in kilograms per meter square while the Y axis represents the concentration of TNF-α in pictograms per millilitre. The scatter plot suggests a weak negative correlation between the two variables suggesting that an increase in BMI values may be associated with a decrease in the concentration of TNF-α in the blood serum of the patients in group 2.

Figure 4.2.1.1d: Scatter plot demonstrating a weak negative correlation between BMI (kg/m²) and TNF-α (pg/ml) value in group 2 (Spearman’s correlation is -0.369, p<0.05)
Figure 4.2.1.1e displays a scatter plot illustrating the observed and linear correlation between the levels of CRP (the Y axis) and the systolic BP of the patients (the X axis) in group 2 (DM control). A weak positive correlation was observed suggesting that a rise in systolic BP in patients in group 2 may increase the values of CRP in the blood serum of those patients (Figure 4.2.1.1e).

**Figure 4.2.1.1e: Scatter plot demonstrating weak positive correlation between systolic BP (mmHg) and CRP (g/l) values in group 2 (Spearman’s correlation is 0.439, p<0.05)**
The scatter plot in Figure 4.2.1.1f, on the other hand demonstrated a weak negative observed correlation between the concentrations of IL-8 and the age of the patients in group 2 (DM control. The scatter plot displays the IL-8 concentrations in pictograms per millilitre on the Y axis, while the age of the patients in years is presented on the X axis. This suggests that an increase in the age of the patients in this group may lead to a decrease in the levels of IL8 concentrations in the blood serum.

Figure 4.2.1.1f: Scatter plot demonstrating weak negative correlation between age (years) and IL-8 (pg/ml) values in group 2 (Spearman’s correlation is -0.374, p<0.05)
### 4.2.1.2. Correlation between dependant variables and neurological characteristics

Interestingly, highly significant correlations were found to be associated with the neurological characteristics of the neuropathy group (group 1) and the levels of CRP, IL-8, and IL-1β. C-reactive protein levels demonstrated significant positive correlations with the increased neuropathy system score (NSS) ($r = 0.693$, $R^2 = 0.48$, $p < 0.01$) (Table 4.2.1.2). This suggests that 48% of the value change in the CRP may be caused by the increased variables in NSS, assuming that co-variants were not involved. Similarly, IL-8 and IL-1β both demonstrated a significant correlation with NSS levels ($r = 0.365$, $R^2 = 0.13$, and $r = 0.462$, $R^2 = 0.21$ respectively) ($p < 0.05$) (Table 4.2.1.2a). Therefore, 13% of the value change in IL-8 may be caused by the increase in the level of NSS scores, and a 21% of the increase in IL-1β may be caused by the increase in the NSS scores in patients with DPN.

#### Table 4.2.1.2a: Spearman’s Correlations between neurological characteristics and pro-inflammatory markers in group 1

<table>
<thead>
<tr>
<th></th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>Spearman Correlation</td>
<td>0.693**</td>
<td>0.269</td>
<td>0.035</td>
<td>0.142</td>
<td>0.365*</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.000</td>
<td>0.150</td>
<td>0.854</td>
<td>0.454</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
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<td>30</td>
<td>30</td>
</tr>
<tr>
<td>NDS</td>
<td>Spearman Correlation</td>
<td>-0.242</td>
<td>0.171</td>
<td>-0.243</td>
<td>-0.014</td>
<td>-0.254</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.197</td>
<td>0.366</td>
<td>0.195</td>
<td>0.941</td>
<td>0.175</td>
</tr>
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<tr>
<td>VPT</td>
<td>Spearman Correlation</td>
<td>-0.190</td>
<td>0.072</td>
<td>-0.357</td>
<td>-0.010</td>
<td>-0.153</td>
</tr>
<tr>
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<td>P-value</td>
<td>0.314</td>
<td>0.707</td>
<td>0.053</td>
<td>0.957</td>
<td>0.419</td>
</tr>
<tr>
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<td>30</td>
<td>30</td>
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</tr>
</tbody>
</table>

*. Correlation is significant at the 0.05 level.
**. Correlation is significant at the 0.01 level.
The other two groups demonstrated no significant correlations between the levels of inflammatory markers and the neurological characteristics (Table 4.2.1.2b, Table 4.2.1.2c).

**Table 4.2.1.2b: Spearman’s correlation between pro-inflammatory markers and neurological manifestations in group 2 (p>0.05)**

<table>
<thead>
<tr>
<th></th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>Spearman Correlation</td>
<td>0.653</td>
<td>0.189</td>
<td>0.092</td>
<td>0.149</td>
<td>-0.368</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.069</td>
<td>0.318</td>
<td>0.629</td>
<td>0.433</td>
<td>0.055</td>
</tr>
<tr>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
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<td>30</td>
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</tr>
<tr>
<td>NDS</td>
<td>Spearman Correlation</td>
<td>0.358</td>
<td>-0.79</td>
<td>-0.325</td>
<td>0.097</td>
<td>-0.229</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.52</td>
<td>0.679</td>
<td>0.08</td>
<td>0.609</td>
<td>0.223</td>
</tr>
<tr>
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</tr>
<tr>
<td>VPT</td>
<td>Spearman Correlation</td>
<td>0.56</td>
<td>-0.060</td>
<td>0.004</td>
<td>-0.408</td>
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<tr>
<td></td>
<td>P-value</td>
<td>0.769</td>
<td>0.752</td>
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</table>

**Table 4.2.1.2c: Spearman’s correlation between pro-inflammatory markers and neurological manifestations in group 3 (p>0.05)**

<table>
<thead>
<tr>
<th></th>
<th>CRP (g/l)</th>
<th>TNF-α (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>IL-8 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>NSS</td>
<td>Spearman Correlation</td>
<td>0.553</td>
<td>0.089</td>
<td>0.102</td>
<td>0.99</td>
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<td>P-value</td>
<td>0.059</td>
<td>0.309</td>
<td>0.529</td>
<td>0.587</td>
<td>0.311</td>
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<tr>
<td>NDS</td>
<td>Spearman Correlation</td>
<td>0.318</td>
<td>-0.61</td>
<td>-0.465</td>
<td>0.106</td>
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<td>P-value</td>
<td>0.72</td>
<td>0.524</td>
<td>0.190</td>
<td>0.489</td>
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</tr>
<tr>
<td>VPT</td>
<td>Spearman Correlation</td>
<td>0.347</td>
<td>0.163</td>
<td>0.281</td>
<td>-0.196</td>
<td>0.216</td>
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<tr>
<td></td>
<td>P-value</td>
<td>0.061</td>
<td>0.390</td>
<td>0.132</td>
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<td>0.252</td>
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</table>
4.2.2. Exploring the correlations between anti-nuclear antibodies and the sample demographics and clinical characteristics.

4.2.2.1. Correlations between dependant variables and sample characteristics

Binary logistic regression was used to explore the correlation between the presence of ANA and the demographical variables and clinical manifestations of peripheral neuropathy in the three groups (Table 4.2.2.1a, Table 4.2.2.1b, and Table 4.2.2.1c). No significant association between the demographics of the subjects and the presence of ANA was encountered in all the three groups ($p>0.05$).

Table 4.2.2.1a: Relationship between ANA values and demographic variables in group 1

<table>
<thead>
<tr>
<th>Demographic Variable</th>
<th>B</th>
<th>P-value</th>
<th>Odd Ratio</th>
<th>Lower</th>
<th>Upper</th>
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</thead>
<tbody>
<tr>
<td>Gender (Male)</td>
<td>0.351</td>
<td>0.708</td>
<td>1.421</td>
<td>0.226</td>
<td>8.947</td>
</tr>
<tr>
<td>Type of DM (Type1)</td>
<td>0.236</td>
<td>0.853</td>
<td>1.266</td>
<td>0.105</td>
<td>15.326</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.028</td>
<td>0.457</td>
<td>1.029</td>
<td>0.950</td>
<td>1.114</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>-0.024</td>
<td>0.660</td>
<td>0.976</td>
<td>0.878</td>
<td>1.086</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.020</td>
<td>0.471</td>
<td>0.980</td>
<td>0.929</td>
<td>1.035</td>
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<tr>
<td>Diastolic BP (mmHg)</td>
<td>0.026</td>
<td>0.483</td>
<td>1.026</td>
<td>0.955</td>
<td>1.103</td>
</tr>
<tr>
<td>RBG (mmol/l)</td>
<td>0.114</td>
<td>0.278</td>
<td>1.121</td>
<td>0.912</td>
<td>1.378</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>0.105</td>
<td>0.178</td>
<td>1.110</td>
<td>0.953</td>
<td>1.293</td>
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</table>
### Table 4.2.2.1b: Relationship between ANA values and demographic variables in group 2

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<tr>
<td>Gender (Male)</td>
<td>0.351</td>
<td>0.708</td>
<td>1.421</td>
<td>0.226</td>
<td>8.947</td>
</tr>
<tr>
<td>Type of DM (Type1)</td>
<td>13.410</td>
<td>0.999</td>
<td>0.848</td>
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<td>0.000</td>
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<td>Age (years)</td>
<td>2.119</td>
<td>0.997</td>
<td>8.320</td>
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</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>1.999</td>
<td>0.997</td>
<td>7.380</td>
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<td>0.000</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>1.145</td>
<td>0.998</td>
<td>3.144</td>
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<td>0.000</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>-1.513</td>
<td>0.999</td>
<td>0.220</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>RBG (mmol/l)</td>
<td>-0.151</td>
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<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Duration (years)</td>
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<td>0.178</td>
<td>1.110</td>
<td>0.953</td>
<td>1.293</td>
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</table>

### Table 4.2.2.1c: Relationship between ANA values and demographic variables in group 3

<table>
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<th>P-value</th>
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<th>Upper</th>
</tr>
</thead>
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<tr>
<td>Gender (Male)</td>
<td>-1.506</td>
<td>0.521</td>
<td>0.222</td>
<td>0.002</td>
<td>21.998</td>
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<tr>
<td>Type of DM (Type1)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.002</td>
<td>0.987</td>
<td>0.998</td>
<td>0.795</td>
<td>1.253</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.307</td>
<td>0.434</td>
<td>1.359</td>
<td>0.630</td>
<td>2.935</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>-0.194</td>
<td>0.325</td>
<td>0.823</td>
<td>0.559</td>
<td>1.213</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>0.320</td>
<td>0.408</td>
<td>1.377</td>
<td>0.646</td>
<td>2.932</td>
</tr>
<tr>
<td>RBG (mmol/l)</td>
<td>0.011</td>
<td>0.991</td>
<td>1.011</td>
<td>0.147</td>
<td>6.968</td>
</tr>
<tr>
<td>Duration (years)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>
4.2.2.2. Correlation between dependant variables and neurological characteristics

Similarly, binary logistic regression was used to explore the correlations between the presence of ANA’s and the neurological manifestations in the three groups. Significant relationship between NSS and the presence of ANA were demonstrated in the neuropathy group (group 1) (Table 4.2.2.2.1). No significant relationships were observed between the presence of ANA’s and the neurological manifestations in groups 2 and 3 (Table 4.2.2.2.2, Table 4.2.2.2.3)

Table 4.2.2.2.1: Relationship between clinical manifestation of peripheral neuropathy and ANA values in group 1

<table>
<thead>
<tr>
<th></th>
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<th>P-value</th>
<th>Odd Ratio</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>2.477</td>
<td>0.041*</td>
<td>11.908</td>
<td>0.891</td>
<td>159.186</td>
</tr>
<tr>
<td>NDS</td>
<td>4.210</td>
<td>0.007*</td>
<td>67.367</td>
<td>3.236</td>
<td>1402.445</td>
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<tr>
<td>VPT</td>
<td>-1.659</td>
<td>0.062</td>
<td>9.190</td>
<td>0.010</td>
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</table>

Table 4.2.2.2.2: Relationship between clinical manifestation of peripheral neuropathy and ANA values in group 2

<table>
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<th></th>
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<th>P-value</th>
<th>Odd Ratio</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>3.115</td>
<td>1.000</td>
<td>0.044</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>NDS</td>
<td>2.787</td>
<td>1.000</td>
<td>16.227</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>VPT</td>
<td>-1.941</td>
<td>0.994</td>
<td>0.144</td>
<td>0.000</td>
<td>-</td>
</tr>
</tbody>
</table>
4.2.2. Relationship between clinical manifestation of peripheral neuropathy and ANA values in group 3

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>P-value</th>
<th>Odd Ratio</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>5.013</td>
<td>1.000</td>
<td>0.068</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>NDS</td>
<td>2.081</td>
<td>1.000</td>
<td>10.317</td>
<td>0.000</td>
<td>-</td>
</tr>
<tr>
<td>VPT</td>
<td>-1.501</td>
<td>0.994</td>
<td>0.210</td>
<td>0.000</td>
<td>-</td>
</tr>
</tbody>
</table>

4.2.3. Exploring potential correlations between sample demographics and clinical manifestations of neuropathy

No significant correlations were observed between the sample demographics and the neuropathy manifestations when the variables were explored in groups 1, 2 and 3 (Table. 4.2.3.1, Table 4.2.3.2, and Table 4.2.3.3).

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>RBG (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>Spearman Correlation</td>
<td>0.179</td>
<td>-0.248</td>
<td>0.044</td>
<td>0.204</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.344</td>
<td>0.187</td>
<td>0.819</td>
<td>0.280</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>NDS</td>
<td>Spearman Correlation</td>
<td>0.097</td>
<td>-0.164</td>
<td>-0.049</td>
<td>-0.211</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.609</td>
<td>0.387</td>
<td>0.796</td>
<td>0.263</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>VPT</td>
<td>Spearman Correlation</td>
<td>0.113</td>
<td>-0.198</td>
<td>-0.128</td>
<td>-0.278</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.552</td>
<td>0.294</td>
<td>0.499</td>
<td>0.137</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
Table 4.2.3: Spearman’s correlation between sample demographics and neurological manifestations in group 2 ($p>0.05$)

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>RBG (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>Spearman Correlation</td>
<td>0.264</td>
<td>0.260</td>
<td>0.148</td>
<td>-0.164</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.158</td>
<td>0.166</td>
<td>0.435</td>
<td>0.386</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>NDS</td>
<td>Spearman Correlation</td>
<td>0.085</td>
<td>0.403</td>
<td>0.249</td>
<td>0.481</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.654</td>
<td>0.077</td>
<td>0.185</td>
<td>0.077</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>VPT</td>
<td>Spearman Correlation</td>
<td>0.459</td>
<td>0.033</td>
<td>-0.282</td>
<td>-0.136</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.081</td>
<td>0.862</td>
<td>0.131</td>
<td>0.474</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.2.3.3: Spearman’s correlation between sample demographics and neurological manifestations in group 3 ($p>0.05$)

<table>
<thead>
<tr>
<th></th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
<th>RGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSS</td>
<td>Spearman Correlation</td>
<td>-0.067</td>
<td>0.280</td>
<td>0.173</td>
<td>0.152</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.726</td>
<td>0.134</td>
<td>0.360</td>
<td>0.422</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>NDS</td>
<td>Spearman Correlation</td>
<td>0.251</td>
<td>-0.039</td>
<td>-0.192</td>
<td>-0.067</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.181</td>
<td>0.839</td>
<td>0.309</td>
<td>0.726</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>VPT</td>
<td>Spearman Correlation</td>
<td>-0.271</td>
<td>0.053</td>
<td>-0.001</td>
<td>0.281</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.063</td>
<td>0.782</td>
<td>0.996</td>
<td>0.132</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
4.2.4. Further analysis exploring the independent relationship between neuropathy and immunological markers

Multiple linear regressions were conducted to explore the possible independent relationships between neuropathy symptoms (NSS, NDS, and VPT) and the dependant variables (Inflammatory markers and ANA’s) (Table 4.2.4.). The multiple correlation coefficient (R) for CRP and the neuropathy variables together was 0.669, suggesting a high degree of correlation between the variables. The regression ANOVA shows that the results are significant ($p<0.05$) (Table 4.2.4). For the independent variables NSS, the probability of the t statistic for the b coefficient is $<0.05$ suggesting a significant relationship when tested with levels of CRP. However, no significant regressions between NDS and VPT were observed when compared with the levels of CRP. The other significant regression was observed between IL-$\beta$ and the NSS ($p<0.05$). However the multiple regression as a whole was insignificant when IL-$\beta$ was tested with NSS, NDS and VPT ($R=0.459, p>0.05$).

Interestingly, significant linear regressions were presented when the dependant variable ANA was correlated with the independent neuropathy symptoms ($p<0.05$). The multiple correlation coefficient (R) was 0.827, demonstrating a significant regression between the variables (Table 4.2.4).
Table 4.2.4: Regression analysis and $p$ values for neuropathy manifestations (NSS, NDS, and VPT) score are from multiple linear regression models with concentrations of immune mediators as dependent variables (*: $p<0.05$).

<table>
<thead>
<tr>
<th>Dependent</th>
<th>R</th>
<th>B</th>
<th>Std. Error</th>
<th>Beta</th>
<th>P</th>
<th>ANOVA p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.669</td>
<td>0.002</td>
<td>0.000</td>
<td>0.737</td>
<td>0.000*</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>0.050</td>
<td>0.000</td>
<td>0.000</td>
<td>-0.157</td>
<td>0.325</td>
<td>0.001*</td>
</tr>
<tr>
<td>VPT</td>
<td>0.040</td>
<td>0.007</td>
<td>0.093</td>
<td>0.556</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.247</td>
<td>0.047</td>
<td>0.038</td>
<td>0.271</td>
<td>0.224</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>-0.004</td>
<td>0.028</td>
<td>-0.026</td>
<td>0.901</td>
<td>0.645</td>
<td></td>
</tr>
<tr>
<td>VPT</td>
<td>0.004</td>
<td>0.007</td>
<td>0.109</td>
<td>0.597</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.370</td>
<td>0.413</td>
<td>0.917</td>
<td>0.094</td>
<td>0.656</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>-0.737</td>
<td>-0.129</td>
<td>0.692</td>
<td>-0.209</td>
<td>0.296</td>
<td>0.272</td>
</tr>
<tr>
<td>VPT</td>
<td>-0.255</td>
<td>0.172</td>
<td>0.129</td>
<td>0.149</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.348</td>
<td>0.129</td>
<td>0.114</td>
<td>0.238</td>
<td>0.269</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>-0.035</td>
<td>0.086</td>
<td>-0.080</td>
<td>0.690</td>
<td>0.332</td>
<td></td>
</tr>
<tr>
<td>VPT</td>
<td>0.039</td>
<td>0.021</td>
<td>0.359</td>
<td>0.080</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.158</td>
<td>-0.507</td>
<td>1.773</td>
<td>-0.064</td>
<td>0.777</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>0.229</td>
<td>1.338</td>
<td>0.036</td>
<td>0.865</td>
<td>0.880</td>
<td></td>
</tr>
<tr>
<td>VPT</td>
<td>-0.269</td>
<td>0.332</td>
<td>-0.168</td>
<td>0.425</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.459</td>
<td>1.212</td>
<td>0.505</td>
<td>0.480</td>
<td>0.024*</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>-0.032</td>
<td>0.381</td>
<td>-0.016</td>
<td>0.934</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>VPT</td>
<td>0.142</td>
<td>0.095</td>
<td>0.280</td>
<td>0.146</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>0.827</td>
<td>0.068</td>
<td>0.027</td>
<td>0.314</td>
<td>0.020*</td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>0.102</td>
<td>0.021</td>
<td>0.582</td>
<td>0.000*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPT</td>
<td>0.010</td>
<td>0.005</td>
<td>0.232</td>
<td>0.051*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.3. Summary of results

From the 500 sample initially selected, 48% were male (n=242) and 52% (n= 258) were female. The mean age was 55 ± 14 years and the mean BMI was 35 ± 9kg/m². Type I DM was present in 8% (n=38) only as opposed to 92% (n=462) who had Type II DM. From the sample randomly selected, 76% (n= 331) of the patients had
other problems associated with DM, the commonest being peripheral neuropathy (26%, n=186) followed by vascular insufficiency (20%, n=141).

When comparing the levels of pro-inflammatory markers and autoimmune antibodies between the three groups, the following was obtained:

- Highly significant differences between group 1, group 2, and group 3 in the levels of IL-6, IL-8, and IL-1β \( (p<0.001) \), highly significant differences in the levels of TNF-α and IFN-ɤ \( (p<0.01) \), and significant differences between the values of CRP \( (p<0.05) \) were noted.

- There were no significant differences in the levels of inflammatory markers between Group 2 and Group 3, suggesting that the increase in the level of markers may be related to the neurological manifestations in Group 1.

- There was highly significant association between the presence and absence of autoimmune antibodies (ANA) when all the three groups were compared. The odds of positive values of ANAs in Group 1 were 50 times higher when compared to Group 2 and Group 3.

Secondary analysis detected a number of correlations between the level of markers and the patients’ demographics. Within Group 1, systolic blood pressure presented a positive relationship with the values of TNF-α, and a negative relationship with the values of IL-6. Type II DM presented a weak positive correlation with the values of CRP in the blood serum of group 1.

Within Group 2, positive correlations were observed between the BMI and the level of CRP, and a negative correlation between BMI and TNF-alpha. In addition, there was a negative correlation between the age of the patient and the values of IL-8, and a positive correlation between systolic blood pressure and the values of CRP.
Interestingly, highly significant correlations were found to be associated with neurological characteristics in group 1 and the levels of CRP, IL-8, and IL-1β. Similarly, significant correlations were observed between the presence of ANA’s and the neurological characteristics in group 1.

Further analysis presented significant linear correlations between the levels of CRP and IL-1β and the NSS in group 1. Significant regressions were also presented between the presence of ANA’s and all the neurological manifestations of neuropathy in group 1 (NSS, NDS, and VPT).
Chapter Five: Discussion

Diabetic peripheral neuropathy is a very complex condition that carries multiple complications, including foot ulceration secondary to gait and microcirculatory changes, and poor quality of life.

This chapter discusses the results of the primary and secondary analysis in light of the study’s limitations. The last section of this chapter discusses the implications of the results and contribution to body of knowledge and clinical practice.

The goals of this chapter are to:

- Explore the demographics and characteristics of the DM population in the RMS-BDF Hospital and compare it to previous studies in Bahrain and the neighbouring countries;
- Discuss the results of hypotheses testing and describe the outcomes in relation to the previously mentioned theories on the pathogenesis of DPN;
- Compare the findings of hypotheses testing to previous research with similar or related hypotheses;
- Identify the relationships observed between the characteristics and demographics of the sample studied and certain inflammatory or autoimmunity markers, and explain the rationale behind it referring to literature review;
- Explain how the hypotheses testing might have been influenced by the relationship between demographics and clinical characteristics of the patients involved;
- Discuss the implication of the study’s findings to theory and clinical practice;
- Discuss the limitations of the study, and provide recommendations for future investigations.

The first section of the research consisted of a cross-sectional analysis aimed at exploring the general demographics and clinical characteristics of DM patients attending the RMS-BDF Hospital. The rationale behind this was mainly to gain an overall understanding of the population of patients at the hospital, and the proportions of DM complications among Bahraini individuals.

The clinical characteristics of the patients with DM attending the diabetic clinic presented a noticeable difference in the percentages of type I and type II DM. From the sample of 500 record cards, type I DM was only present in 8% (n=38) of the total population, while type II DM was present in 92% (n=462) (Results). This is in agreement with the epidemiological study conducted by Al-Mahroos and Al-Roomi (2007) who investigated the prevalence of diabetic complications in a cross-sectional study of 1477 DM patients in Bahrain, of whom 93% had type II DM. This supports the evidence that type II DM may now be one of the lead causes of adult morbidity in the Arabian Peninsula (IDF 2009).

Complications associated with DM were observed in more than half of the population sample, the highest being peripheral neuropathy followed by vascular insufficiency (26% (n=186), 20% (n=141), respectively). This was quite similar to the findings of Al-Mahroos and Al-Roomi (2007) where the overall prevalence rate of diabetic neuropathy was 32.3% in men and 38.1% in women, and the rates of peripheral vascular disease were 12.1% in men and 11.6% in women. The increased prevalence of DPN in the country justifies the importance of investigating this complication of DM complication.

The prevalence rate of DPN in Bahrain is considered high and similar to rates observed in other countries in the MER, with the highest incidence in Egypt (61.3%), followed by Jordan (57.5%), Lebanon (53.9%) and the Gulf States (37.1%) (Jambart
et al. 2011). These prevalence rates were higher than those from patients in the Western countries (United Kingdom and the United States), which reported prevalence rates of 15-20% (Boulton et al. 2004; Davies et al. 2006; Sadosky et al. 2008; and Veves et al. 2008). This may reflect the better management and DM care in Western countries from that currently practiced in the MER. A more recent study, however, investigated the prevalence of painful diabetic neuropathy in a large community-based diabetic population in the United Kingdom and noted that one-third of all community-based DM patients have painful neuropathy symptoms, regardless of their neuropathic deficit (Abbott et al. 2011). This rate was similar to the prevalence rate of neuropathy in DM patients attending primary health care centres in Bahrain (Al-Mahroos and Al-Roomi 2007).

The prevalence of DPN can vary widely, depending on the patient populations studied and the criteria used to define DPN (Tabatabaei-Malazy 2011). Higher rates are typically described in studies involving patients with longer duration of DM, and in patients with type 1 DM (Veves et al. 2008). It should be noted that the prevalence of neuropathy in patients with DM depends on the application of clinical examination and the electrophysiological criteria used in the administered diagnostic technique (Tabatabaei-Malazy 2011). In a 25 years follow up trial performed on 4400 patients with DM, the prevalence rate of neuropathy based on diagnostic criteria, such as, the absence of Achilles tendon reflex and the abnormal perception sensation was reported to be 7.5% at baseline. This rate reached up to 50% by the end of study (Pirart 1978). In the population-based Rochester Diabetic Neuropathy study, the prevalence of neuropathy was 54% in type I DM, and 45% in type II DM. DPN was diagnosed based on clinical examination, nerve conduction velocity measurement, and autonomic nervous system testing (Dyck et al. 1993).

Epidemiological cross-sectional studies are the most appropriate studies to draw valid conclusions regarding the prevalence of diabetic neuropathy if they are population-based and can obtain response rates (Ahmed 2010). In contrast, hospital-based studies may not reflect the true prevalence of this complication. It is estimated from a comprehensive collection of epidemiological studies that the prevalence of
neuropathy in patients with DM is approximately 30% in hospital patients and 20% in community patients (Shaw and Zimmet 1999). The current study may have been limited to one hospital in Bahrain; however, it was not designed to investigate the true prevalence of DM complications in the country. As previously explained, the aim of this part of the analysis was mainly to increase awareness of the DM complications within the population of DM patients attending the RMS-BDF Hospital. Furthermore, all the data obtained for this part of the research was collected from patients’ record cards, as described in Chapter two. Hence no clinical examinations were conducted at this point to further evaluate the presence of undiagnosed complications.

The most likely explanation for the higher prevalence of DPN in MER DM populations is that they might differ from Western populations in key clinical features that constitute risk factors for DPN development. For example, poor glycaemic control has been reported to be a significant risk factor for diabetic neuropathy in general and, specifically, for painful DPN both in Western studies (Harris et al., 1993 Smith & Singleton 2008) and in those conducted in the MER (Akbar et al. 2000; Al-Mahroos and Al-Roomi 2007). Random Blood Glucose in the DM population at the RMS-BDF Hospital displayed a mean of 10 ± 4 mmol/L, which is above the normal range (RBG normal values are between 4-7 mmol/L according to the ADA guidelines). This confirms the need for optimal care of DM patients in preventing complications. Moreover, the increased risk of neuropathy associated with hyperglycaemia is likely to account for the high proportion of cases of foot ulcerations in DM patients in Bahrain (Al-Mahroos and Al-Roomi 2007). The strong association between the presence of diabetic neuropathy and high levels of glycated haemoglobin suggests that poor glycaemic control is an important risk factor for diabetic neuropathy and foot ulcerations among Bahrainis (and likely other Arabian Gulf communities) (King et al. 2010).

Several studies have reported that diabetes-related knowledge is low and poor glycaemic control is common (exceeding 50%) among patients with DM in the MER (Habib and aslam 2003; Youssef et al. 2006; Al-Eqla 2009). In a cross-sectional
study that involved newly diagnosed type II DM patients, diabetic neuropathy was not related to levels of glycaemic control defined by glycated haemoglobin (Barbosa et al. 2001). Among Bahrainis, however, glycated haemoglobin remained a significant risk factor for diabetic neuropathy, even after adjustment for all other risk factors in the multivariate model performed by Al-Mahroos and Al-Roomi (2007).

Body Mass Index for the total population in the present study displayed a mean of 35± 9 kg/m². Most of the patients diagnosed with DM complications had a body mass index of more than 30 kg/m² (classified as obese). Obesity accompanying type II DM and hypertension are known to be closely linked with insulin resistance and elevated sympathetic nervous activity. It has been well documented that obesity, hypertension, and DM are high risk factors for subsequent cardiovascular and renal complications in DM (Masuo et al. 2010).

Many patients develop type II DM and hypertension, while they are obese, but not all DM patients have hypertension, indicating that insulin resistance is not only a mechanism for blood pressure elevation in DM-hypertensive patients (Tesfaye et al. 2010). Blood pressure measurements were within normal ranges with a mean of 147± 21 mmHg for systolic pressure, and 73 ± 15 mmHg for diastolic pressure, which was mostly controlled by antihypertensive medication. Several investigators have reported that sympathetic nervous activation relates to cardiovascular complications in patients with hypertension, DM, and obesity, and that sympathetic nerve activity accompanying insulin resistance is closely linked with left ventricular hypertrophy in healthy subjects. In addition, sympathetic nerve activation may predict future renal injury in healthy normotensive subjects (Masuo et al. 2010). Obesity, however, was not a significant risk factor for DPN in logistic regression analysis conducted by Katulanda et al. (2012) who investigated prevalence, patterns, and predictors of DPN in developing countries. Several studies from Asian countries have also reported similar results or no association between obesity and presence of DPN (Morkrid et al. 2010). Hence, further studies are required to define the role of body weight in DPN in the Asian population.
The majority of Arab countries are engaged in a multi-dimensional transition (demographic, economic, epidemiologic and geographic), as explained in Chapter one, section 13.2, and Chapter two, section 2.2. This 4-dimensional transition engendered many direct and indirect factors explaining the high prevalence of DM (Al-Mahroos and Al-Roomi 2007). The shift from rural to urban dominance leads to sedentary life and less physical exercise. The socioeconomic development allowed for higher income and more consumption especially of fast-food and western diet. Socio-cultural habits in the Arab population, including family or work related gatherings, might have also encouraged high calorie intake (Al-Mahroos and Al-Roomi 2007).

The rising rates of obesity and diabetes mellitus constitute a real challenge in the Arab region (Al-Mahroos and Al-Roomi 2007). In order to alleviate the burden of DM, preventive strategies are needed, based essentially on a more healthy diet with regular exercise. Health authorities are also obliged to provide populations with appropriate health-care and early diagnosis to avoid the high burden caused by complications of DM in terms of mortality and morbidity, and the increased loss of economic productivity.

5.2. Part 2 – Case Control analysis

The case control analysis aimed to investigate the relationship between DPN and pro-inflammatory and autoimmune markers by comparing the levels of specific markers in the blood serum obtained from the three groups. The study results demonstrated significant differences between the levels of pro-inflammatory markers in the blood serum obtained from the samples in the neuropathy group when compared to the two control groups. Similarly, there was a significant presence of seropositive autoimmune antibodies in the neuropathy group when compared to the other two control groups.
Despite the fact that an attempt was made to match the three groups’ demographics, recruiting participants with the exact similar variables in the limited time was unattainable. Significant differences in age, BMI and systolic BP were observed when the three groups were compared. Group 1 and group 2, however, matched in almost all variables, except the RBG and duration of DM. Comparisons between the duration of DM and the random blood glucose values between the three groups was not possible since samples in group 3 (healthy control) did not have diabetes mellitus. Accordingly, values were expectedly higher in groups one and two (DM groups) when compared with group 3 (healthy control).

The demographics of the samples obtained in this study matched the demographics of the samples in the study by Doupis et al. (2009) where BMI levels were higher in the neuropathy group when compared to the control groups (32.3 kg/m² in the neuropathy group, 31.2 kg/m² in the DM control, and 27.2 kg/m² in the healthy control group). Similarly, systolic BP measurements in their study were higher in the neuropathy group when compared to the two control groups (146±19 mmHg in neuropathy group, 126±16 mmHg in DM control, and 122±16 mmHg in healthy control).

5.2.1. DPN and pro-inflammatory markers

One of the main findings of the present study was that DM patients with peripheral neuropathy had increased serum levels of pro-inflammatory markers. The findings revealed significant difference between group 1, group 2, and group 3 in the levels of IL-6, IL-8, and IL-1β (p<0.001), significant differences in the levels of TNF-α and IFN-γ (p<0.01), and a significant difference between the values of CRP (p<0.05) (rejecting null hypothesis one). When each two groups were further evaluated, the levels of the pro-inflammatory markers were significantly different between group 1 (DM neuropathy group) and group 2 (DM Control) and between group 1 and group 3 (healthy control). The differences in the level of markers were not noticeable when groups 2 and 3 (DM control and healthy control) were compared. This suggests a
possible relationship between the levels of pro-inflammatory markers and the neurological features of the sample in group one.

As previously discussed in Chapter two, chronic inflammation is thought to be a characteristic feature seen at sites of DM complications (Forbes and Cooper 2013). In clinical studies, circulating inflammatory markers were increased in patients with type I and type II DM (Younger et al. 1996), and the levels of these markers appear to predict the onset and progression of DM complications (Forbes & Cooper 2013).

Inflammatory mediators such as cytokines and C-reactive proteins have been linked to many forms of neuropathic disorders (Navarro and Mora 2005). Hyperglycaemia is known to stimulate the release of inflammatory cytokines from various cell types and can lead to the induction and secretion of acute-phase reactants by adipocytes (Pradhan et al. 2001; Spranger et al. 2003). Marvidis et al. (2008) have reported that patients with metabolic syndrome have a significantly higher hsCRP level than those without.

Pro-inflammatory cytokines are produced locally by resident and infiltrating cells (Forbes and Cooper 2013). These molecules exhibit pleiotropic effects on homeostasis of glia and neurons in the central, peripheral and autonomic nervous systems, as discussed in Chapter two. Changes induced by chronic hyperglycaemia may lead to dysregulation of these cytokines. An increase in the levels of cytokines may ultimately lead to the development of inflammation. It has been demonstrated that endogenous TNF-α production, for example, is accelerated in micro-vascular and neural tissues, which may undergo increased micro-vascular permeability, hypercoagulability and nerve damage, thus initiating and promoting the development of characteristic lesions of diabetic peripheral neuropathy (Kolla et al. 2009).

A number of experimental and clinical studies have demonstrated that DPN exhibits signs of inflammation (Tesfaye et al. 2010). As discussed earlier in Chapter two, three recent papers support the fact that inflammation may play a significant role in
the pathogenesis of DPN (Kolla et al. 2009; Doupis et al. 2009; Herder et al. 2009). This will be further illustrated in the sections below.

5.2.1.1. C-reactive protein and DPN

C-reactive protein measurements have been used for decades to evaluate the level of inflammation (Kang et al. 2005). The serum high sensitivity CRP (hsCRP) levels in DM patients are known to be higher than in normal subjects (Schalkwijk et al. 1999). In this study, the median value of serum hsCRP concentration in group one (neuropathy group) was significantly higher than the two control groups (5 mg/L in group one, and below 1 mg/L in groups two and three). This is similar to the results documented by Doupis et al. (2009) and Herder et al. (2009) where CRP levels were elevated in the neuropathy groups when compared to the control groups.

C-reactive protein is a type of circulating protein that is released by the liver in response to inflammation (Yeh 2005). It is a pattern recognition receptor whose physiological role is to activate the complement system (Thompson et al. 1999). C-reactive protein is thought to be a sensitive biomarker for cardiovascular disease (CVD) (Pepys et al. 2006), but it has also been suggested that this molecule could be selectively targeted as a therapy for CVD (Pepys et al. 2006).

According to NHANES III (the Third National Health and Nutrition Examination Survey), the mean serum hsCRP concentration in adults over 20 years is 4.14 mg/L (Ross 1993). Studies which investigated the values of hsCRP in healthy subjects reported that the median serum hsCRP level varies between 0.5mg/L to 4mg/L (Hashimoto et al. 2001; Kang et al. 2005). A number of studies presented similar values of CRP when the study was conducted among people with similar ethnicity suggesting there might be ethnical differences in the serum hsCRP level (Hashimoto et al. 2001; Kang et al. 2005).
The association between the serum hsCRP levels and the micro-vascular complications of DM, such as retinopathy, neuropathy and nephropathy was evaluated by Kang et al. (2005). There were no differences between the mean hsCRP levels of those with and without retinopathy in their study. Likewise, there were no differences between the mean hsCRP levels of those with and without neuropathy. However, significant differences between the mean hsCRP levels of those with and or without nephropathy were noted.

There are many factors that affect the serum CRP levels. Sesmilo et al. (2000) reported that C-reactive protein is known to be associated with DM and cardiovascular diseases. An investigation by King et al. (2003) demonstrated that a higher HbA1c is significantly associated with a greater likelihood of higher CRP among adults with DM. In their study, the likelihood of elevated CRP concentrations increased with increasing HbA1c levels, suggesting an association between glycaemic control and systemic inflammation in people with established DM. Other studies (Han et al. 2002; Tamakoshi et al. 2003) have emphasized the concept that a pro-inflammatory state is one component of the metabolic syndrome in DM. The reasons for the link between inflammation and DM are not fully understood. One possible mechanism is that adipocytes in obese patients with DM release into the circulation high amounts of TNF-α and IL-6 (Pickup et al. 1997), which stimulates the production of CRP by the liver and induce insulin resistance. Insulin resistance itself is responsible for the higher level of cytokine production (Mclaughlin et al. 2002). In the present study, no significant differences were detected between the DM control and the healthy control groups. These findings are in agreement with King et al. (2003) who suggested that raised CRP levels are associated with high levels of HbA1C and therefore are only elevated in uncontrolled DM patients. The mean RBG levels in group 2 (DM control) was 10±4 mmol/l, with values ranging between 6 and 18mmol/l. According to American Diabetes Association, a non-fasting RBG of 10mmol/l in patients with DM is considered controlled DM (ADA 2006).
One might argue that the raised inflammatory markers such as CRP might only be evident in the early stages of the diagnosis and the development of the disease. However, the results of Kings et al. (2003) showed that among people with established DM, at successively higher levels of HbA1c the percentage of people with hsCRP >0.30 mg/l was significantly higher. They suggested that inflammation may not only be implicated in the development of DM, but also in ongoing levels of hyperglycaemia once DM is established.

Kang et al. (2005) also reported that smokers have high levels of CRP, IL-6, and soluble intercellular adhesion molecule type-1 which decrease after the cessation of smoking. Smith et al. (1999) reported that the CRP level decreased by 53% after long-term exercise in those at risk of developing ischemic heart disease. C-reactive protein levels were also found to increase after growth hormone replacement therapy (HRT) for the treatment of growth hormone deficiency (Kang et al. 2005). In general, oral postmenopausal hormone therapy (HT) increases CRP levels, whereas transdermal HT does not (Sowers et al. 2003; Hemelaar et al. 2007). To date it is unclear as to whether the rise in CRP observed after oral HT is actually caused by the HRT or by the hormonal imbalance in menopausal status. The effects of endogenous sex hormones on CRP are less well characterized (Crandall et al. 2006). A recent review, however, concluded that changes in CRP across the menopausal transition could not clearly be attributed to hormonal changes (Stork et al. 2008). Fat tissue expresses aromatase, and conversion of testosterone to oestrogen by aromatase is one of the main sources for endogenous oestrogen in postmenopausal women (Davidson et al. 2003). The evaluation of direct effects of endogenous oestrogen levels on CRP is complicated because adipose tissue produces inflammatory mediators that increase CRP-production in the liver (Purohit and Reed 2002). Some studies found that increased levels of CRP were associated with body fat, but not with menopausal status (Sites et al. 2002).

Various medications also have several effects on the CRP levels. Medications such as 5-Acetylsalicylic acid (aspirin) (Takeda et al. 2003), 3-hydroxy-3-methylglutaryl (HMG) CoA reductase inhibitor (statin) (Ridker et al. 1998; Takida et al. 2003),
angiotensin-converting enzyme inhibitor (ACEI) (Di Napoli and Papa 2003), and thiazolidinedione (Fuell et al. 2001) decrease the CRP levels, while HRT in postmenopausal women increases them (Cushman et al. 1999). One of the limitations of the present study was that although patients on such medications were excluded from the study, one could not control the effect of external variables such as the medications used for the control of DM. Antidiabetic agents reduce CRP concentration in addition to their primary effect of lowering blood glucose (Dandona 2008).

5.2.1.2 Cytokines and DPN

Peripheral nerve complications due to irregular cytokine production are one of the eminent factors in many inflammatory diseases (Manandhar et al. 2002; Miesse et al. 2004). Studies have identified polymorphisms in cytokine gene regulatory regions that correlated to intra-individual variations in cytokine production (Hutchinson et al. 1999; Sankaran et al. 1999). Over twenty years ago, it was proposed that the differential production of cytokines will eventually amend the downstream signalling processes that could directly or indirectly affect nerve functions which could lead to neurodegeneration (Hartung 1993). These mediators are pro-inflammatory cytokines that share several biological properties. They normally are practically absent from the nervous system but can become upregulated under neuropathic or inflammatory conditions (Jansky et al., 1995).

5.2.1.2.1 Interleukins

Interleukins (IL) are a group of cytokines named for their ability to communicate between leucocytes. In recent years, research has shown their involvement in cell signalling in a number of other cell types and tissues (Wilson and Wright 2011). There are more than 30 interleukin isoforms currently identified that can have pro-inflammatory (IL-1, IL-6 and IL-8) or anti-inflammatory (IL-4 and IL-10) actions.
Interleukins have been shown to be involved in a number of different neuropathic conditions in both animal and human studies (Uceyler et al. 2010).

The results of the present study confirm the hypothesis that interleukins are elevated in patients with DPN (group 1 was significantly higher than group 2 and group 3) \( (p<0.001) \). In group 2 (DM control) the levels of interleukin-8 was higher than the levels observed in group 3 (healthy control) proposing that this marker may also be related to pathogenesis of DM which has been confirmed by previous studies (Huseynova et al. 2009). Interleukin-6 and interleukin-1β, however, displayed lower values in the DM group when compared to the healthy control. This might be a consequence of the pharmacological management of DM patients which may contribute to the reduction in the levels of pro-inflammatory cytokines as has been proposed by Kewcharoenwong (2013).

While significant research has been undertaken in other neuropathic conditions, research on the role interleukins play in DPN is just beginning to be explored. Rodent studies utilizing streptozotocin (STZ) injections to induce DPN have revealed increased in IL-6 mRNA levels in both the DRG and sciatic nerve compared to non-diabetic mice (Bierhaus et al. 2004; Yamakawa et al. 2011). Similarly, clinical findings utilizing sural nerve biopsies from DPN patients demonstrated increased IL-6 expression compared to healthy controls (Bierhaus et al. 2004).
Associations of CRP and IL-6 with cardiac autonomic neuropathy have been previously reported in two small studies in patients with type 1 DM (Gonzalez-Clemente et al. 2007; Lanza et al. 2007). Furthermore, significant associations of subclinical inflammation with some of the individual neuropathic impairments were described by Herder et al. (2009). Patients with diabetic peripheral neuropathy in their study had higher levels of C-reactive protein (CRP) ($p < 0.013$), interleukin (IL)-6 ($p < 0.0091$), and interferon-γ inducible protein-10 ($p < 0.039$) compared with those in patients without diabetic peripheral neuropathy, whereas leucocyte count and levels of serum amyloid A, IL-18, tumour necrosis factor-α, adiponectin, IL-8, and monocyte chemo-attractant protein-1 did not differ significantly.

Herder et al. (2009) reported that both CRP and IL-6 were highly significantly associated with the continuous neuropathic scores according to the MNSI score system in univariate analyses and in multiple linear regression models that adjusted for anthropometric, metabolic, and lifestyle factors. The most consistent associations with individual neuropathic deficits were observed for CRP and IL-6, and some associations between IL-18 and neuropathic deficits were found. High levels of CRP or IL-6 were associated with impaired ankle reflex, high vibration perception threshold, abnormal appearance of feet, and impaired pain perception (pinprick) (Herder et al. 2009).

Interestingly, impaired temperature perception and pain or discomforts in the lower limbs were not associated with any of the measured immune mediators in the study by Herder et al. (2009). The authors indicated that the association between subclinical inflammation and diabetic peripheral neuropathy may only affect certain components of DPN, whereas others may be independent of immune activation (Herder et al. 2009).

The association between inflammation and DPN appears relatively complex because although in the present study CRP, IL-6, IL-8, and IL-1β were higher in the neuropathy group, Herder et al. (2009) in his study noted that CRP and IL-6 levels were associated with DPN, whereas for IL-18, an inverse association was found. C-
reactive protein’s main inducer is IL-6 (Kang et al. 2005). This relationship was reflected by the high degree of correlation between these two mediators in their study.

Clinical studies in small-fibre neuropathy patients demonstrated a two-fold increase in circulating IL-2 mRNA levels in peripheral blood compared to healthy controls (Uceyler et al. 2010). Skin samples from the affected area were collected from patients, which showed increased IL-6 and IL-8 mRNA levels compared to controls. Further analysis comparing the skin affected by neuropathy with the control revealed increases in IL-1β, IL-6, and IL-8 mRNA levels. This increase was twofold, greater than 200 fold, and greater than 500 fold, respectively, when compared to the control (Uceyler et al. 2010). Patient selection in this study, however, included different kinds of peripheral neuropathies including, even the ones not associated with DM. Expansion of these findings with particular focus on how interleukins contribute to the symptoms of DPN such as nerve conduction velocity and fibre loss is still needed to further understand their roles in DPN pathogenesis.

5.2.1.2.2. Tumour Necrosis Factor α (TNF-α)

Tumour necrosis factor-α (TNF-α) was originally discovered as a monokine produced by macrophages (Wilson and Wright 2011). It was subsequently revealed that various cells, such as fibroblasts, epithelial cells, adipocytes, and myocytes, also produce TNF-α, which has a variety of biological activities (Wilson and Wright 2011). It has been indicated that TNF-α plays a role in the pathogenesis of not only type 1 diabetes mellitus (Rabinovitch 1994) but also type II diabetes mellitus (Hotamisligil and Spiegelman 1994). Studies have further disclosed that this peptide contributes to the development of DM complications (King and Brownlee 1996).

Tumour necrosis factor-α is a pro-inflammatory cytokine that has been implicated in neuropathic and inflammatory nociceptive conditions for a number of years (Wilson and Wright 2011). This link stems from the ability of TNF-α administration to induce
ectopic firing in sensory neurons (Sorkin et al. 1997) and mechanical and thermal nociceptive behaviour (Wagner and Myers 1996) in rodents. More confirmation of the link between TNF-α and nociceptive behaviour is evidenced by the fact that TNF-α mRNA expression corresponds to the time course of the development of thermal and mechanical behaviour in a neuropathic pain model using chronic constriction injury (Okamoto et al. 2001).

Recently TNF-α has also been linked to diabetic neuropathy. Clinical studies have shown increased TNF-α plasma protein and mRNA levels in DM patients compared to controls (Navarro et al. 2006; Purwata 2011). Increased TNF-α macrophage expression and plasma levels were also demonstrated in DN patients compared to controls and was correlated to pain intensity (Purwata 2011).

Exploration of these clinical findings in rodent models has been key to understanding its role in DM (Wilson and Wright, 2011). STZ induced DPN in rodents results in increased circulating levels of TNF-α (Sharma et al. 2007) and the consequence of increased TNF-α expression was explored in STZ-injected rats. Moreover, TNF-α administration into the sciatic nerve induced a reduction in motor nerve conduction velocity (MNCV), a symptom commonly witnessed in patients with DN (Satoh et al. 2003).

The role of TNF-α in the symptoms of DPN has been validated in studies utilising TNF-α null mutant mice. TNF-α deficient diabetic mice failed to develop changes in nociceptive behaviour, MNCV, and sensory nerve conduction velocity (SNCV) compared to diabetic mice with wild type TNF-α expression (Yamakawa et al. 2011). To further characterize TNF-α role in diabetic neuropathy, neutralization studies were conducted using the TNF-α neutralising anti-body, infliximab. Infliximab treatment following STZ injections recovered MNCV and SNCV losses, tail flick nociceptive behaviour, and prevented a loss of epidermal nerve fibres compared to STZ-injected control animals (Yamakawa et al. 2011). Treatment with the TNF-α neutralising antibody was also effective in reducing circulating TNF-α serum levels and TNF-α mRNA expression back to normal animal levels (Yamakawa et al. 2011).
Thus, these important and neutralizing studies highlight a likely important role of TNF-α in the development of DPN.

TNF-α levels in the current study showed significant increase in the neuropathy group when compared to the other two groups. Although levels in the DM control group were still higher than the healthy control it was still significantly lower than the levels obtained in group one. This is similar to the results obtained from a study by Doupis et al. (2009) where the levels of TNF-α were 7.1 mg/L in the neuropathy group, 5.1 mg/L in DM control group, and 4.7 mg/L in healthy control.

Inflammation, as previously described in Chapter two, is a well-known risk factor for the development of macro-vascular disease. Studies have shown the vascular reactivity of the skin microcirculation (which includes both endothelium-dependent and -independent vasodilation) is impaired in patients with type II DM and in subjects at risk of developing type II DM, and this impairment is associated with inflammatory cytokines (Doupis et al. 2009). Doupis et al. (2009) also indicate an association between a variety of inflammation markers and the development of peripheral diabetic neuropathy.

Tumour necrosis factor-α overproduction has demonstrated the pathogenicity in diabetic neuropathy (Satoh et al. 2003). It has been suggested that its overproduction stimulates the synthesis of other pro-inflammatory cytokines such as interferon gamma (IFN-γ) which has a pivotal role in the induction of immune mediated inflammatory response (Billiau et al. 1998). TNF-α has been found to induce neuronal damage (Nilsson et al. 1998) and is considered as a major initiator of inflammation. In pre progression stage of diabetic peripheral neuropathy circulating levels of TNF-α are elevated in type II DM (Nilsson et al. 1998).

Furthermore, tumour necrosis factor-α has been implicated in contributing to insulin resistance in obesity due to increased expression in adipose tissue (Uysal et al. 1997). Obese mice with TNF-α mutation displayed improved insulin sensitivity and lowered circulation fatty acids, improving obesity-induced glucose tolerance in a study by
Uysal et al. (1997). Increased plasma TNF-α is also proposed to be associated with the progression of diabetic nephropathy, suggesting continued expression of these cytokines contribute to diabetic micro-vascular complications (Purwata 2011). Similar experiments evaluating TNF-α in null mice, however, showed that they are less susceptible to developing diabetic complications (Gao 2007). Targeting TNF-α through pharmacological means may potentially reverse the deleterious effects of DPN and other DM complications (Wilson and Wright 2011).

5.2.1.2.3. Interferon-gamma (IFN-ɤ)

In the present study, IFN-ɤ was significantly higher in group 1 (DM neuropathy) when compared to the two control groups ($p<0.001$), and the values in group 2 and 3 were almost identical.

Interferon-gamma is a pivotal pro-inflammatory cytokine that has a role in the induction of immune mediated inflammatory response (Billiau et al. 1998). It has been noted that polymorphism plays a fundamental role in the induction of IFN-ɤ production (Govan Vandana et al. 2002). The T allele of IFN-ɤ provides a binding site for the transcription factor NF-kβ (nuclear factor kappa B), which is able to regulate IFN-ɤ expression (Pravica et al. 2000). This protein plays an important role in the transcriptional regulation of IFN-ɤ gene (Regarajan et al. 2002). It is possible that low IFN-ɤ production will facilitate an immune response against inflammation rendering these individuals more susceptible to the disease as the downstream process would eventually lead to nerve damage. Studies investigating IFN-ɤ and its contribution to diabetic complications are however limited.

A previous report from the North Indian population evaluating the expression and polymorphism of IFN-ɤ in patients with cervical cancer detected a profound increase in the distribution of IFN-ɤ homozygotes in patients with diabetic neuropathy demonstrating a twofold increased risk and suggested that this could be a risk factor
for susceptibility to diabetic peripheral neuropathy in patients with DM (Kordi Tamandani et al. 2008).

5.2.2. DPN and autoimmune antibodies

The most significant finding was that the present study demonstrates for the first time at a population level, that autoimmune antibodies may be associated with the presence of DPN. Further studies will be needed to investigate the cause and effect relationship between DPN and Autoimmune antibodies. There was a highly significant difference between the percentages of positive and negative autoimmune antibodies (ANA) when all the three groups were compared. The odds of positive values of ANAs in group one (neuropathy group) was 50 times higher when compared to group two and group three.

To our knowledge, there are no studies investigating the presence of autoimmune antibodies in the blood serum of patients with DPN after excluding other causes. A few examples of diabetic neuropathy confirmed by nerve tissue obtained at biopsy or post-mortem examination was available to allow clinic-pathological correlation of the different neuropathic syndromes (Vinik et al. 2005). In 1996, Younger (2010) reported clinic-pathological and immune-histochemical findings in 20 patients with heterogeneous forms of diabetic neuropathy. This was followed at a later stage to include a total of 107 patients who underwent detailed clinic-pathological assessment (Younger 2010). Nerve biopsy in this study revealed the underlying histopathology, including cell and humoral-mediated immunological lesions in the majority of patients. When combined with clinical and laboratory studies, nerve biopsy has shown to have the potential to assist in the selection of patients who may benefit from immunomodulatory therapy. The sample of the patients included in this study were DM patients with different types of neuropathies including: DPN, DLRPN, and MNM. Despite the fact that Younger (2010) suggested an autoimmune pathological mechanism associated with DM patients diagnosed with neuropathy, it was still not clear whether DPN on its own was associated with autoimmunity.
Autoimmunity is the major cause of type 1 DM, which results from an autoimmune destruction of pancreatic β-cells (Winer et al. 2003). Neurons and pancreatic β-cells are neuroectodermal derivatives and therefore share common antigens, especially in the early stages of cellular evolution (Winer et al. 2003). Winer et al. (2003) stated that the pancreatic islets of Langerhans are surrounded by a Schwann cell sheath. There may be a direct destruction of neurons by the same autoimmune process in DM. Components of the peri-islet Schwann cells include GAD (Donev 1984). There is an early appearance of anti-GAD65–specific T-cells in type 1 DM, and is therefore a strong predictive marker for the onset of type 1 DM (Winer et al. 2003). Presence of this antibody in patients with recent-onset type 1 DM is associated with worse glycaemic control and worse peripheral nerve function, suggesting a common mechanism for β-cell and neuronal damage (Hoeldtke et al. 1997).

Patients with high GAD65 antibodies were shown to have positive correlation with reduced motor nerve conduction velocities, prolonged F wave latencies, increased thermal threshold detection, and reduced cardiovascular autonomic function (Hoeldtke et al. 2000). However, many studies have failed to show any significant relation of GAD antibodies to the development of neuropathy (Vinik et al. 2005). Jaeger et al. (1997), for example, concluded that GAD antibodies had no effects on residual β-cell function or diabetic neuropathy, and that there is no association between GAD antibodies or even islet-associated protein cell antibody with autoimmunity to nervous tissue structures or cardiac autonomic functions (Jaeger et al. 1997).

Pittenger et al. (1995) however reported in their study that serum collected from type 1 DM patients is toxic to neuroblastoma cells. About two-thirds of this toxicity was due to autoimmune serum factors (Pittenger et al. 1995). One of the components of this serum that mediates immune destruction of neuroblastoma cells in cultures was found to be Fas-specific IgG antibodies. These antibodies bind to Fas-ligand on the surface of neuroblastoma cells and induce apoptosis (Pittenger et al. 1995).
Moreover, serum from patients with diabetic neuropathy contains an activator of Fas-regulated apoptosis that may contribute to the pathogenesis of diabetic neuropathy (Pittenger et al. 1997). Furthermore, Vinik et al. (1995) previously found that sera with high titers of phospholipase antibody inhibited the growth and differentiation of neuroblastoma cells in culture. Unfortunately, this is so common that the issue has been raised that phospholipase antibodies do not directly contribute to nerve damage and that they are formed as a result of antigen release from tissue damage.

The relationship between autoimmunity and development of autonomic neuropathy has historically been stronger than somatic neuropathies and was first suggested in the early 1980s with the report of coincident autoimmune iridocyclitis and diabetic autonomic neuropathy (Guy et al. 1984). Granberg et al. (2005) provide epidemiological data to support the implication of autoimmunity in autonomic neuropathy. They examined 41 patients for a period of 14 years and assessed, among other measures, heart rate variability, vasoconstrictor response to cooling, and acceleration of the brake index, which was measured as the heart rate reaction to postural change at intervals over 14 years. Fifty-six percent of patients had antibodies to autonomic nervous system of some sort: sympathetic ganglion, vagus nerve, or adrenal medulla. What the authors show is that an index of autonomic neuropathy is 7.5 times more likely to become abnormal in patients who are autoantibody positive than those who are autoantibody negative. However, the authors did not determine whether the patients had autonomic nervous system antibodies in their first visit, and whether this correlated to the progression of autonomic neuropathy. In certain subtypes of type 1 DM, antibodies that are present in the beginning subsequently disappear. It is difficult to argue post hoc ergo propter hoc because antibodies may be secondary to damage to the autonomic nervous system that is occurring for other reasons. Nonetheless, this is a very interesting report suggesting that antibodies predict the evolution and development of autonomic nervous system dysfunction. The authors fall short of demonstrating that the course of autonomic neuropathy can be reversed by therapies directed at autoimmune neuropathy and, for this reason, the authors believed that their results do not fulfill the criteria for cause and effect evident with proximal neuropathies (Granberg et al. 2005).
Since type I DM is an autoimmune disease one would assume that all the positive ANAs detected were in the blood serum of patients diagnosed with type I DM (type I DM was 43\%(n=13)) in the neuropathy group. Interestingly, however, only 7 out of the 13 type I DM patients had positive ANA, while the other positive results were obtained from 11 of the type II DM patients in this group. The possible rationale behind this could be the fact that the patients attending DM clinic were diagnosed in the system with type I DM based on their insulin uptake despite the fact that they did not carry an autoimmune disease, and that they were misclassified into type I DM when they should have been type II DM. Moreover, a number of patients with type II DM are GAD antibody positive (Radtke et al. 2009). These individuals have been referred to as having LADA (latent autoimmune diabetes in adults) or type 1.5 DM (Carlsson et al. 2007). However, there is limited information regarding the phenotypic and metabolic characteristics of these patients in comparison to the majority of patients with type II DM who are GAD antibody negative (Romkens et al. 2006).

Autoimmunity is assumed to be the major cause of LADA because this category of diabetes shares biochemical markers of β-cells directed autoimmunity (Carlsson et al. 2007). LADA is considered a “mild” form of type I DM. Mild indicates the fact that patients with LADA do not by clinical judgment need insulin from the time of diagnosis. However, within the first few years after the diagnosis of diabetes a need for insulin treatment develops in many patients with LADA (Zinman et al. 2004). This distinguishes patients with LADA from those with non-autoimmune type II DM where a need for insulin treatment for them develops later than in those with LADA (Turner et al. 1997). An early need for insulin treatment in patients with LADA points to ongoing autoimmune mediated destruction of β-cells in these patients (Zinman et al. 2004).

Since ANA profile analysis was not conducted in this investigation due to time and financial restriction, it was not possible to distinguish which types of anti-nuclear antibodies were detected in the sample of patients with DPN. It remains
questionable whether the ANAs detected in the neuropathy sample were in fact antibodies related to DM itself or antibodies related to neuropathy.

In addition, multiple studies have shown ANA positivity to be highly prevalent in both the general and various patient populations (Abeles and Abeles 2013). In a Belgian study by Verstegen et al. (2009) 42.6% of 6422 consecutively tested patients at a large community hospital were ANA-positive, and Marin et al. (2009) demonstrated the prevalence of ANA positivity in a group of 304 healthy individuals to be 54.3%. Fernandez et al. (2003) demonstrated ANA positivity in 22.6% of normal blood donors, and Peene et al. (2001) found that 23.5% of 10,550 consecutive patients referred for ANA testing at a single medical centre had a positive result. Hayashi et al. (2008) notes that even when the cut off for ANA positivity is defined more rigorously the rate of ANA positivity in the general population remains fairly high (at approximately 9.5%) (Hayashi et al 2008).

Abeles and Abeles (2013) noted that a test that is positive in approximately half of the population results in an unacceptably high rate of false positivity, and a poor positive predictive value when ordered in the face of relatively low pre-test probability. This could be another limitation of the present study.

5.3. Part 3- Exploring correlations between immunological markers and sample demographics

This section of the study looked at whether the levels of pro-inflammatory and autoimmune markers were associated with the sample demographics and neurological features and hence influenced the study results. Secondary analysis through correlation co-efficient detected a number of correlations between the levels of markers and patient’s demographics and neurological features which will be discussed shortly.
5.3.1. Pro-inflammatory markers and sample demographics.

As described earlier in section 4.2 in Chapter four, systolic BP readings were slightly higher in the sample in group one (neuropathy group) in comparison to the two control groups. Correlations co-efficient analysis further demonstrated a weak positive correlation between the values of systolic BP and levels of TNF-α, and a weak negative relationship with the values of IL-6 in group one (neuropathy group) ($p<0.05$).

Tumour necrosis factor-α plays a significant role in the initial activation of the immune system, and its release is stimulated by several factors, including IL-1β and bacterial endotoxin (Kofler et al. 2005). Of note, intra-arterial TNF-α has been shown to cause an acute local vascular inflammation that is associated with impaired endothelium-dependent relaxation (Chia et al. 2003).

There are a number of studies to date that have looked into the potential association between increasing TNF-α and hypertension. Seven reported a positive association with TNF-α and hypertension (Yudkin et al. 1999; Ito et al. 2001; Fernandez-Real et al. 2002; Furumoto et al. 2002; Chrysohoou et al. 2004; Bautista et al. 2005 and Stumpf et al. 2005) with two reporting negative results (Mendal et al. 1997; and Sheu et al. 2000). There is also evidence to support the synthesis of TNF-α in adipose tissue (Bullo et al. 2003). This may contribute to both the maintenance of a chronic low-grade inflammatory state in obese patients and to the associated co-morbidities, such as hypertension (Bullo et al. 2003; Ferroni et al. 2004). In one study of 368 individuals, the ratio of soluble TNF-α receptors, correlated positively with both systolic and diastolic blood pressure, and reduction in blood pressure equated with a fall in this ratio (Ohta et al. 1998).

The values of the systolic BP readings in group one however does not necessarily imply a hypertensive phase since the rise in systolic BP was not significant for the diagnosis of hypertension (Chobanian et al. 2003) and the diastolic BP was within the normal range. Of interest, the Attica study demonstrated an association between
elevated TNF-α levels and pre-hypertension, even after correcting for multiple comparisons and adjusting for age, body mass index, blood lipids, glucose, food groups consumed, and other potential confounders (Chrysohoou et al. 2004).

Interleukin-6 stimulates the synthesis of several acute-phase reaction proteins, including CRP, Serum amyloid-A and fibrinogen, and counter regulates TNF-α and IL-1β (Tilg et al. 1997). It is also known that IL-6 prolongs endothelial dysfunction, which may in turn lead to increasing peripheral vascular resistance and consequent hypertension (Brevetti et al. 2003). However, the data linking increased IL-6 to hypertension is less convincing than that for TNF-α, and of the published studies, some have reported a positive association between IL-6 and hypertension (Fernandez-Real et al. 2002; Stumpf et al. 2005; and Bautista et al. 2005) with three reporting negative results (Mendal et al. 1997; Refai et al. 1999; and Furumoto et al. 2002). Thus, the role of raised systolic BP in increasing the levels of IL-6 remains controversial.

A positive correlation between systolic blood pressure and the value of CRP was also presented in group two (DM control) sample. There is a large body of evidence indicating that inflammation plays a crucial role in all steps characterizing the atherosclerotic process. C-reactive protein is a circulating marker of inflammation which recently emerged as a powerful independent determinant of cardiovascular events (Rost et al. 2001). Hypertension is closely linked to inflammation. Experimental data and results from cross-sectional studies in humans indicate a relationship between CRP levels and blood pressure. In particular, CRP seems to be related with markers of arterial stiffness, thus suggesting a specific interaction between CRP and systolic blood pressure (Virdis et al. 2007).

The evidence to support an independent association between CRP and hypertension is also overwhelming, and far stronger than for any other studied inflammatory marker (Boos and Lip 2006). In contrast, published data negating this association are derived from only four studies, which have included low numbers of hypertensive patients (Mandel et al. 1997; Hak et al. 1999; Onat et al. 2001; Bautista et al. 2005).
The relationship between elevated hsCRP and hypertension is not exclusive to blood pressure within the so-called ‘hypertensive range’ (Boos and Lip 2006). Several studies have recently provided further support to the inflammation/hypertension association by demonstrating higher hsCRP levels among patients with blood pressure in the ‘pre-hypertension’ stage (that is, systolic blood pressure (BP) 120-139mmHg or diastolic BP 80-89 mm Hg) when compared with normotensive subjects (Chobanian et al. 2003; Chrysohoou et al. 2004; King et al. 2004). In a large cross sectional study, for example, King et al. (2004) demonstrated that pre-hypertensive participants (systolic BP of 120-139 mmHg and/or diastolic BP 80-89 mmHg) had a higher prevalence of elevated hsCRP levels than normotensive subjects.

Some studies have examined the inter-relationships of the different inflammatory markers in hypertension. For example, Bautista et al. (2005) examined the cross-sectional relationship between IL-6, TNF-α, and hsCRP and hypertension in a random sample of 196 healthy subjects and showed that after adjusting for other risk factors, IL-6 and TNF-α, hsCRP were not significantly associated with hypertension. Whilst this was a relatively small study and only includes 79 hypertensive patients, it is the only study to date to specifically analyse the potentially confounding relationship between several inflammatory markers and hypertension.

Interestingly, although systolic BP in group 1 was higher than group 2, no association between systolic BP and CRP levels in group 1 were noted. This might be related to the anti-diabetic medications taken in this group which might have been lowering the levels of CRP in the blood serum.

Positive correlations were observed between the BMI values and the levels of CRP in group two (DM control). This was similar to the study by Visser et al. (1999) who noted a higher prevalence of low-grade systemic inflammation (detected by increased CRP levels) which was observed in overweight and obese persons when compared with normal-weight persons in a population of young adults aged 17 to 39
years, in whom the prevalence of any confounding subclinical disease is generally very low. Similarly, more recent observational analyses showed a strong, positive association between circulating CRP and BMI (change in BMI for a doubling in log CRP of 1.03 kg/m², \( p<0.0001 \)) (Timpson et al 2011). These data suggest that the observed association between circulating CRP and measured BMI is likely to be driven by BMI, with CRP being a marker of elevated adiposity. Both groups 1 and 2 sample in the current study consisted of patients within the overweight and obese BMI ranges. C-reactive protein levels in group one (neuropathy group), however, demonstrated no correlations with increased BMI levels.

A negative correlation between BMI and TNF-\( \alpha \) was observed in group two (DM control) sample. This was in disagreement with previous studies which have revealed a positive correlation between obesity and the levels of inflammatory markers including TNF-\( \alpha \) in DM patients suggesting an inflammatory mechanism in DM, especially, type II DM (Rodriguez-Moran and Guerrero-Romero 2004; Nystrom et al. 2006; Mirza et al. 2012). Hotamisligil et al. (1995) first demonstrated that mRNA for TNF-\( \alpha \) is elevated in adipose tissue of obese humans and that over two hour incubation there was greater release of TNF-\( \alpha \) by explants of adipose tissue from obese sample. However, Bruun et al. (2002) reported that obese humans with an average BMI of 39 kg/m² had a significant fall in plasma TNF-\( \alpha \) after 20 weeks of weight reduction. Moreover, Mohamed-Ali et al. (1999) could not find any TNF-\( \alpha \) in a vein draining subcutaneous adipose tissue suggesting that TNF-\( \alpha \) is not elevated by adipose tissue. Fain et al. (2004) indicated that the vast majority of TNF-\( \alpha \) released by human adipose tissue explants comes from the non-fat cells of adipose tissue, and that the average contribution of adipocytes to total TNF-\( \alpha \) release was only 9%. The finding of a negative correlation between BMI and TNF-\( \alpha \) concentration may be related to the negative correlation between adiponectin release and TNF-\( \alpha \) release by human adipose tissue. Kern et al. (2003) found a negative correlation of -0.48 (\( p<0.05 \)) between the level of adiponectin mRNA and that of TNF-\( \alpha \) in human subcutaneous adipose tissue.
A positive correlation between type II DM, and the level of CRP was noted in the samples in group 1 (DM neuropathy). Studies on western populations have shown low grade systemic inflammation to be one of the mechanisms by which known risk factors such as obesity, smoking and hypertension promote the development of type II DM (Pradhan et al. 2001; Pfutzner et al. 2006). Mohan et al. (2003) and Wild et al. (2004) noted that CRP levels were elevated in DM subjects compared with non-DM subjects in their studies. Several studies have also shown that CRP predicts DM in western populations (Pradhan et al. 2001; Haffeiner 2003; Hanley et al. 2004) and can therefore be used as a biomarker of inflammation to detect the development of type II DM.

Interestingly, highly significant correlations were found to be associated with neurological symptoms (NSS) of group 1 and the levels of CRP, IL-8, and IL-1β, and the presence of ANA’s. This goes in line with the results of Doupis et al. (2009) where they suggested peripheral diabetic neuropathy to be associated with increased markers of inflammation and endothelial dysfunction, and that painful neuropathy was associated with further increase in inflammation and markers of endothelial dysfunction. Their results showed patients with painful neuropathy had higher sICAM-1, and CRP levels when compared to painless neuropathy.

Results of linear regression, which was carried out to test whether neuropathy was independently related to the increased levels of inflammatory markers, demonstrated that CRP and IL-1β were the only markers significantly correlated with NSS. Although all the inflammatory markers tested were significantly higher in the neuropathy group (Table 4.1.2.3.1a in Chapter four), cytokines were not independently correlated with NSS, NDS or VPT, after adjustment for multiple confounders. Therefore, immune activation in DPN cannot be explained solely as a consequence of hyperglycaemia or other metabolic disturbances. Furthermore, neuropathy symptoms score (NSS) is a scoring symptom involved with the symptoms of pain, burning sensation, lancinating or stabbing pain and hyperesthesia. Since CRP and IL-1β were exclusively correlated with NSS, this raises the question of whether increased inflammatory markers were associated with the pain symptom
of neuropathy rather than the disease itself. Herder et al. (2009) showed that impaired temperature perception and pain or discomfort in the lower limbs were not associated with any of the measured immune mediators in their study, indicating that the association between clinical inflammation and DPN may only affect certain components of diabetic neuropathy, whereas others may be independent of immune activation.

Neuropathic pain encompasses a series of heterogeneous conditions with some similar clinical manifestations (Lees et al. 2013). Dysegulation of cytokines has been implicated in a variety of neuropathic conditions in both humans and animals. For example, differential expression of blood and cerebrospinal fluid cytokines has been demonstrated in patients with painful neuropathies. Compared to healthy controls, these patients showed higher levels of pro-inflammatory cytokines, such as, IL-1β and TNF-α, and lower levels of anti-inflammatory cytokines (Uceyler et al. 2007; Backonja et al. 2008). Nerve injury is known to increase expression and secretion of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-6. And IFN-γ, all of which are required for the development of pain hypersensitivity (Costigan et al. 2009). In case of peripheral nerve injury, the local inflammatory response is followed by a proximal response in both the dorsal root ganglion and the spinal cord. Shortly after injury to the sciatic nerve, mRNA coding for the cytokine TNF-α is rapidly elevated in the sciatic nerve (within hours of injury), and subsequently in the dorsal root ganglia (1-3 days following injury) (Sacerdote et al. 2008). TNF-α alters the excitability of neurons and promotes continued inflammation. Dubovy et al. (2006) stated that for at least two weeks, TNF-α display elevated expression in the ipsilateral and contralateral dorsal root ganglion of nerve-injured animals. This will be followed by activation and upregulation of other cytokines, such as, IL-6 and IL-1β.

Despite intense research over the last years, debate is still ongoing regarding the nature of neuropathic pain, including controversy as to whether such pain is peripheral or central in origin (Leung 2010), and as to whether it is related to inflammation and autoimmunity. Increasing evidence however provided better understanding of the roles of both immune and pro-inflammatory mediators (for
example, the interleukins, TNF-α, complement components, ATP and the chemokines) in the mechanisms of both peripheral and central neuropathic pain (Campbell and Meyer 2006; Moalem and Tracey 2005).

As regards to the linear regression analysis conducted on ANA’s and neuropathy manifestations, significant correlations were observed between the presence of ANA’s and all the variables of neuropathy (NSS, NDS, VPT). This suggests that autoimmunity may be independently associated with the neurological manifestations of DPN. Further studies however need to investigate this hypothesis before a clear conclusion can be made.

5.4. **Summary of the discussion for the cross-sectional and case-control analysis**

The cross-sectional analysis in part one, in agreement with previous literature, demonstrated that DM (most commonly type II DM) is a common health problem, and complications of DM are common in Bahrain. The prevalence rates of these complications in Bahrain and the MER region are higher than rates observed in studies from Western countries.

The majority of Arab countries are engaged in a multi-dimensional transition (demographical, economical, epidemiological and geographical). The shift from rural to urban dominance led to a sedentary lifestyle with minimum physical activity. The socioeconomic development allowed for higher income and increased consumption of fast-food and western diet. Socio-cultural changes may have also contributed to the high calorie intake. Furthermore, with such epidemiological and demographical transitions, the change of dietary intake and social activities fostered the development of obesity and impaired glucose tolerance.
The present hospital-based study on Bahraini DM patients in the RMS-BDF Hospital demonstrated that a large proportion of DM patients have neuropathic complications and are therefore, at substantial risk of developing foot ulceration. The study also identified some important risk factors for DM neuropathy, including poor glycaemic control, long duration of DM, and obesity which was witnessed in a large number of the sample selected.

The rising rates of obesity, IGT and DM constitute a real challenge in the Arab region. In order to alleviate the burden of DM complications, preventive strategies are needed, based essentially on adopting a more healthy diet with regular exercise. Health authorities are also required to provide populations with appropriate health care and early diagnosis to avoid the high burden caused by complications of DM.

The case control analysis which compared the levels of inflammatory markers between DM patients with DPN, DM controls, and healthy controls supported the previous studies conducted on both animal and human subjects which suggested the involvement of an inflammatory mechanism in the pathogenesis of diabetic peripheral neuropathy. The levels of pro-inflammatory cytokines and C-reactive protein were significantly higher in the neuropathy group when compared to the two control groups.

Cytokines play a major role in the pathophysiology of the development of nerve damage in many inflammatory diseases. The present study focused on investigating six of the pro-inflammatory cytokines commonly linked to neuropathies in type I and type II DM patients. To the best of knowledge, this is the first report from the Middle East region demonstrating the significant associations of TNF-α, IFN-γ, IL-6, IL-8, and IL-1β with diabetic peripheral neuropathy. Despite the fact that diabetes mellitus, especially type II DM, has been considered an inflammatory disease by many previous authors, the low levels of inflammatory markers detected in the DM control group in comparison to the neuropathy group suggests that the inflammatory phase of DM is not consistent throughout the course of the disease, and may only be present early at diagnosis.
Taken together, the data indicate that DPN and some of its individual impairments are significantly associated with inflammation, correlation co-efficient within the secondary analysis revealed some associations between a rise in systolic BP and the values of TNF-α, and IL-6 in group one, and systolic BP and CRP values in group two. Similarly, an increase in body mass index may have been related to an increase in CRP levels, and a decrease in TNF-α levels within group two (DM control). Further analysis detected a correlation between Neuropathy Symptom Score and the levels of CRP, IL-8, and IL-1β in the neuropathy group, suggesting that these markers may be further elevated in painful neuropathy. Prospective studies will therefore be required to assess the time course and causal relevance of clinical inflammation in the development of DPN independently without the influence of other factors in order to test whether immunomodulation could become a treatment option for patients with DPN.

The most interesting findings of this study were the significant differences in the percentages of positive autoimmune antibodies detected in the blood serum of DM patients with peripheral neuropathy when compared with the DM control and healthy control subjects. Autoimmunity has been linked to different types of neurological conditions some which are related to DM. However, this was the first study to date to investigate the relationship between DPN and autoimmunity, with the exclusion of other factors. Secondary analysis confirmed that the presence of antinuclear antibodies may independently be associated with DPN since significant correlations were presented between the ANA’s and all the neuropathy manifestations.

In conclusion, the present study demonstrated that human DPN is associated with increased biochemical markers of inflammation and antinuclear antibodies. Furthermore, painful neuropathy is associated with a further increase in inflammatory markers. These results indicate that inflammation and autoimmunity may be important contributors to the development of peripheral neuropathy in diabetes mellitus.
5.5. Theoretical and clinical implications of the study

The present study investigated the relationship between inflammation and/or autoimmunity and DPN in human subjects. Although previous studies have indicated that there is a link between inflammation and DPN, this relationship was not confirmed due to a number of limitations that were encountered within those studies as discussed in Chapter two. Furthermore, despite the arguments that some authors noted regarding the possibility of DPN being an autoimmune disease, to knowledge there were no studies directly investigating the presence of autoimmune antibodies in the blood serum of patients with DPN with the exclusion of other causes.

Several theoretical and clinical implications emerged from the results of this study. DPN is a complex disorder with a variety of pathological theories contributing to its pathogenesis. The association between inflammation and/or autoimmunity and DPN although investigated by several authors, was never confirmed as one of the theories behind the development of DPN.

Diabetic micro-vascular complications progress due to inflammation which originated from multiple pathways and mechanisms. This complexity warrants the need for effective therapies that target more than one signalling cascade. Inhibition of both inflammatory cytokines and their activators/regulators may provide additional coverage to treating DM complications. As further studies emerge to address current limitations, improved therapies targeting DM micro-vascular complications may ultimately shift the focus from treating the pathology to prevention.

It is evident from the scope of this study that the pathogenesis mechanism involved in diabetic peripheral neuropathy is complicated as depicted by the number of pathways implicated. Good glycaemic control combined with minimising the risk factors associated with DM complications has stabilized the level of morbidity and mortality associated with DM in most developed countries. However, good
glycaemic control alone has been shown to be insufficient to prevent DM complications. In particular, the concern is that a vast number of new cases of DM are now originating from developing countries, and hence, it is likely that less strict management in these nations due to resource issues and social habits may result in a greater incidence of DM complications worldwide.

The ultimate goal is to prevent or reverse these complications seen in individuals with DM. In particular, it is critical that one not only understands the mechanisms that lead to disease development and progression, but also how these changes occur in a temporal manner.

Animal studies are believed to be powerful tools in establishing patterns of progression of a disease, and implicating the involvement of particular molecules in treatment or prevention. Indeed, studying the early development of complications of a disease in animal models may provide clues as to the initiators and early promoters of a disease. However, the results obtained from animal studies may present limitations when compared to the actual human model. Studies involving human subjects may therefore be necessary to confirm what has already been established in animal models.

To date, research focused on improving the treatment of chronic pain has largely ignored the role of inflammation-associated factors in nociceptive pathways in the peripheral nervous system in DM subjects. This preliminary study suggests that inflammatory markers might play an essential role in the pathogenesis of DPN. Hence, the results from this study may suggest that painful diabetic neuropathy may possibly be considered, at least in part, a ‘neuro-inflammatory’ condition. A better understanding of the role of these inflammatory and pro-nociceptive markers may therefore lead to the development of novel analgesic targets which may target those symptoms effectively.

Treatment of peripheral neuropathy involves; controlling the underlying disease process, and treating painful symptoms. This is usually achieved by eliminating the
causative factors, such as toxins or medications; correcting a nutritional deficiency; or treating the underlying disease (e.g., corticosteroid therapy for immune-mediated neuropathy). These steps are important to control the progression of neuropathy, and they may improve symptoms.

Selecting an appropriate DPN therapy remains challenging due to the differences in effectiveness and harms associated with a large number of available therapeutic options. Taking into account the results of this study, investigations on a bigger scale should be conducted in the future to investigate the effects of new therapeutic alternatives that would target inflammation and/or autoimmunity in reducing the symptoms of DPN. Hence, a more controlled and guided management plan for such patients might eventually be available.

Although many medicines are used for treatment of neuropathy's symptoms, not all are yet officially FDA-licensed for such use. However, doctors have wide latitude in such off-label prescribing, and several prescription medications have passed safety inspection, and are now being evaluated for their efficacy as neuropathy treatments. New research such as the current investigation has shed light on other possible causes of this common DM complication, and may ultimately offer a way to reverse it.

The understanding of the pathogenesis mechanism could be extremely important not just for treating diabetic neuropathy but for other conditions that cause chronic pain, such as nerve injuries from accidents or wounds. It is extremely important to understand possible factors that might contribute to the pathogenesis of DPN in order to discover new treatments because of the growing prevalence of DM and the limitations of existing options. In some ways, one can consider it as going back to the baseline to uncover what might have been missed.

Furthermore, if the basis of hypotheses were correct, and the release of inflammatory markers and autoimmune antibodies are involved in the development and progression of DPN, then measurement of such markers may be used to detect the
activity of the disease, or to predict the developing of DPN in DM patients with raised levels of such markers. Further studies will be needed to reveal the function of immunological markers in the progression of DPN.

Of even greater importance is the possibility that inhibitors of pro-inflammatory markers could be of benefit in clinical practice. Several inhibitors of TNF-α and interleukins, for example, are available as research tools and have been used to attenuate inflammatory arthritis and osteolysis in experimental animals. More immediately, however, there could be therapeutic potential for short-term use of high dose systemic glucocorticoids (which are known to decrease expression of pro-inflammatory cytokines) or of TNF-α antagonists (such as infliximab or etanercept), which are currently used for clinical management of conditions such as rheumatoid arthritis. Nevertheless, the relative risks and benefits of such therapies would need to be carefully considered.

5.6. Limitations of the study

It should be emphasized that the above observed measurements are correlative and do not necessarily indicate causality. Although the design of the current study, as is the case with the majority of human studies, does not allow definite conclusions about possible causality, one would believe that there are some indications that the observed results may be related to the development of peripheral neuropathy. The presence of major differences between the DPN group and the two control groups possibly indicates that the observed changes may be associated with the presence of neuropathy rather than DM itself.

As discussed earlier in Chapter two, the design of the study, the inclusion of two control groups, the extensive immunological investigations, and the multiple statistical analyses represent strengths of this study. This study also has limitations. First, although the exclusion of other neuropathic causes were carefully conducted by
reviewing the patient’s records and personally enquiring about other symptoms in the interview prior to blood investigation, in some cases neuropathy may not have been due to DM alone. However, an exclusion of other potential causes of neuropathy through the use of other investigations for this study sample was not feasible.

Second, NSS and NDS were used to assess the neurological manifestations of the sample in order to confirm the diagnosis of DPN, and to rule out undiagnosed neuropathies in the control groups. Another limitation might be the fact that this study did not use electrophysiological and autonomic function measurements for the diagnosis of diabetic neuropathy. According to a recent statement by the American Diabetes Association, however, an accurate diagnosis of DPN can be made without the use of the above tests (ADA 2010).

Third, although frequency matching was utilized to ensure homogeneity between the three groups, the non neuropathic and neuropathic groups were not matched for the duration of DM which might have biased the results. However, it should be pointed out that the duration of DM is rather long in both groups; more than five years. In addition, the BMI values were elevated in group one (DPN group) and group two (DM control) when compared to the third control group. Nevertheless, searching for patients with DM and DPN with low BMI levels was unattainable in the limited time available, especially since most of the DM patients attending the diabetic clinic had high BMI values.

Fourth, the study was limited to single measurements of immune mediators and at different stages of the disease. Fifth, the study was not aimed at assessing neuropathic pain in detail; DPN group was not separated into painful and painless. Thus, further studies are necessary to clarify the association between inflammation and/or autoimmunity and this specific neuropathic symptom after adjusting for other variables.

Sixth, in this study, women undergoing HRT, and patients taking aspirin, statins, ACEI, or thiazolidinediones were excluded to rule out the effects of these
medications, as they have been proven to have an effect on increasing serum inflammatory markers. However, the effects of the other drugs related to DM control were not evaluated and not excluded. Moreover, hormonal imbalances as seen in pre and post menopause may influence the levels of inflammatory markers. However, it is still not clear whether post menopausal state is directly responsible for the increased in the levels of pro-inflammatory markers.

Seventh, the cross-sectional design of the study does not permit the evaluation of a cause and effect relationship between the two variables. It is therefore not possible to confirm whether DPN leads to inflammation and autoimmunity or whether inflammation and autoimmunity contribute to the development of DPN. Further studies are needed to evaluate this relationship. However, either direction of causality would have important implications. If DPN leads to inflammation and autoimmunity, then better control of DPN should lower inflammation and autoimmunity, and therefore lower the risk of cardiovascular complications. If inflammation and autoimmunity leads to the development of DPN, then treatment of inflammation and autoimmunity may help improve neuropathy symptoms.

Finally, as previously noted, the amount of time and funding available for this investigation was not sufficient enough to conduct a large study with a large enough sample size, and therefore was regarded as a pilot study which can be used as a reference for further research to take place in the future.

5.7. Future research

As mentioned previously, further studies are required to investigate the relationship between DPN and inflammation, and DPN and autoimmunity. The study conducted was restricted with the available time and funding dedicated for this type of research. Hence, larger studies from multiple geographical locations with a larger sample size
and for a longer follow up period are thus essential to develop further evidence related to these hypotheses.

The presence of other possible confounding factors that could be contributing to the differences in inflammatory markers levels and the presence of ANA’s in painful and painless neuropathy patients should be studied and controlled. For example, depression, which has also been shown to correspond with increased cytokine levels, could be contributing to the differences witnessed, since it has been proven that a significant number of patients diagnosed with chronic painful neuropathy have suffered from depression (Uceyler et al. 2010). This assumption is made stronger with the evidence that higher TNF-α level were present in depressed painful neuropathy patients compared to those without depression (Uceyler et al. 2010). Whether depression is the determining factor for differences in cytokine levels, or if the decreases in quality of life resulting from increased pain itself are contributing to the depression is unclear.

Another variable associated with the time of sample collection and duration of illness needs to be addressed. Taking this into account, the initial immune response would be early and probably provide a minor contribution to when the samples were collected. Similarly, the types of medications DM patients are receiving to control their DM might have an effect of the level of markers and thus should be investigated. Taking into account the outlined confounding variables that could be contributing, follow-up studies with a greater number of patients and with samples from a number of time points would further validate these findings.

Furthermore, with the discovery that inflammatory mediators are increased in DPN, researchers should begin to focus on therapeutic treatments that could target these inflammatory mediators. Therapeutic treatments aimed at targeting inflammatory mediators in DPN have shown effects when the therapeutic is administered at the time of DPN induction. There is a lack of evidence to demonstrate their effectiveness after the development of DPN in reversing any of the symptoms of DPN such as reductions in nerve conduction velocities or nociceptive behaviour. It is highly
unlikely the initiation of therapeutic treatment would coincide with the initial development of DPN in patients. Therefore, studies investigating the time course of anti-inflammatory therapeutics are needed. While current studies have not addressed reversal of DPN, a notable finding from a limited number of studies is that treatment effects were ineffective in initial time points, but instead the statistically significant beneficial effects were only evident after weeks of treatment. This temporal aspect may suggest the inflammatory component of DPN might not develop until later time points. Therefore, administration of therapeutics at later time points might still be effective, but further studies are needed to validate these findings.

The increase of inflammatory mediators in a number of neuropathic pain states has been well documented (Wilson and Wright 2011). The potential role of these mediators in DPN is just beginning to be explored. Further investigation into additional inflammatory mediators such as interleukins and chemokines as well as anti-inflammatory cytokines is warranted. Anti-inflammatory cytokines block the process or at least suppress the intensity of the pro-inflammatory cascade. Cytokines such as IL-4, IL-10, IL-13, and transforming growth factor (TGF)-b suppress the production of IL-1, TNF-α, chemokines such as IL-8, and vascular adhesion molecules. Therefore, a “balance” between the effects of pro-inflammatory and anti-inflammatory cytokines is thought to determine the outcome of disease, whether in the short term or long term. In fact, some studies have data suggesting that susceptibility to disease is genetically determined by the balance or expression of either pro-inflammatory or anti-inflammatory cytokines. Investigating the balance between pro-inflammatory and anti-inflammatory cytokines in DPN is therefore essential to better understand the mechanism of their actions. Rodent studies are also required to explore the mechanisms by which these proteins could result in a number of DPN symptoms, including nerve conduction velocity and epidermal innervation deficits. Further translational studies are needed to confirm findings in animal models, especially expanding on the therapeutic potential of targeting inflammatory mediators.
Additionally, follow-up studies determining whether differences in cytokine profile expression continue when comparing painful versus painless diabetic neuropathy patients is an interesting aspect to explore. If similar results are evident in DPN patients, it could be beneficial in determining enhanced personalized treatments for diabetic neuropathy patients.

Moreover, since the only autoimmune investigation performed in this study was to detect the presence of ANA’s in the blood serum, more complex autoimmune investigations, one that would detect the type of antibodies witnessed in the blood of DPN patients is strongly recommended.

Finally, one must not overlook that within the body, glucose abnormalities with relative insulin deficiency are the key determinants of DPN. Indeed, more research should be targeted toward elucidating the initial functional and structural patterns altered by the common changes in glucose uptake and trafficking that occur at sites of DM complications. Such investigations may provide answers to particular genetic susceptibility associated with these disorders. Hence, identification of why certain persons with DM progress to complications whereas others remain remarkably resistant to developing these disorders is of paramount importance.
Chapter Six: Conclusion

6.1. The summary

It is well established that DPN is a complex condition that has many consequences. There are varied clinical presentations of diabetic neuropathy with involvement of proximal or distal peripheral sensory and motor nerves, as well as autonomic nerves. The early recognition of neuropathy is important to reduce morbidity and mortality and to improve quality of life.

The findings of the present hospital-based study demonstrated that a large proportion of DM patients have neuropathic complications and are therefore, at substantial risk of developing foot complications. From the sample randomly selected, 76% of the patients had complications associated with DM, the commonest being peripheral neuropathy followed by vascular insufficiency.

DPN is not a single entity but a set of different syndromes reflecting the heterogeneity of the problem. Classification of DPN can be difficult due to frequent co-existence of these forms and their clinical and electro-diagnostic overlap. Symmetric distal sensory or sensorimotor polyneuropathy, also known as DPN, is the most common form of neuropathy, affecting almost half of the patients diagnosed with DM. Currently, early detection of DPN is the only means of slowing the progression of this complication. It is foreseeable that an increasing understanding of the pathogenetic mechanisms will lead to effective treatment in the future.

Most of the current knowledge on the pathogenesis of diabetic DPN has been derived from studies of various animal models of diabetic neuropathy. The results of the Diabetes Control and Complications Trial link neuropathy to hyperglycaemia. Hyperglycaemia is thought to induce neurotoxicity through four pathways; metabolic, vascular, neurotrophic growth factor–deficiency, and recently an immunologic–autoimmune pathway has been suggest.
The present investigation was therefore conducted to test the hypotheses that propose a relationship between pro-inflammatory markers and autoimmune markers and the development of DPN. The case control analysis which compared the levels of inflammatory marker between DM patients with DPN, DM controls, and healthy controls supported the previous studies conducted on both animal and human subjects which suggested the involvement of an inflammatory mechanism in the pathogenesis of diabetic peripheral neuropathy. Within the 90 sample selected (30 in each group), the levels of pro-inflammatory cytokines and C-reactive proteins were significantly higher in the neuropathy group.

With regard to autoimmunity, the present study investigated, for the first time, the presence of anti-nuclear antibodies in the blood serum of patients with DPN after exclusion of other causes. One of the most remarkable findings of this study was that highly significant differences between the percentages of positive and negative autoimmune antibodies (ANA) was witnessed when all the three groups were compared, proposing the possibility of an autoimmune factor involvement in the pathogenesis of DPN.

Secondary analysis detected a number of associations between the levels of pro-inflammatory markers and the demographics of the sample selected. Most importantly, significant associations were evident between pro-inflammatory markers and neurological manifestations of DPN. Such markers were further elevated in patients with raised Neuropathy Symptom Scores, suggesting that pro-inflammatory markers are further elevated in painful DPN.

One cannot overlook the fact that the results obtained might have been influenced by confounding factors. However, the significant increase in all the inflammatory markers evaluated and the presence of autoimmune antibodies in 60 percent of the patients diagnosed with DPN support previous literatures that proposed such hypotheses.
The role of inflammation and autoimmunity in DPN is starting to unfold. Metabolic and vascular pathways damage the neuronal unit and this may cause antigenic leakage with resulting activation of the immune system. Autoimmunity in diabetic neuropathy has always been questioned but no studies investigated its relationship with DPN. The ultimate proof of the relevance of circulating antibodies to neuronal structures will rest with identification of the specific antigen and reversal of diabetic neuropathies with neutralization of the antibody to the antigen.

Although this study did not examine treatment choices made for individual patients in the sample, the present findings of elevated pro-inflammatory markers and the presence of autoimmune markers in the blood serum of patients with DPN may be a clue to an underlying autoimmune-immunological pathogenesis in diabetic peripheral neuropathy. Despite relative lack of success of interventional agents to reverse or slow established DPN, there is still hope to find some effective agents.

In summary, the understanding of the pathogenesis of diabetes mellitus has changed in the last few years, with immunological pathways playing pivotal roles in the development and progression of DM complications. These new findings lead to a consideration of new therapeutic approaches. Inflammation and autoimmunity in the setting of diabetes mellitus is nowadays a matter of great interest. It is possible that in the coming years the hope of new therapeutic strategies based on anti-inflammatory and immunological properties with beneficial actions on DM complications may be converted to real clinical treatments.
6.2. The thesis journey within the professional doctorate programme

The Professional Doctorate programme is designed for experienced professionals in health and social sciences with an aim of contributing to the advancement of professional practice. In the author’s point of view, undertaking the current research facilitated the development of the individual’s professional practice on many aspects as described below.

Within the last years, the author has witnessed a great number of DPN patients attending the podiatry department seeking answers hoping to find a cure for this devastating health problem. It has been a difficult challenge for the author and other health professionals who have been working with such patients who have tried most of the treatments available for this condition, yet still lived with pain.

Choosing to investigate the pathogenesis of DPN was simply influenced by the rise in the epidemiology of DM and its complications in the Arab countries, and especially Bahrain. Diabetes mellitus is one of the most rapidly growing health problems in the developing countries and DPN is one of the most common complications of diabetes mellitus, accounting for substantial morbidity and mortality and resulting in huge health care costs.

One might argue that this investigation will not provide DPN patients with a conclusive answer to their problems at the time being, and thus might not improve the management of such patients immediately. However, investigating the theories that open doors for further research to take place to eventually find a cure or a preventative method for DPN is nevertheless rewarding not just for the patients, but for the health professionals as well.

Research advances present clinicians with opportunities to provide better patient care. These advances typically involve modest changes in the treatment of chronic
diseases. Research that improves the therapeutic precision with established treatments is usually easy to incorporate into practice. Major changes in therapeutics however tend to emerge over 5 to 10 years, and may require clinicians to develop a new understanding of an otherwise familiar disease, as exemplified by the current thesis.

The thesis journey facilitated personal and professional development, and formed the last part of the professional doctorate work. On a personal perspective, studying the pathogenesis of DPN and investigating whether other factors are involved in this complex complication produced a professional who is confident and determined to discover new treatments for such patients. In addition, studying the physiology and pathomechanics of this disease expanded the scope of knowledge by understanding the disease from a different angle to what is usually practiced in the medical field.

Some may claim that the depth of this research may be beyond the scope of Podiatry, since it is investigating a neurological condition from physiological and immunological aspects. The author however believes that in order to discover an answer for a complicated matter one must consider investigating all aspects of the pathology. Investigations that seemed totally irrelevant to practice, for example, have yielded most of the major discoveries of medicine. X-rays for example were discovered by a physicist observing charges in vacuum tubes; penicillin came from enzyme studies of bacterial lysis and the polio vaccine from learning how to grow cells in culture. Cisplatin, a widely used drug in cancer chemotherapy, was an accidental discovery of how electric fields affect the growth of bacteria. All these discoveries have come from the pursuit of curiosity and the determination to find an answer.

It has been said of the Professional Doctorate that it trains the individual to be ‘a researching professional’, rather than the PhD which trains the ‘professional researcher’. This was evident in the curiosity, eagerness, and determination to expose the unknown and find an answer to this overwhelming complication in order to provide the best possible practice to patients.
On a professional perspective, undertaking this research helped in building up professional relationships with other professions interested in this disease. The collaboration with neurology and internal medicine physicians inside and outside the hospital has opened doors for the possibility of multidisciplinary team approach management for patients with DPN to be discussed in the future. Moreover, revealing interests in this condition helped in promoting and advancing the podiatry profession among other medical professionals, especially since podiatry has only recently been established as a new medical profession in Bahrain.

This research process involved the contribution of team members from departments outside the podiatry circle. The diabetic clinic staff, lab technicians, hospital administration, neurology physicians and medical students all contributed in different ways. Leadership, team work and collaboration, and inter-professional working were strongly witnessed throughout this research journey with the hope of making a difference.

In summary, it can be said that DPN wears many faces but none of these faces wear a smile. It can be a devastating complication of DM. A determined practitioner and a motivated patient can certainly be an effective team in controlling the progression of DPN. This requires aggressiveness, a high index of suspicion, early intervention, taking on the role of teacher and motivator and being a true patient advocate. Last but not least, improving the basic knowledge and exploring further options that might contribute to improving the management of such patients is a task worth considering.

“We are here for a reason; we are here to make a difference”
References


Dear Ms. Noor,

Greetings,

It gives me a great pleasure to inform you that your research proposal “The role of Pro-inflammatory Cytokines and Autoimmune Antibodies in Diabetic Peripheral Neuropathy” has been accepted for funding by Dr. Ali Al-Khalifa Medical Research Fund.

You will be awarded the maximum funding of BD 2000/-. 

Rules for Dr. Ali Al-Khalifa Medical Research Fund:

1. The research is expected to be completed within a year
2. 50% of the payment will be given initially
3. Progress report should be submitted after data collection and the second 50% of the payment will be given
4. We would appreciate receiving a copy of the final results of your research (after publication).
4. Would you be willing to submit your paper for publication at the Bahrain Medical Bulletin? Please let us know by replying by email.

Wish you best luck,

Fadheela Al-Mahroos, MD, MHPE
Consultant Pediatrician, Sulmanyia Medical Complex,
Ministry of Health
Associate Professor – College of Medicine & Medical Sciences,
Arabian Gulf University
Director, Dr. Ali Al-Khalifa Medical Research Fund
Bahrain
Appendix 2: Approval of ethics application from the RMS-BDF Hospital Ethics Committee

Ms. Noor Janahi
Podiatry Specialist
Orthopaedic Department
BDF- Hospital

RE: The role of Pro-inflammatory Cytokines and Autoimmune Antibodies in the Pathogenesis of Peripheral Neuropathy in Type1 and Type2 Diabetes:-

Dear Ms. Noor,

Thank you so much for submitting your aforementioned research project proposal to the Research Ethics Committee (REC) of the Royal Medical Services of the Bahrain Defence Force (RMS-BDF) Hospital which is planned to be undertaken in our hospital.

The REC of the RMS-BDF has reviewed your application (Delegated Review) and we would like to inform you that: it is ethically acceptable as is.

We expect your research to commence within the next 12-Months and please be kind enough to inform all related parties in your proposed study to this decision.

Also please note that all information collected from the RMS-BDF should remain totally & absolutely confidential – any patient identifying numbers (that can trace a patient which include but not limited to hospital numbers, dates of birth, Bahrain CPR numbers, addresses etc.) should be destroyed within 1-years time of completion of your research project.

Please feel free to contact us for any further assistance.

Many Thanks

[Signature]

Mr. Mohammad A Al Maharraki
MBChB (Drd.), BDS (Drd.), MDSc (Drd.), MRCS (Glas.), FFD RCS (Irel.), MFDS RCS (Eng.)
Chairman of the Research Committee
Consultant Oral & Maxillofacial Surgeon, Oral Physician
Royal Medical Services – Bahrain Defence Force
Appendix 3: Approval of ethics application from QMU Research Ethics Committee

DIVISION OF DIETETICS, NUTRITION, BIOLOGICAL SCIENCES, PHYSIOTHERAPY, PODIATRY and
RADIOGRAPHY

STUDENT PROJECT RELEASE FORM

This form is designed to notify each student of the DivREC response to individual
Dissertation Proposals. A copy of the form will also be retained by the Committee to record
each decision and to monitor resource requirements. Students Please complete a – d below

a. PROJECT TITLE: The role of Pro-inflammatory Cytokines and Autoimmune
   Antibodies in Peripheral Neuropathy in patients with Type 1 and Type 2
   Diabetes
b. STUDENT(S): Noor Mohamed Janahi
c. SUPERVISOR: Dr. Mairghread Ellis, Christine Blyth
d. SITE FOR DATA COLLECTION Royal Medical Services- Bahrain Defence Force Hospital-
   Bahrain
e. APPROXIMATE DATES FOR DATA COLLECTION: April/May 2012

All students should refer to Committee Response below and Comments overleaf

COMMITTEE RESPONSE

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<tr>
<th>Decision</th>
<th>✓ / X</th>
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<tr>
<td>1. Project proposal and Ethical approval granted</td>
<td>✓</td>
<td>8/5/2012</td>
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<tr>
<td>2. Proceed with minor modifications to the project proposal (as noted in response overleaf)</td>
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<td>3. Resubmit revised proposal by (insert date...........)</td>
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<td>4. Resubmit revised ethics by (insert date ...........)</td>
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<tr>
<td>5. Submit for further ethics scrutiny (QMU / external)</td>
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<td>ASAP</td>
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<td>6. Project documentation incomplete</td>
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Please note – you can not proceed to dissertation unless response box 1 or boxes 1 and 2
ticked ✓

GENERAL COMMENTS:

QMU DivREC approval has been granted for this project. This project may now proceed and
be submitted for further ethical consideration at your local ethics committee in Bahrain.
This form will only be signed by Head of Division once project and ethical approval granted

Signature DiVREC member: __Dr Derek Santos__    DATE_08/05/2012___

SIGNATURE OF HEAD OF DIVISION: ______________________    DATE: ______

Submission 1     Date 10/2/2012
Submission 2     Date 01/05/2012
Wednesday, 01 August 2012

Dear Noor

RE: Revision to approved ethics application 1/5/2012 (The role of Pro-inflammatory Cytokines and Autoimmune Antibodies in Peripheral Neuropathy in patients with Type 1 and Type 2 Diabetes)

We have received your application regarding a minor change to your previous accepted application that was approved on 1/5/2012. This change related to the possibility of recruiting from an addition centre using the previously approved methodology. We are happy to accept this change and you may proceed with this change provided your local ethics committee has also approved it.

Yours sincerely

Dr Derek Santos
Senior Lecturer
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**Risk assessment form**

**Needlestick injury**

- **Operator:**
  - **Conducting patient:**
  - **Hazard:**
  - **Relevant measures:**

---

**Table: Risk assessment**

- **Total Number Exposed to Risk:** 30
- **Assessed by:**
  - **Job Title:**
  - **Department:**
  - **School of Health and Social Care:**
  - **Location:**
  - **Date:** 07/07/2012

---

**Queen Margaret University**
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- Emotional Fear
- From study at any
- Subjects can withdraw
- Will be consulted 1
- Any loss of Hospital
- School workers
- Line

Note: RV value may be adjusted based on the above factors.
Appendix 5: Information sheet for potential participants with diabetes mellitus and control group

Research Information Sheet for Potential Participants (patients with diabetes mellitus)

My name is Noor Janahi and I am a Podiatrist working at the RMS-BDF Hospital. I am also a part time student on the Professional Doctorate Programme from the school of Health and Social Sciences’ at Queen Margaret University in Edinburgh, UK. As part of this programme, I am undertaking a research project for my thesis dissertation.

The title of my project is:

‘The role of Pro-inflammatory Cytokines and Autoimmune Antibodies in diabetic peripheral neuropathy’

What is this study designed for?

This study is designed to look into factors that might be involved with the development of diabetic peripheral neuropathy (which is the lack of sensation and motor functions in the feet) in diabetic patients.

The study will investigate whether certain elements that are related to inflammation and autoimmunity are present in the blood serum of patients with diabetic peripheral neuropathy.
How will we benefit for the findings of this study?

The findings of the project will be useful because if our study proved that there is a link, this could lead to the adoption of new markers for the activity of the disease, and of effective new therapies to develop in the future, and certainly filling a gap in the literature and improving the management of such patients.

Who will be involved in this study?

A sample of subjects will be selected from the population of diabetic patients attending the diabetic clinic at the RMS-BDF Hospital for their routine follow ups. This sample will be further examined to ensure they match the inclusion and exclusion criteria of our study. Once samples have been initially selected, the participants will be contacted and informed about the details of the investigations.

What are the inclusion and exclusion criteria?

The inclusion / exclusion criteria for the group of subjects required for this study are described in details below.

The inclusion criteria will thus include:
- Patients with Type 1 and Type 2 diabetes attending the Diabetic Clinic at the RMS-BDF Hospital who will agree to participate in this study and will sign the consent form.
- Healthy subjects with no known underlying disease who will agree to participate in this study and will sign the consent form.

The exclusion criteria will include:
- Symptomatic peripheral arterial disease (ankle brachial index < 0.65 and/or symptoms of claudication),
- Congestive heart failure, cardiac arrhythmias, stroke or transient ischemic attack,
- end stage renal failure
- uncontrolled hypertension,
- severe dyslipidemia
- chronic liver disease
- other severe chronic medical condition requiring active treatment
- Diabetic patients with inflammatory neuropathies including chronic inflammatory demyelinating polyneuropathy (CIDP), Proximal diabetes neuropathy, and autonomic neuropathies.
- Patients with other types of neuropathies not associated with diabetes such as B12 deficiency, hypothyroidism, and uremia.
- Subjects older than 70 years, or younger than 20 years
- Obese Patients
- Subjects over the age of 70 and under 20
- Subjects with any acute infection or inflammation
- Pregnancy

What is required from me if I accept to be a part of this study?

If you agree to participate in the study, you will be asked to attend the clinic for a full examination which will include history taking, vital measurements, drug history, and a thorough neurological and vascular examination of both feet. The participants will then be asked to return to the clinic on a given date for a fasting blood investigation.

What is a blood investigation?

A blood test is a medical procedure that allows a nurse/collector to take blood from a person for the purpose of examining it. A blood test is the removal of blood from a vein or artery. It is usually removed from the arm of a patient near the elbow. The reason why the elbow area is used is because it does not have many nerves. Other sites that can be used are the wrist, hand and foot.

What happens during a blood test?

Initially a cord (tourniquet) is tied around the upper arm of the person to make the vein prominent. The tightness of the cord makes it much easier to remove the blood. The arm is cleaned and then a needle is inserted through the skin into the vein. The necessary amount of blood is extracted into a special tube or tubes, the needle is removed and a little ball of cotton wool is held over the wound. This should be pressed for one to two minutes before applying a band aid. The tubes are sent to a laboratory where the blood is analysed.

How much blood will be taken for the purpose of this investigation?

The amount of blood taken will be the same amount they usually obtain for your routine blood tests however two extra tubes will be used to test for chemicals in your blood that might suggest inflammatory and/or autoimmune reactions. Total amount of blood collected will not exceed 15ml.

Are there any risks associated with this procedure?

The researcher is not aware of any risks associated with the procedure. The whole procedure should take no longer than 30 minutes within the first visit, and 30 minutes within the second scheduled appointment time.

What is required from me if I needed to withdraw from the study?

You will be free to withdraw from the study at any stage and you would not have to give a reason. Your health care will not be affected in any way if you choose to withdraw from this study.
Will my personal details be mentioned in the study?

All data gathered from your medical records and blood investigations will be confidentially kept between the people involved in the study. No mention of names or personal details will be mentioned during the write up of the study. The results may be published in a journal or presented at a conference.

If you have read and understood this information sheet, any questions you had have been answered, and you would like to be a participant in the study, please now see the consent form.

Contact details of the researcher

Name of researcher: Noor Mohamed Janahi
Address: Orthopaedic Department
         BDF Hospital
         Bahrain
Email / Tel: n.janahi1@hotmail.com/ +97336090444

Details of Independent Advisor

Name: Nouf Al Qahtan (Practical Nurse- Surgical Department)
Address: Surgical Department
         BDF Hospital
         Bahrain
Emails/ Tel: +97339449567
Research Information Sheet for Potential Participants (Control Group)

My name is Noor Janahi and I am a Podiatrist working at the RMS-BDF Hospital. I am also a part time student on the Professional Doctorate Programme from the school of Health and Social Sciences’ at Queen Margaret University in Edinburgh, UK. As part of this programme, I am undertaking a research project for my thesis dissertation.

The title of my project is:

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What is this study designed for?

This study is designed to look into factors that might be involved with the development of diabetic peripheral neuropathy (which is the lack of sensation and motor functions in the feet) in diabetic patients.

The study will investigate whether certain elements that are related to inflammation and autoimmunity are present in the blood serum of patients with diabetic peripheral neuropathy.

How will we benefit for the findings of this study?

The findings of the project will be useful because if our study proved that there is a link, this could lead to the adoption of new markers for the activity of the disease, and of effective new therapies to develop in the future, and certainly filling a gap in the literature and improving the management of such patients.
Who will be involved in this study?

A sample of subjects will be selected from the population of diabetic patients attending the diabetic clinic at the RMS-BDF Hospital for their routine follow ups. A sample of volunteers to participate in the project as part of the control group (non-diabetic subjects).

The control group will consist of healthy individuals between the age of 20-70, with no known medical problems.

This sample will be further examined to ensure they match the inclusion and exclusion criteria of our study. Once samples have been initially selected, the participants will be contacted and informed about the details of the investigations.

What are the inclusion and exclusion criteria?

The inclusion / exclusion criteria for the group of subjects required for this study are described in details below.

The inclusion criteria will thus include:
- Patients with Type 1 and Type 2 diabetes attending the Diabetic Clinic at the RMS-BDF Hospital who will agree to participate in this study and will sign the consent form.
- Healthy subjects with no known underlying disease who will agree to participate in this study and will sign the consent form.

The exclusion criteria will include:
- Symptomatic peripheral arterial disease (ankle brachial index < 0.65 and/or symptoms of claudication),
- Congestive heart failure, cardiac arrhythmias, stroke or transient ischemic attack,
- end stage renal failure
- uncontrolled hypertension,
- severe dyslipidemia
- chronic liver disease
- other severe chronic medical condition requiring active treatment
- Diabetic patients with inflammatory neuropathies including chronic inflammatory demyelinating polyneuropathy (CIDP), Proximal diabetes neuropathy, and autonomic neuropathies.
- Patients with other types of neuropathies not associated with diabetes such as B12 deficiency, hypothyroidism, and uremia.
- Subjects older than 70 years, or younger than 20 years
- Obese Patients
- Subjects over the age of 70 and under 20
- Subjects with any acute infection or inflammation
- Pregnancy
What is required from me if I accept to be a part of this study?

If you agree to participate in the study, you will be asked to attend the clinic for a full examination which will include history taking, vital measurements, drug history, and a thorough neurological and vascular examination of both feet. The participants will then be asked to return to the clinic on a given date for a fasting blood investigation.

What is a blood investigation?

A blood test is a medical procedure that allows a nurse/collector to take blood from a person for the purpose of examining it. A blood test is the removal of blood from a vein or artery. It is usually removed from the arm of a patient near the elbow. The reason why the elbow area is used is because it does not have many nerves. Other sites that can be used are the wrist, hand and foot.

What happens during a blood test?

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How much blood will be taken for the purpose of this investigation?

Total amount of blood collected will not exceed 15 ml.

Are there any risks associated with this procedure?

The researcher is not aware of any risks associated with the procedure. The whole procedure should take no longer than 30 minutes within the first visit, and 30 minutes within the second scheduled appointment time.

What is required from me if I needed to withdraw from the study?

You will be free to withdraw from the study at any stage and you would not have to give a reason.

Will my personal details be mentioned in the study?

All data gathered from your medical records and blood investigations will be confidentially kept between the people involved in the study. No mention of names
or personal details will be mentioned during the write up of the study. The results may be published in a journal or presented at a conference.

If you have read and understood this information sheet, any questions you had have been answered, and you would like to be a participant in the study, please now see the consent form.

Contact details of the researcher

Name of researcher: Noor Mohamed Janahi  
Address: Orthopaedic Department  
          BDF Hospital  
          Bahrain  
Email / Tel: n.janahi1@hotmail.com/ +97336090444

Details of Independent Advisor

Name: Nouf Al Qahtan (Practical Nurse- Surgical Department)  
Address: Surgical Department  
          BDF Hospital  
          Bahrain  
Emails/ Tel: +97339449567
Appendix 6: Consent form

Research Subjects Consent Form

“The role of Pro-inflammatory Cytokines and Autoimmune Antibodies in Diabetic Peripheral Neuropathy”

I have read and understood the information sheet and this consent form. □
I have had an opportunity to ask questions about my participation. □
I understand that I am under no obligation to take part in this study. □
I understand that I have the right to withdraw from this study at any stage without giving any reason. □

I agree to participate in this study.

Name of participant: ________________________________
Signature of participant: ________________________________
Signature of researcher: ________________________________

Date: ______________
Contact details of the researcher

Name of researcher: Noor Mohamed Janahi

Address: Orthopaedic Department
         BDF Hospital
         Riffa

Email / Tel: n.janahi1@hotmail.com/ +97336090444

Details of Independent Advisor

Name: Nouf Al Qahtan (Practical Nurse- Surgical Department)

Address: Surgical Department
         BDF Hospital
         Bahrain

Emails/ Tel: +97339449567
Appendix 7: C-reactive protein analysis

510(k) SUBSTANTIAL EQUIVALENCE DETERMINATION
DECISION SUMMARY
ASSAY ONLY TEMPLATE
A. 510(k) Number:
K053603
B. Purpose for Submission:
New assay
C. Measurand:
C-Reactive Protein
D. Type of Test:
Particle enhanced turbidimetric assay.
E. Applicant:
ROCHE DIAGNOSTICS CORP.
F. Proprietary and Established Names:
C-REACTIVE PROTEIN (LATEX) HIGH SENSITIVE TEST SYSTEM FOR COBAS INTEGRA INSTRUMENTS
G. Regulatory Information:
1. Regulation section:
21CFR Sec- 866.5270-C-reactive protein immunological test system.
2. Classification:
3. Product code:
NQD - CARDIAC C-REACTIVE PROTEIN, ANTIGEN, ANTISERUM, AND CONTROL
4. Panel:
Immunology (82)
H. Intended Use:
1. Intended use(s):
The CRP (Latex) High Sensitive Immunoturbidimetric assay is for the in vitro quantitative determination of C-reactive protein (CRP) in human serum and plasma on Roche automated clinical chemistry analyzers. Measurement of CRP is of use for the detection and evaluation of inflammatory disorders and associated diseases, infection and tissue injury. Highly sensitive measurement of CRP may also be used as an aid in the assessment of the risk of future coronary heart disease. When used as an adjunct to other laboratory evaluation methods of acute coronary syndromes, it may also be an additional independent indicator of recurrent event prognosis in patients with stable coronary disease or acute coronary syndrome.
2. Indication(s) for use:
The CRP (Latex) High Sensitive Immunoturbidimetric assay is for the in vitro quantitative determination of C-reactive protein (CRP) in human serum and plasma on Roche automated clinical chemistry analyzers. Measurement of CRP is of use for the detection and evaluation of inflammatory disorders and associated diseases, infection and tissue injury. Highly sensitive measurement of CRP may also be used as an aid in the assessment of the risk of future coronary heart disease. When used as an adjunct to other laboratory evaluation methods of acute coronary syndromes, it may also be an additional independent indicator of recurrent event prognosis in patients with stable coronary disease or acute coronary syndrome.
3. Special conditions for use statement(s):
Prescription use
4. Special instrument requirements:
The CRP (Latex) HS assay is intended for use on Roche automated clinical chemistry analyzers.
This submission describes applications for the Integra family of analyzers; namely, the Integra 400, 400 plus, 700, and 800. The Integra family of analyzers is cleared under K951595.

I. Device Description:
The CRP (latex) HS Test System is a latex particle-enhanced immunoturbidimetric test for the quantitative measurement of C-reactive protein in human serum or plasma. Human CRP agglutinates with latex particles coated with monoclonal anti-CRP antibodies. The precipitate is determined turbidimetrically. The calibrator is the Calibrator for automated systems (C.f.a.s). Proteins; and the recommended control materials are CRP T Control N and Precinorm Protein. The reagents are for use on the Integra 400, 400 plus, 700 and 800 analyzers.

J. Substantial Equivalence Information:
1. Predicate device name(s):
   Roche Tina-quant® CRP (latex) HS Test System, Dade Behring N High Sensitivity CRP
2. Predicate 510(k) number(s):
   K042485, K033908 respectively
3. Comparison with predicate:
   The CRP (latex) HS Test System for COBAS Integra instruments is compared to the currently marketed Roche Tina-quant® CRP (latex) HS Test System cleared under K042485. For purposes of cardiac risk assessment, the CRP (latex) HS system is also equivalent to the Dade Behring N High Sensitivity CRP (K033908). The Tina-quant® CRP (Latex) High Sensitive Immunoturbidimetric assay is for the in vitro quantitative determination of C-reactive protein (CRP) in human serum and plasma on Roche automated clinical chemistry analyzers. Highly sensitive measurement of CRP is of use for the detection and evaluation of inflammatory disorders and associated diseases, infection and tissue injury.
   Measurement of CRP may also be used as an aid in the assessment of the risk of future coronary heart disease. When used as an adjunct to other laboratory evaluation methods of acute coronary syndromes, it may also be an additional independent indicator of recurrent event prognosis in patients with stable coronary disease or acute coronary syndrome.
   Both test systems are intended for the in vitro quantitative determination of C-reactive protein in human serum and plasma and have the same indications for use. Both share the same test principle - they are both latex-particle enhanced immunoturbidimetric assays. The reagents are quite similar; the active ingredients and antibodies are the same. Both are ultimately traceable to the same reference material (CRM 470).
   The new test system has similar imprecision, known interferences, comparable standards and calibrators, and is comparable in absolute values to the predicate device. They share expected values and instructions for result interpretation.
   This test system is intended for use on the COBAS Integra family of analyzers while the predicate device was intended for use on the Roche/Hitachi family of analyzers. There are some very minor differences in reagent composition.
   Compared to the predicate device, this test system has some slight differences in specific performance characteristics.

K. Standard/Guidance Document Referenced (if applicable):
Guidance for Industry - Review Criteria for Assessment of C-Reactive Protein (CRP), High Sensitivity C-Reactive Protein (hsCRP) and Cardiac C-Reactive Protein (cCRP) Assays: http://www.fda.gov/cdrh/oivd/guidance/1246.html

L. Test Principle:
The Immunoturbidimetric methodology used in this assay is well-established and is the basis for the already cleared Integra CRP assay (K981897). During the reaction, anti-CRP antibodies coupled with latex microparticles react with CRP in the sample to form an antigen-antibody agglutinate, which is measured turbidimetrically.

M. Performance Characteristics (if/when applicable):
1. Analytical performance:
   a. Precision/Reproducibility:
   Reproducibility was determined using human samples and controls (within run n = 21, between run n = 21).
   Within-run Between-run
   Sample Mean CV Mean CV
   mg/L (nmol/L) % mg/L (nmol/L) %
   Control Level 1 3.3 (31.4) 0.9 3.3 (31.4) 3.5
   Control Level 2 8.0 (76.2) 0.7 8.0 (76.2) 2.2
   Human pool 1 1.6 (15.2) 1.3 1.5 (14.3) 3.1
   Human pool 2 11.4 (109) 0.6 11.4 (109) 2.3
   b. Linearity/assay reportable range:
   To determine linearity of the CRP (Latex) HS test system, three dilution series of different analyte concentrations were measured as samples using the CRP (Latex) Test system on the Integra 700. The three dilution series covered the low end (0-3 mg/L); midrange (0-30 mg/L); and extended measuring ranges (0-306 mg/L). The attached files show the results of these measurements. The linearity data support a linear range of 0 up to 306 mg/L. Comparable results were found on the Integra 400.
   c. Traceability, Stability, Expected values (controls, calibrators, or methods):
   The calibrators and controls including stability claims have all been previously cleared and used with CRP test systems. Their composition has not been modified for use with the CRP (Latex) HS test system. All calibrators and controls are traceable to the reference preparation CRM 470. All value assignments were performed under standardized conditions using CRP (Latex) HS reagents.
   d. Detection limit:
   Analytical sensitivity (lower detection limit)
   0.1 mg/L (0.952 nmol/L)
   The detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying three standard deviations above that of a zero sample (zero sample + 3 SD, within run precision, n = 21).
   Functional sensitivity (limit of quantitation)
   0.3 mg/L (2.96 nmol/L)
   The functional sensitivity (limit of quantitation) is the lowest CRP concentration that can be reproducibly measured with an inter-assay coefficient of variation of <10%.
   e. Analytical specificity:
   Interference
   Criterion: Recovery within ±10% of initial value.
   Serum, plasma
   Hemolysis - No interference up to 10 g/L or 621 μmol/L hemoglobin.
   Icterus - No interference up to 0.6 g/L or 1030 μmol/L bilirubin.
   Lipemia(Intralipid) - No significant interference up to a triglycerides level of 5 g/L at 2 mg/L or 19 nmol/L CRP.
   High-dose hook effect - Does not occur at CRP concentrations below 40 mg/L or 380 nmol/L. Samples with concentrations >40 mg/L are flagged either >TEST RNG or “HIGH ACT”.
   Rheumatoid factors - No interference up to 1200 IU/mL.
Dysproteinemia - In very rare cases, monoclonal gammopathy may lead to false CRP values due to formation of turbidity or direct interaction of the monoclonal antibody in the specimen with the test system.

HAMA - Although measures were taken to minimize interference caused by human anti-mouse antibodies, erroneous findings may be obtained from samples taken from patients who have been treated with monoclonal mouse antibodies or have received them for diagnostic purposes.

f. Assay cut-off:
Not Applicable

2. Comparison studies:

a. Method comparison with predicate device:
Method comparison

CRP values for human serum and plasma samples obtained on a COBAS INTEGRA 700 analyzer with the COBAS INTEGRA C-Reactive Protein (Latex) cassette High Sensitive Assay (CRPHS) were compared to two commercially available alternative automated systems. Sample size (n) represents all replicates.

Roche Tina-quant® CRP (latex) HS Test System:
Values ranged from 0.2 to 16.3 mg/L (1.9 to 15.5 nmol/L).

Passing/Bablok30 Linear regression
\[ y = 1.0548x + 0.0414 \]
\[ y = 0.9877x + 0.1264 \]
\[ \tau = 0.956 \]
\[ r = 0.996 \]

Number of samples measured: 58

Dade Behring N High Sensitivity CRP:
Values ranged from 0.1 to 9.0 mg/L (1.0 to 8.6 nmol/L).

Passing/Bablok30 Linear regression
\[ y = 0.9715x + 0.0211 \]
\[ y = 0.9941x + 0.0295 \]
\[ \tau = 0.935 \]
\[ r = 0.998 \]

Number of samples measured: 54

b. Matrix comparison:
The studies described in this submission were all performed using serum samples. To validate the use of the additional sample types Li-heparin and K2-EDTA plasma with the CRP (Latex) HS assay; parallel samples were collected in serum, Li-heparin plasma, and K2-EDTA collection tubes and analyzed on the Integra 700 analyzer using the CRP(Latex) HS test system.
The serum sample was used as the reference sample and for each plasma tube type, the deviation from the reference sample was noted. For samples < 1 mg/L CRP, the deviation was expressed in absolute terms; and for samples > 1 mg/L CRP the deviation was expressed as a percentage. The plasma sample types were considered acceptable if the average deviation for samples < 1 mg/L was < 0.1 mg/L; or 10% for samples > 1mg/L. As can be seen by the attached data, these criteria were met, supporting the method sheet recommendation of Li-heparin and K2-EDTA plasma as acceptable specimen types.

3. Clinical studies:

a. Clinical Sensitivity:
Not Applicable

b. Clinical specificity:
Not Applicable

c. Other clinical supportive data (when a. and b. are not applicable):
Not Applicable

4. Clinical cut-off:
Not Applicable
5. Expected values/Reference range:
Consensus reference interval for adults:
<5.0 mg/L
IFCC/CRM 470
The CDC/AHA recommended the following hsCRP cut-off points (tertiles) for CVD risk assessment:
<1.0 mg/L low
1.0–3.0 mg/L average
>3.0 mg/L high
5-95% reference intervals of neonates and children:
Neonates (0-3 weeks): 0.1-4.1 mg/L
Children (2 months-15 years): 0.1-2.8 mg/L
N. Proposed Labeling:
The labeling is sufficient and it satisfies the requirements of 21 CFR Part 809.10.
O. Conclusion:
The submitted information in this premarket notification is complete and supports a substantial equivalence decision.
Appendix 8: Sensitivity, Precision and linearity for IL-6, IL-8, IL-1β, TNF-α and IFN-γ (Catalogue manual)

IL-6

Quantikine Human IL-6 ELISA Catalogue # HS600B

<table>
<thead>
<tr>
<th>Assay Type:</th>
<th>Solid Phase Sandwich ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Format:</td>
<td>96-well strip plate</td>
</tr>
<tr>
<td>Assay Length:</td>
<td>5.5 hours</td>
</tr>
<tr>
<td>Sample Type &amp; Volume Required Per Well:</td>
<td>Serum (100 μL), EDTA Plasma (100 μL), Citrate Plasma (100 μL), Urine (100 μL)</td>
</tr>
<tr>
<td>Sensitivity:</td>
<td>0.11 pg/mL</td>
</tr>
<tr>
<td>Assay Range:</td>
<td>0.156 - 10 pg/mL (Serum, Citrate Plasma, EDTA Plasma, Urine)</td>
</tr>
</tbody>
</table>

Specificity: Natural and recombinant human IL-6

Cross-reactivity: ≤ 0.5% cross-reactivity observed with available related molecules. < 50% cross-species reactivity observed with species tested.

Interference: Interference observed with 1 or more available related molecules.

Product Summary:
The Quantikine HS Human IL-6 Immunoassay is a 5.5 hour solid-phase ELISA designed to measure human IL-6 in serum, plasma, and urine. It contains E. coli-expressed recombinant human IL-6 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human IL-6 showed linear curves that were parallel to the standard curves obtained using the Quantikine HS kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring IL-6.

Precision:
Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.
**Linearity:**
To assess the linearity of the assay, samples spiked with high concentrations of IL-6 were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

![Graph showing linearity](image)

**Sample(s) Tested:** homogenized human colon tissue.


**Sample(s) Tested:** rabbit serum. Ara, T. *et al.* (2009) Interleukin-6 in the bone marrow microenvironment promotes the growth and survival of neuroblastoma cells. Cancer Res. **69**:329.

**Sample(s) Tested:** human serum, human bone marrow, human neuroblastoma cell culture supernate.

IL-8

Quantikine Human CXCL8/IL-8 ELISA Catalogue # DY208

**Assay Type:** Solid Phase Sandwich ELISA  
**Format:** 96-well strip plate  
**Assay Length:** 4.5 hours  
**Sample Type & Volume Required Per Well:** Serum (100 μL), EDTA Plasma (100 μL), Heparin Plasma (100 μL)  
**Sensitivity:** 0.4 pg/mL  
**Assay Range:** 1 - 64 pg/mL (Serum, Heparin Plasma, EDTA Plasma)

**Specificity:** Natural and recombinant human IL-8

**Cross-reactivity:** < 0.5% cross-reactivity observed with available related molecules. < 50% cross-species reactivity observed with species tested.

**Interference:** No significant interference observed with available related molecules.

**Product Summary:**  
The Quantikine HS Human IL-8 Immunoassay is a 4.5 hour solid phase ELISA designed to measure IL-8 levels in serum and plasma. It contains E. coli-expressed recombinant human IL-8 and antibodies raised against the recombinant factor. The immunoassay has been shown to quantitate recombinant human IL-8 accurately. Results obtained using natural human IL-8 showed linear curves that were parallel to the standard curves obtained using the Quantikine HS kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IL-8.

**Precision:**  
**Intra-Assay Precision (Precision within an assay)** Three samples of known concentration were tested on one plate to assess intra-assay precision.  
**Inter-Assay Precision (Precision between assays)** Three samples of known concentration were tested in separate assays to assess inter-assay precision.

**Linearity:**  
To assess the linearity of the assay, samples spiked with high concentrations of IL-8 were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.
Validated Sample Type(s): cell culture supernate, plasma (citrate, EDTA, heparin), serum.


Sample(s) Tested: human serum.

Sample(s) Tested: human plasma (EDTA).

Sample(s) Tested: human peripheral blood mononuclear cell culture supernate.


Sample(s) Tested: novel human bronchial epithelial cell culture supernate

IL-1β/IL-1F2

Quantikine Human IL-1β/IL-1F2 ELISA Catalogue # DLB50

| Assay Type: | Solid Phase Sandwich ELISA |
| Format: | 96-well strip plate |
| Assay Length: | 6.5 hours |
| Sample Type & Volume Required Per Well: | Serum (150 µL), EDTA Plasma (150 µL), Heparin Plasma (150 µL) |
| Sensitivity: | 0.14 pg/mL |
| Assay Range: | 0.125 - 8 pg/mL (Serum, Heparin Plasma, EDTA Plasma) |

**Specificity:** Natural and recombinant human IL-1 beta

**Cross-reactivity:** < 0.5% cross-reactivity observed with available related molecules.

**Interference:** No significant interference observed with available related molecules.

**Product Summary:**
The Quantikine HS Human IL-1 beta Immnoassay is a 6.5 hour solid phase ELISA designed to measure human IL-1 beta levels in serum and plasma. It contains E. coli-expressed recombinant human IL-1 beta and antibodies raised against the recombinant factor and has been shown to accurately quantitate recombinant human IL-1 beta. Results obtained using natural IL-1 beta showed linear curves that were parallel to the standard curves obtained using the Quantikine HS kit standards. These results indicate that this kit can be used to determine relative mass values for natural IL-1 beta. Reports indicate that ELISA kits calibrated using mature IL-1 beta as a standard will detect, but considerably underestimate, the unprocessed IL-1 beta precursor present in samples. In biological samples other than cell lysates, the precursor form of IL-1 beta is usually not the predominant form of IL-1 beta present and, additionally, is not biologically active. Therefore, results obtained using this kit should provide a useful measure of the levels of active IL-1 beta present in serum and plasma.

Sample(s) Tested: human plasma (citrate).

Sample(s) Tested: human monocyte cell culture supernate.

Sample(s) Tested: homogenized human nasal polyp tissue.
Sample(s) Tested: human serum.

Sample(s) Tested: baboon plasma (EDTA).

**TNF-α**

**Quantikine Human TNF-α ELISA Catalogue # DTA00C**

| Assay Type: Solid Phase Sandwich ELISA |
| Format: 96-well strip plate |
| Assay Length: 6.5 hours |
| Sample Type & Volume Required Per Well: Serum (200 µL), EDTA Plasma (200 µL), Heparin Plasma (200 µL), Citrate Plasma (200 µL) |
| Sensitivity: 0.191 pg/mL |
| Assay Range: 0.5 - 32 pg/mL (Serum, Heparin Plasma, Citrate Plasma, EDTA Plasma) |

**Specificity:** Natural and recombinant human TNF-alpha  
**Cross-reactivity:** < 0.5% cross-reactivity observed with available related molecules.  
< 30% cross-species reactivity observed with species tested.  
**Interference:** No significant interference observed with available related molecules.

**Product Summary:**
The Quantikine HS Human TNF-alpha Immunoassay is a 6.5 hour solid phase ELISA designed to measure TNF-alpha in serum and plasma. It contains E. coli-derived recombinant human TNF-alpha and antibodies raised against the recombinant factor. It has been shown to accurately quantitate recombinant human TNF-alpha. Results obtained with naturally occurring TNF-alpha samples showed linear curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural TNF-alpha. Since the measurement of TNF-alpha is insensitive to the addition of recombinant forms of either of the two types of soluble receptors, it is probable that this measurement detects the total amount of TNF-alpha in samples, i.e., the total amount of free TNF-alpha plus the amount of TNF-alpha bound to soluble receptors.
Validated Sample Type(s): cell culture supernate, plasma (citrate, EDTA, heparin), serum.


Sample(s) Tested: human plasma (citrate).

Sample(s) Tested: human T cell culture supernate.

Sample(s) Tested: human BALF, human synovial fluid.

Sample(s) Tested: rabbit serum.
IFN-γ

**Human IFN-γ ELISA Catalogue # D1F50**

<table>
<thead>
<tr>
<th><strong>Assay Type:</strong></th>
<th>Solid Phase Sandwich ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Format:</strong></td>
<td>96-well strip plate</td>
</tr>
<tr>
<td><strong>Assay Length:</strong></td>
<td>4.5 hours</td>
</tr>
<tr>
<td><strong>Sample Type &amp; Volume Required Per Well:</strong></td>
<td>Cell Culture Supernates (100 µL), Serum (100 µL), EDTA Plasma (100 µL)</td>
</tr>
<tr>
<td><strong>Sensitivity:</strong></td>
<td>8 pg/mL</td>
</tr>
<tr>
<td><strong>Assay Range:</strong></td>
<td>15.6 - 1,000 pg/mL (Serum, Cell Culture Supernates, EDTA Plasma)</td>
</tr>
</tbody>
</table>

**Specificity:** Natural and recombinant human IFN-gamma

**Cross-reactivity:** < 0.5% cross-reactivity observed with available related molecules. < 50% cross-species reactivity observed with species tested.

**Interference:** No significant interference observed with available related molecules.

**Product Summary:**
The Quantikine Human IFN-gamma Immunoassay is a 4.5 hour solid phase ELISA designed to measure IFN-gamma levels in cell culture supernatants, serum, and plasma. It contains E. coli-expressed recombinant human IFN-gamma and antibodies raised against the recombinant factor. Results obtained for naturally occurring human IFN-gamma samples showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for natural human IFN-gamma.

**Precision:**
- **Intra-Assay Precision (Precision within an assay):** Three samples of known concentration were tested on one plate to assess intra-assay precision.
- **Inter-Assay Precision (Precision between assays):** Three samples of known concentration were tested in separate assays to assess inter-assay precision.

**Linearity:**
To assess the linearity of the assay, five samples were spiked with high concentrations of IFN-gamma in various matrices and diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.
Validated Sample Type(s): cell culture supernate.

Sample(s) Tested: human natural killer cell culture supernate.

Sample(s) Tested: human plasma.
Sample(s) Tested: rhesus macaque peripheral blood mononuclear cell culture supernate.
Appendix 9:

Normality tests for the dependant variables

Kolmogorov-Smirnov and Shapiro-Wilk tests for the dependant variables per group

Group 1

<table>
<thead>
<tr>
<th>Tests of Normality(^b)</th>
<th>Group</th>
<th>Kolmogorov-Smirnov(^a)</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>CRP (g/l)</td>
<td>Group 1</td>
<td>.314</td>
<td>30</td>
</tr>
<tr>
<td>TNF-Alpha (pg/ml)</td>
<td>Group 1</td>
<td>.152</td>
<td>30</td>
</tr>
<tr>
<td>Inf-Gamma (pg/ml)</td>
<td>Group 1</td>
<td>.191</td>
<td>30</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>Group 1</td>
<td>.166</td>
<td>30</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>Group 1</td>
<td>.251</td>
<td>30</td>
</tr>
<tr>
<td>IL-1Beta (pg/ml)</td>
<td>Group 1</td>
<td>.174</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) Lilliefors Significance Correction

\(^b\) Group = Group 1

Group 2

<table>
<thead>
<tr>
<th>Tests of Normality(^b)</th>
<th>Group</th>
<th>Kolmogorov-Smirnov(^a)</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>CRP (g/l)</td>
<td>Group 2</td>
<td>.475</td>
<td>30</td>
</tr>
<tr>
<td>TNF-Alpha (pg/ml)</td>
<td>Group 2</td>
<td>.113</td>
<td>30</td>
</tr>
<tr>
<td>Inf-Gamma (pg/ml)</td>
<td>Group 2</td>
<td>.188</td>
<td>30</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>Group 2</td>
<td>.263</td>
<td>30</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>Group 2</td>
<td>.425</td>
<td>30</td>
</tr>
<tr>
<td>IL-1Beta (pg/ml)</td>
<td>Group 2</td>
<td>.245</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^a\) Lilliefors Significance Correction

\(^*\) This is a lower bound of the true significance.

\(^b\) Group = Group 2
Group 3

<table>
<thead>
<tr>
<th>Test</th>
<th>Group</th>
<th>Kolmogorov-Smirnov</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>CRP (g/l)</td>
<td>Group 3</td>
<td>.493</td>
<td>30</td>
</tr>
<tr>
<td>TNF-Alpha (pg/ml)</td>
<td>Group 3</td>
<td>.239</td>
<td>30</td>
</tr>
<tr>
<td>Inf-Gamma (pg/ml)</td>
<td>Group 3</td>
<td>.258</td>
<td>30</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>Group 3</td>
<td>.193</td>
<td>30</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>Group 3</td>
<td>.386</td>
<td>30</td>
</tr>
<tr>
<td>IL-1Beta (pg/ml)</td>
<td>Group 3</td>
<td>.159</td>
<td>30</td>
</tr>
</tbody>
</table>

a. Lilliefors Significance Correction  
b. Group = Group 3

Histograms (Left) and P-P plots (right) demonstrating non-normally distributed variables in group 1
Box-plots demonstrating non-normally distributed variables in group 1
Histograms (left) and P-P plots demonstrating non-normally distributed variables in group 2.
Box-plots demonstrating non-normally distributed variables in group 2
Histograms (lefts) and P-P plots demonstrating non-normally distributed data in group 3.
Box-plots demonstrating non-normally distributed variables in group 3
Normality tests for the independent variables

Kolmogorov-Smirnov and Shapiro-Wilk tests for the independent variables per group

Group 1

<table>
<thead>
<tr>
<th>Tests of Normality&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Kolmogorov-Smirnov&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>Age</td>
<td>.159</td>
<td>30</td>
</tr>
<tr>
<td>BMI</td>
<td>.152</td>
<td>30</td>
</tr>
<tr>
<td>Systolic BP Last Visit</td>
<td>.088</td>
<td>30</td>
</tr>
<tr>
<td>Diastolic BP Last Visit</td>
<td>.155</td>
<td>30</td>
</tr>
<tr>
<td>RBG</td>
<td>.113</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lilliefors Significance Correction

<sup>b</sup> This is a lower bound of the true significance.

b. Group = Group 1

Group 2

<table>
<thead>
<tr>
<th>Tests of Normality&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Kolmogorov-Smirnov&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>Age</td>
<td>.110</td>
<td>30</td>
</tr>
<tr>
<td>BMI</td>
<td>.180</td>
<td>30</td>
</tr>
<tr>
<td>Systolic BP Last Visit</td>
<td>.089</td>
<td>30</td>
</tr>
<tr>
<td>Diastolic BP Last Visit</td>
<td>.133</td>
<td>30</td>
</tr>
<tr>
<td>RBG</td>
<td>.164</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lilliefors Significance Correction

<sup>b</sup> This is a lower bound of the true significance.

b. Group = Group 2
Group 3

Tests of Normality\(^b\)

<table>
<thead>
<tr>
<th></th>
<th>Kolmogorov-Smirnov(^a)</th>
<th>Shapiro-Wilk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statistic</td>
<td>df</td>
</tr>
<tr>
<td>Age</td>
<td>.132</td>
<td>30</td>
</tr>
<tr>
<td>BMI</td>
<td>.268</td>
<td>30</td>
</tr>
<tr>
<td>Systolic BP Last Visit</td>
<td>.102</td>
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</tr>
<tr>
<td>Diastolic BP Last Visit</td>
<td>.136</td>
<td>30</td>
</tr>
<tr>
<td>RBG</td>
<td>.093</td>
<td>30</td>
</tr>
</tbody>
</table>

\(a\). Lilliefors Significance Correction

\(^*\). This is a lower bound of the true significance.

\(b\). Group = Group 3