Distal sensory polyneuropathy in South Africans infected with human immunodeficiency virus: a cross-sectional analysis of a community cohort.

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Declaration

I, Jean Maritz, hereby declare that the work on which this dissertation is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work or any part of it has been, is being, or is to be submitted for another degree is this or any other university.

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Abbreviations

ADSP – Asymptomatic Distal Sensory Polyneuropathy

AIDS – Acquired Immunodeficiency Syndrome

ART – Antiretroviral Therapy

ATN – Antiretroviral Toxic Neuropathy

BMI – Body Mass Index

bp – Basepair

CIPN – Chemotherapy-induced peripheral neuropathy

DNA – Deoxyribonucleic Acid

dNRTI – dideoxynucleoside Reverse Transcriptase Inhibitor

dNTPs – Deoxyribonucleotide Triphosphates

DRG – Dorsal Root Ganglion

DSP – Distal Sensory Polyneuropathy

EDTA – Ethylene diaminetetraacetic acid

ENFD – Epidermal Nerve Fibre Density

gp120 – glycoprotein 120

HIV – Human Immunodeficiency Virus

Hz – Hertz

IFN – Interferon

IL – Interleukin

IV – Intravenous

kD – Kilodalton

LPS – lipopolysaccharide

mtDNA– Mitochondrial DNA

NCBI – National Centre for Biotechnology Information

NK – natural killer

NSAID – Non-Steroidal Anti-Inflammatory Drugs

OGTT – Oral Glucose Tolerance Test

PCR – Polymerase Chain Reaction

PGP – Protein Gene Product

RANTES – Regulated upon Activation, Normal T-cell Expressed and Secreted

rpm – Revolutions Per Minute

RNA – Ribonucleic Acid

SDSP – Symptomatic Distal Sensory Polyneuropathy

SFN – Small Fibre Neuropathy

SNP – Single Nucleotide Polymorphism

TACE – Tumour Necrosis Factor-Alpha Converting Enzyme

Tm – melting temperature

TNF – Tumour Necrosis Factor

TNF-R – Tumour Necrosis Factor Receptor

w/v – Weight per volume

WHO – World Health Organization

Abstract

Introduction

Distal sensory polyneuropathy (DSP), the most common neurological complication of HIV infection, is related to either HIV or antiretroviral therapy (ART). Dideoxynucleoside reverse transcriptase inhibitors such as stavudine are widely used in resource-poor countries and often associated with neuropathy. The prevalence of DSP in developed countries range from 21% to 63%; little data is available from Africa. We aimed to estimate the prevalence of DSP in a South African community clinic-based population and to investigate associated risk factors.

Methods

In a cross-sectional study, DSP status was determined in 598 HIV-infected adults using validated tools (Brief Peripheral Neuropathy Screen and a modified version of the Total Neuropathy Score) to categorize subjects. Symptomatic DSP required the presence of at least two neuropathic signs together with at least one symptom. Asymptomatic DSP required the presence of two neuropathic signs. Clinical, anthropometric, quality of life and laboratory evaluations were prospectively performed. Information about CD4 counts, antiretroviral therapy (ART) and questionnaires regarding previous tuberculosis (TB) and alcohol exposure was retrospectively collected

Results

Approximately half (49%) of the study population were diagnosed with DSP (30% symptomatic DSP). In the ART-naïve group 37% had evidence of neuropathy (23%

symptomatic) compared to 63% of the ART-exposed subjects (39% symptomatic). Overall, subjects with DSP were older ($p<0.001$) and had lower CD4 counts ($p<0.001$) compared to those without neuropathy. Previously treated TB infection ($p<0.001$) and ART use ($p<0.001$) showed strong associations with DSP. In multivariate analyses the odds (95% confidence interval) of developing DSP was independently associated with ART use (OR 1.7, 1.0-2.9), age (per 10 year increments) (OR 1.7, 1.4-2.2) and previously treated TB infection (OR 2.0, 1.3-3.0). Although stavudine significantly associated with DSP, the duration of exposure was similar irrespective of neuropathy status. Pain or paresthesiae was reported by 69% of those with symptomatic DSP and rated as at least moderate to severe. ART-exposed subjects had a tendency towards lower pain scores compared to ART-naïves ($p=0.032$).

Conclusions

DSP is a clinically significant problem in urban HIV-infected Africans. The findings of this study raise the possibility that with avoidance of stavudine-containing regimens in older subjects, especially those with a history of previously treated TB infection, the prevalence of DSP may be reduced.

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Chapter 1

Background

1.1 Distal sensory polyneuropathy background

1.1.1 Introduction

Human immunodeficiency virus (HIV) infection and the acquired immunodeficiency syndrome (AIDS) currently have a devastating impact in South Africa. In 2006, 11% of the South African population or about 5.4 million South Africans was infected with HIV, of which about 600 000 people were suffering from AIDS (Dorrington *et al.,* 2006).

Distal sensory polyneuropathy (DSP) is recognized as the most common neurological complication of HIV infection (Barohn *et al.,* 1993, Tagliati *et al.,* 1999). Clinically it presents as a painful neuropathy, although autonomic dysfunction and thermal hypoesthesia can also occur (Devigili *et al.,* 2008). DSP may have a drastic impact on quality of life due to neuropathic pain and potentially limits activities of daily living (as measured by a quality of life score) due to neuropathic symptoms (Pettersen *et al.,* 2006). Amongst HIV-infected subjects, living with pain also increases the frequency of visits to outpatient services, putting additional strain on medical resources (Dobalian *et al.,* 2004). It may consequently also have psychosocial implications as a result of unemployment or depression.

DSP in the context of HIV infection may arise either as a direct consequence of the viral infection itself (probably related to HIV-triggered immune activation) or as an adverse effect of antiretroviral therapy (ART) (see *1.1.2 Categorization of HIV-associated DSP*). We refer to the former as HIV-DSP and the latter as antiretroviral toxic neuropathy (ATN). The more generic term DSP is used when the distinction between these two pathophysiological mechanisms is not being made.

Current treatment modalities for DSP are also frequently ineffective. Agents such as non-steroidal anti-inflammatory drugs, opioid analgesics and tricyclic

antidepressants offer relief only in a minority of patients (Wulff *et al.,* 2000). Anticonvulsants such as gabapentin, which has shown therapeutic benefit, are not available in the state healthcare sector (Verma *et al.,* 2004). Lamotrigine, which is widely available, has only limited efficacy (Silver *et al.,* 2007), and carbamazepine is contraindicated with antiretroviral therapy.

No data on either the prevalence of HIV-associated DSP or associations thereof in African subjects have been published to date. As DSP may impose a significant problem on our HIV-infected population and on already-limited medical resources, we aimed to describe the prevalence of HIV-associated DSP in an urban African population and investigate the association between HIV-associated DSP and possible risk factors. We also investigated the association between selected functional cytokine gene promoter polymorphisms and the occurrence of HIV-associated DSP.

1.1.2 Categorization of HIV-associated DSP

HIV-associated DSP can be categorized into two groups based on etiology. It can occur either as a direct consequence of HIV infection (HIV-DSP) or related to antiretroviral therapy (ART), termed antiretroviral toxic neuropathy (ATN). ATN is clinically and electrophysiologically indistinguishable from HIV-DSP (Cherry *et al.,* 2006), although early studies suggest a more rapid onset and progression of ATN compared to HIV-DSP, particularly in individuals with moderate to severe immune suppression and at higher doses of neurotoxic drugs (Berger *et al.,* 1993). HIV-associated DSP is a predominantly small fibre length-dependant sensory polyneuropathy that may manifest as pain, paresthesiae, numbness or cramps. Symptoms are bilateral and symmetrical, and predominantly affect the lower legs although the hands may also become involved as the disease progresses.

HIV-associated DSP can also be classified based on the presence or absence of symptoms. Individuals with symptoms of small fibre dysfunction in addition to signs are referred to as having symptomatic DSP (SDSP), whereas those with signs but

without symptoms are referred to as having asymptomatic DSP (ADSP). Signs of DSP would be either deep tendon reflex changes with a distal-to-proximal gradient of hypo- or areflexia, as well as vibration sense loss or pinprick sensation loss in a glove-and-stocking distribution. Motor weakness is an uncommon finding, and usually only affects the intrinsic foot muscles (Wulff *et al.,* 2000). It is not yet clear whether the development of asymptomatic- and symptomatic DSP represent a continuum from a neuropathy-free state. Schifitto *et al.* demonstrated that a diagnosis of asymptomatic DSP was not a significant predictor of subsequent development of symptomatic DSP (Schifitto *et al.,* 2002). Morgello *et al.* found that risk factors such as demographic variables, dideoxynucleoside reverse transcriptase inhibitor (dNRTI) exposure, plasma viral load and $CD4^+$ count did not significantly differ between individuals with symptomatic or asymptomatic DSP (Morgello *et al.,* 2004). Epidermal nerve fibre density (EFND, see *1.1.7 Diagnostic Methods*) quantification has also been used in an attempt to better define asymptomatic DSP. Herrmann *et al.* showed that subjects with a diagnosis of both symptomatic- or asymptomatic DSP had reduced ENFDs compared to subjects without DSP and HIV negative controls (Herrmann *et al.,* 2004) and also that individuals with reduced ENFD have a 14-fold increased risk of developing symptomatic DSP (Herrmann *et al.,* 2006).

1.1.3 Pathogenesis of HIV-associated DSP

HIV-associated neuropathy is a small fibre neuropathy that predominantly affects small unmyelinated C-fibres and possibly thinly myelinated Aδ-fibres (Devigili *et al.,* 2008). A 'dying back' pattern of degeneration is seen in sensory neurons, which is similar to the degeneration seen in diabetic and alcoholic polyneuropathies (Melli *et al.,* 2006). Clinically, a length-dependant pattern of sensory loss, with or without allodynia, is noted in a glove-and-stocking distribution.

1.1.3.1 Pathogenesis of HIV-DSP

Several mechanisms have been proposed for the pathogenesis of HIV-DSP. Reports of axonal localizing HIV-1 in peripheral nerve tissue are limited, and in situ hybridization has detected HIV-1 RNA in only a few isolated perivascular mononuclear cells in dorsal root ganglia (DRG) in a minority of patients with AIDS (Yoshioka *et al.,* 1994). Very few of these patients had any evidence of DSP (Yoshioka *et al.,* 1994). Since direct neurotoxicity seemed unlikely as a primary cause for DSP, the focus shifted to the investigation of secreted viral proteins and cytokine-mediated injury.

HIV entry into target cells are facilitated by the membrane-bound chemokine receptors CXCR4 and CCR5 (Keswani *et al.,* 2003). These receptors can also be ligated by viral proteins, as it has been shown that the HIV-1 envelope glycoprotein 120 (gp120) in its soluble form can bind to CXCR4 and CCR5 receptors on axonal membranes and exert pathologic effects in nerves and DRGs (Keswani *et al.,* 2003, Melli *et al.,* 2006).

CXCR4 and CCR5 receptors are present on a variety of cell types, including Schwann cells. Exposure of Schwann cells to gp120 causes ligation of CXCR4 receptors and subsequent secretion of chemokines such as RANTES (regulated upon activation, normal T-cell expressed and secreted). RANTES causes neurons to release tumour necrosis factor alpha (TNF-α), which induces neuronal apoptosis via TNF-receptor-1 signalling (Keswani *et al.,* 2003). Both RANTES and TNF-α previously have been linked to neuropathic pain, which may explain the painful nature of DSP (Keswani *et al.,* 2003).

In vitro experiments have shown gp120 to be pathogenic both to sensory neuronal cell bodies in the presence of Schwann cells, and also directly to nerve axons (Melli *et al.,* 2006, Keswani *et al.,* 2003*)*. In cell bodies, the binding of gp120 to receptors on Schwann cells caused a reduction in neurite branching and neurite length per neuron, as well as neuronal mitochondrial membrane depolarization, the latter begin a crucial event early in the process of programmed cell death, or apoptosis (Keswani *et al.,* 2003). In axons, binding of gp120 to CXCR4 receptors results in

mitochondrial dysfunction and ultimately in decreased axon length, but not apoptosis (Melli *et al.,* 2006). Similar dysfunction of axonal mitochondria has also been demonstrated in diabetic polyneuropathy (Melli *et al.,* 2006).

The proposed sources of gp120 are HIV-infected macrophages infiltrating DRGs and peripheral nerve axons, or circulating gp120 due to the absence of an appreciable blood-nerve barrier at terminal axons (Keswani *et al.,* 2003). In support of the causative role of gp120 in DSP, studies have demonstrated a decrease in ENFD (see *1.1.7 Diagnostic Methods*) in rats treated with perineural gp120 (Wallace *et al.,* 2007).

In conclusion, the pathogenesis of HIV-DSP is not fully understood but viral protein and host cytokine responses may participate in a complex interplay resulting in nerve fibre toxicity and loss.

1.1.3.2 Pathogenesis and history of Antiretroviral Toxic Neuropathy (ATN)

The exact pathogenesis of ATN is not yet established. It is proposed that ATN caused by the dNRTI-class of drugs may occur through dysfunction or toxicity of mitochondrial metabolism (Berger *et al.,* 1993, Keswani *et al.,* 2002). Based on an animal model investigating neurotoxic antiretroviral drugs, it has been proposed that ATN may also be a synergistic consequence of both HIV infection and ART neurotoxicity (Pettersen *et al.,* 2006).

Dideoxynucleoside analogues contain azido groups that compete with natural thymidine triphosphate as substrates of DNA polymerase γ, the host enzyme responsible for mitochondrial DNA synthesis. This competitive inhibition of DNA polymerase γ may disrupt mitochondrial DNA (mtDNA) synthesis and ultimately result in decreased levels of mtDNA (in Hung *et al.,* 2008). Plasma mtDNA depletion was not found to be present in all subjects with clinical evidence of mitochondrial toxicity (including those with peripheral neuropathy), and surprisingly, found to be increased in subjects exposed to dNRTIs for more than 5 years (McComsey *et al.,* 2005). The authors postulated that blood levels of mtDNA may reflect an

overreplication of mtDNA due to mitochondrial dysfunction after long-standing dNRTI exposure (McComsey *et al.,* 2005).

ATN was the dose-limiting toxicity in phase I trials of several agents used to treat HIV infection. Stavudine (d4T) (Browne *et al.,* 1993), didanosine (ddI) (Lambert *et al.,* 1990) and zalcitabine (ddC) (Berger *et al.,* 1993), all dNRTIs, have been shown to cause ATN (Cherry *et al.,* 2006, Smyth *et al.,* 2007). In phase I studies on stavudine, ATN occurred as early as five weeks after exposure to the drug (Browne *et al.,* 1993), and symptomatic peripheral neuropathy was the dose-limiting toxicity associated with increased doses and more frequent dosing intervals (Browne *et al.,* 1993, Berger *et al.,* 1993).

A WHO survey on ART use in resource-limited countries showed that 69% of first-line ART regimens included stavudine, representing 53% of all patients on ART in 23 developing countries, or an estimated 851 000 individuals (Renaud-Théry *et al.,* 2006). Stavudine is still widely used as a first-line agent in South African ART regimens, and can thus still have a considerable impact on prevalence rates of ATN in our HIV-infected population.

In keeping with the WHO recommendations (Antiretroviral Therapy Recommendations [WHO], 2006), South African ART regimens do not contain more than one dNRTI-class drug due to possible or probable overlapping toxicity (Renaud-Théry *et al.,* 2006). The combination of stavudine and didanosine compared to stavudine monotherapy has an increased DSP prevalence of 2.5-fold (Moore *et al.,* 2000). Combination therapy compared to didanosine monotherapy increased DSP prevalence 3.5-fold (Moore *et al.,* 2000), and didanosine is occasionally used as a second-line agent in South Africa (National Antiretroviral Treatment Guidelines 2004). Although fewer patients are on second-line ART than first-line ART, 60% of individuals in developing countries are still receiving a ddI-containing second-line ART regimen (Renaud-Théry *et al.,* 2006).

It is not yet clear what duration of dNRTI exposure confers maximum risk of developing toxicity to nerves, and conversely, not all studies have shown an association between the use of dNRTIs and the development of neuropathy.

Longitudinal studies have shown that exposure to dNRTIs were associated with a decreased risk of developing DSP and even a trend towards protection against symptomatic DSP (Schifitto *et al.,* 2002, Simpson *et al.,* 2006). The authors suggested that, although seemingly paradoxical, maintaining a certain degree of immune function through dNRTI use possibly outweighs the drugs' potential neurotoxicity (Schifitto *et al.,* 2002). More recent studies confirmed that neither exposure to dNRTIs at baseline nor their cumulative use was associated with progression of neuropathy (Simpson *et al.,* 2006, Arenas-Pinto *et al.,* 2008). Based on these findings, several researchers have hypothesized that dNRTI neurotoxicity occurs early during the agents' use in susceptible subjects who then discontinue the agents, as well as a survival bias in those subjects who tolerate the drug's effects (Simpson *et al.,* 2006, Hung *et al.,* 2008, Arenas-Pinto *et al.,* 2008).

Arenas-Pinto *et al.* found that the risk for DSP peaked at 90 days of exposure to dNRTIs, where after the risk decreased (Arenas-Pinto *et al.,* 2008). It was also suggested that individuals who remain neuropathy free after 12 months of exposure to dNRTIs will tolerate continued therapy without an increased risk of worsening neuropathy signs or symptoms (Hung *et al.,* 2008). Morgello *et al.* concluded that dNRTI use *per se* in the era of highly active antiretroviral therapy (HAART) was not a risk factor for the development of DSP, but that its contribution to the development of DSP is reliant on the underlying metabolic and immunologic status of the individual (Morgello *et al.,* 2004).

Other classes of ART drugs, such as the protease inhibitors (indinavir, saquinavir and ritonavir - Pettersen *et al.,* 2006, Smyth *et al.,* 2007), have also been associated with the development of neuropathy. Fusion inhibitors such as enfuvirtide are new drugs used for the treatment of HIV infection and are also currently under investigation for possible neurotoxic effects (Cherry *et al.,* 2008a).

1.1.4 Risk factors for HIV-associated DSP

Known etiologies for small fibre neuropathies other than HIV or ART include diabetes and the pre-diabetic state, vitamin B_{12} deficiency, hypothyroidism, statin therapy, isoniazid therapy, immune-mediated and connective tissue disorders, paraneoplastic syndromes and opportunistic infections such as cytomegalovirus (CMV) infection (Tagliati *et al.,* 1999, Pardo *et al.,* 2001, Devigili *et al.,* 2008). In a proportion of subjects a cause cannot be identified.

HIV-associated DSP has previously been associated with older age, patient height and BMI, poor nutrition, hepatitis C co-infection, plasma lactate level and advanced HIV disease with CD4 counts below 200 cells/µl (Tagliati *et al.,* 1999, Childs *et al.,* 1999, Morgello *et al.,* 2004, Cherry *et al.,* 2005, Cherry *et al.,* 2006, Simpson *et al.,* 2006, Smyth *et al.,* 2007).

Demographic variables such as male sex and white race have been reported to be associated with DSP (Tagliati *et al.,* 1999, Morgello *et al.,* 2004), but not consistently (Schifitto *et al.,* 2002). Although age older than 40 years is generally accepted as being particularly important in the development of DSP (Morgello *et al.,* 2004, Cherry *et al.,* 2006), one study reported that it was only associated with the presence of DSP, not the progression thereof (Simpson *et al.,* 2006). When measured in 5-year increments, older age did not predict a worsening in the outcome measure (Total Neuropathy Score, see *1.1.7 Diagnostic Methods)* over a 48 week period (Simpson *et al.,* 2006).

As a measure of HIV severity, high plasma HIV RNA levels have been associated with the presence and severity of DSP (Simpson *et al.,* 2002). Studies reported higher HIV load and lower CD4 counts to be predictors of particularly symptomatic DSP (Childs *et al.,* 1999, Watters *et al.,* 2004). Nadir CD4 counts was found to be a predictor of HIV-associated DSP, but not the current CD4 count (at the time of study) in a cross-sectional cohort (Lichtenstein *et al.,* 2005). Either of these measures of HIV severity (CD4 counts and viral load) have failed to correlate with either the presence of progression of neuropathy consistently, although selection bias by study parameters may be at fault (Simpson *et al.,* 2006). An early study on

HIV-associated neuropathy on about 800 North American subjects reported that no individuals with $CD4^+$ counts greater than 300 cells/µl had any evidence of neuropathy (Barohn *et al.,* 1993).

The role of alcohol exposure as a risk factor is poorly defined at present. Studies have reported a positive association between alcohol use and the development of symptomatic DSP (Morgello *et al.,* 2004) while others failed to find an association, and another study reported that alcohol use was more prevalent in neuropathy-free subjects than in subjects with neuropathy (Pettersen *et al.,* 2006). One study found impairment in physical function to be a predictor for symptomatic DSP (Schifitto *et al.,* 2002) but it seems more likely that the association comes from a limitation in activities of daily living due to painful neuropathy.

Vitamin B_{12} levels have not been associated significantly with DSP in HIV-infected individuals (Tagliati *et al.,* 1999). A recent study from an urban African population on TB therapy reported a 57% reduction in peripheral neuropathy in those supplemented with various micronutrients (including vitamin B complex), irrespective of HIV status (Villamor *et al.,* 2008).

1.1.5 Prevalence of HIV-associated DSP in developed countries

The prevalence of HIV-associated DSP (combined HIV-DSP and ATN) from cross-sectional studies has been reported in the literature to range from 21% (Marra *et al.,* 1998) to 53% and 63% (Morgello *et al.,* 2004, Cherry *et al.,* 2006). Longitudinal studies report a high conversion rate from baseline, as 34% to 52% of neuropathy free patients developed symptomatic DSP at 1 year follow-up (Schifitto *et al.,* 2002, Simpson *et al.,* 2006). In these studies, dNRTI use ranged from 29% to 83%. It is important, however, to keep in mind that some of the published studies were based on highly selected populations, such as a referral neurology clinic (Simpson *et al.,* 2006) or the HIV Brain Bank project (Morgello *et al.,* 2004) and might not be truly generalisable to the community.

A population-based (non-referral) study from a military medical centre reported a 16% DSP prevalence rate – the majority of subjects had early HIV disease as the diagnosis was made in a mandatory screening program, and therefore may be more representative of the background prevalence in a general population of young men (Barohn *et al.,* 1993).

Since the advent of HAART in 1996, incidence rates of CNS opportunistic infections and dementia have decreased (Sacktor *et al.,* 2001, Brodt *et al.,* 1997) but the incidence of HIV-associated DSP has increased with rates exceeding 50% per year (Bacellar *et al.,* 1994, Morgello *et al.,* 2004, Watters *et al.,* 2004). Although it was thought that development and progression of neuropathy correlated with progression of HIV disease and that longer survival with severe immunosuppression might explain the observed increases in incidence rates, the effect remained after adjusting for CD4 cell count.

1.1.6 Data on HIV-associated DSP in Africa and other developing countries

Limited data is available from African populations. A Medline search (using 'Africa', 'HIV' and 'neuropathy' as parameters) failed to show studies documenting data of a systematic examination for neuropathy using validated clinical tools, although a few reports mentioning neuropathy prevalence in the context of adverse effects of ART were found. In the majority of these studies, diagnostic criteria for neuropathy were not defined and the diagnosis was frequently based mainly on symptom reporting (Jamisse *et al.,* 2007, Hawkins *et al.,* 2007). Jamisse *et al.* investigated ART-related toxicities in urban pregnant women in Mozambique, and found an 18% symptomatic neuropathy prevalence rate based on self-reporting of acute numbness, pain or paresthesiae with no reference made to the presence of signs (Jamisse *et al.,* 2007). Such symptoms were not associated with $CD4^+$ count, and neither with AZT- or stavudine-containing ART regimens (Jamisse *et al.,* 2007). A study on ART tolerability in an urban Kenyan population reported a 21% HIV-associated neuropathy prevalence, but no reference as to which criteria were used for the diagnosis (Hawkins *et al.,* 2007). Recently a South African study evaluated

the tolerability of non-dNRTI regimens in an urban South African cohort and reported a DSP incidence of 8 per 100-person years. The diagnosis was based on reported symptoms (numbness or paresthesiae in the lower limbs), or the presence of decreased sensation to light touch or attenuated ankle deep tendon reflexes on physician examination (Hoffmann *et al.,* 2008). A large multicentre study from the Asia-Pacific region reported a 19.7% prevalence of symptomatic DSP based on the presence of symptoms and at least 1 neuropathic sign consistent with DSP (Wright *et al.,* 2008).

1.1.7 Diagnostic methods

Various neurophysiologic and neuropathologic techniques have been used in the diagnosis of small fibre neuropathy. Commonly used tools include questionnaires on sensory symptoms (for symptomatic DSP), sensory signs, ankle deep tendon reflexes, quantitative sensory threshold testing (QST), sural sensory nerve action potentials (SNAP) and epidermal nerve fibre density (ENFD) quantification on skin biopsy. Routine nerve conduction studies cannot reliably identify pure small fibre neuropathy due to the lack of myelination of small sensory nerve fibres (Lauria *et al.,* 2008).

There is not currently a recognised clinical definition for small fibre neuropathy for use in research trials, which poses a problem in comparing reported neuropathy rates and risk factors. Neurophysiologic studies such as QST and SNAPs require specialized equipment and thus have practical limitations in field studies in resource-poor settings. ENFD measurements on skin biopsy can be reliably quantified and is an objective measure of small fibre neuropathy in the clinical setting (Lauria, 2005), but is impractical in field studies. Because of these limitations, field or epidemiological studies therefore rely on the availability and validity of clinical tools to detect DSP, and even more so in resource-limited settings where there is a lack of specialised diagnostic equipment.

1.1.7.1 Proposed definitions

In 2005, a set of definitions for distal symmetrical polyneuropathy for clinical research were proposed by a collaboration between the American Academy of Neurology, the American Association of Electrodiagnostic Medicine and the American Academy of Physical Medicine and Rehabilitation (England *et al.,* 2005). This definition is dependent on the presence of neuropathic symptoms, including numbness, burning, paresthesiae, dysesthesiae and allodynia, and the presence of signs, including attenuated deep tendon reflexes, decreased pin sensation or decreased vibration sense, recognising that deep tendon reflexes abnormalities may not be necessary for a diagnosis of small fibre neuropathy (England *et al.,* 2005). Different definitions for clinical research studies and epidemiologic studies were proposed, and definitions were ranked by estimated ordinal likelihood of disease.

For field studies, the highest ranked definitions included a combination of symptoms, attenuated deep tendon reflexes and decreased sensation (pin or vibration), although the task force recognized that pure small fibre neuropathy may not necessarily have abnormal deep tendon reflexes. The collaboration concluded that evidence-based literature for pure small fibre neuropathy is insufficient at present to provide an adequate case definition (England *et al.,* 2005).

1.1.7.2 Epidermal Nerve Fibre Density (ENFD)

Skin biopsy with determination of ENFD is the most reliable method of diagnosing small fibre neuropathy and has been used in the examination of patients with suspected small fibre neuropathy since the availability of antibodies against protein-gene-product 9.5 (PGP9.5) which allows immunocytochemical staining of the extensive innervation of the epidermis (McArthur *et al.,* 1998, Lauria, 2005, Devigili *et al.,* 2008). This technique was also used to demonstrate that small fibres, which penetrates to the stratum spinosum layer in the epidermis (McArthur *et al.,* 1998), undergo morphologic change early in the course of neuropathies associated with HIV infection and that this can be a predictor of progression to a more diffuse

neuropathy (Herrmann *et al.,* 2006, Devigili *et al.,* 2008). Studies showed a significant reduction in the ENFD in subjects with small fibre neuropathy in both the thigh and distal leg, but as expected more fibre loss in the distal leg due to the "dying-back" pattern of degeneration, and a negative correlation was demonstrated between ENFD and neuropathic pain intensity as measured by a validated indicator of pain severity (Zhou *et al.,* 2007, Polydefkis *et al.,* 2002). As briefly mentioned earlier, it has been shown that a reduction in ENFD confers a 14-fold increased risk of developing symptomatic DSP (Herrmann *et al.,* 2006).

Despite the proven accuracy of ENFD quantification, its use is unfortunately limited in a resource-poor research settings. Skin sampling by punch biopsy can be done with little pain or discomfort in the clinic, but expertise on the histological techniques is not widely available.

1.1.7.3 Clinical tools

The Brief Peripheral Neuropathy Screen (BPNS) (see *Appendix A*) is a screening tool for DSP that was developed for the clinical diagnosis of HIV-associated neuropathies and has been used by the AIDS Clinical Trial Group (North America) in study protocols (McArthur, 1998, Cherry *et al.,* 2005). A diagnosis of symptomatic DSP on an adapted version of the BPNS (only requiring 1 sign for the diagnosis of symptomatic DSP) has been shown to correlate well with decreased ENFD measurements as well as increased thresholds on QST as a diagnostic tool for DSP (Cherry *et al.,* 2005). The BPNS was also validated to differentiate between cases with symptoms and signs consistent with DSP, and those only reporting symptoms without the presence of signs. The latter group do not meet the criteria for DSP on the BPNS (previously labeled as being indeterminate), and was shown to have ENFD and QST measurements similar to subjects without symptoms or signs suggestive of DSP (Cherry *et al.,* 2005).

The Total Neuropathy Score (TNS) is another scoring system for distal length-dependant axonal neuropathy, and has been shown to be a valid measure of

peripheral nerve function with excellent inter- and intrarater reliability (Cornblath *et al.,* 1999). It was validated in diabetic polyneuropathy against various other recognised clinical measures of peripheral nerve function, as well as sensory- and motor nerve biopsies, and can also be used to measure the progression of neuropathy longitudinally (Cornblath *et al.,* 1999). A reduced version of the TNS was developed specifically for the diagnosis of chemotherapy-induced peripheral neuropathy (CIPN), a painful sensory polyneuropathy (Cavaletti *et al.,* 2003). This version did not included vibration QST testing, and also did not score autonomic or motor symptoms, but was validated against the same clinical tools as the original TNS (Cavaletti *et al.,* 2003). In addition it showed good concordance with ENFD measurement, endorsing it as a reliable tool in screening for sensory polyneuropathies (Zhou *et al.,* 2007).

Another modified version of the TNS was developed, only incorporating clinical parameters (TNSc). This scoring system grades symptoms (sensory, motor and autonomic) as well as clinical signs, namely pin sensibility, vibration sensibility, deep tendon reflexes and strength. It was developed as a tool that could be applied at the bedside without the need for special equipment. The TNSc was validated in a multicentre study against the same scoring systems as the original TNS and the reduced TNS in the diagnosis of CIPN, and was found to have good correlation with the aforementioned tools (Cavaletti *et al.,* 2007).

1.1.8 Aims

Concerns exist about the frequent use of stavudine in a population already at risk of developing polyneuropathy based on the high prevalence of malnutrition, alcohol use, prior anti-tuberculous therapy and HIV infection itself. Interaction of these risk factors can possibly lead to an epidemic of antiretroviral toxic neuropathy, and these concerns have provided the impetus for this cross-sectional study with the following aims:

1) To establish the prevalence of neuropathy amongst HIV-infected subjects including both HIV-DSP and ATN, in a community-based cohort, and

2) to identify relevant risk factors that may contribute to the development of distal sensory polyneuropathy in this HIV-infected population.

1.2 Tumour necrosis factor background

1.2.1 Introduction

Tumour necrosis factor alpha (TNF-α) is a potent immune modulator and pro-inflammatory cytokine, which has been implicated in the pathogenesis of autoimmune and infectious diseases (Herbein and Khan 2008, in Abbas *et al.,* 2007). It was originally identified as a substance present in the serum of animals treated with lipopolysaccharide (LPS, a bacterial endotoxin) which caused the necrosis of tumours *in vivo* (in Thomson and Lotze, 2003). In low concentrations, it confers resistance to certain types of infection, but at higher levels (as caused by severe infection) it can be the cause of pathological complications (in Thomson and Lotze, 2003, in Abbas *et al.,* 2007).

1.2.2 Gene and protein structure

TNF-α is synthesized as a 26 kilodalton (kD) non-glycosylated type II membrane protein and is expressed as a homotrimer on the membranes of mononuclear phagocytes (in Abbas *et al.,* 2007, in Thomson and Lotze, 2003). The membrane form of TNF-α is cleaved by a membrane-associated metalloproteinase disintegrin named tumour necrosis factor-alpha converting enzyme (TACE), releasing a 17-kD polypeptide. Circulating TNF-α is a 51-kD protein formed by polymerization of three of these polypeptides. It assumes a triangular shape, and can simultaneously bind to three receptor molecules (in Abbas *et al.,* 2007).

1.2.3 Function

TNF-α exerts effects on a various immune cell types, vascular endothelium, adipocytes, fibroblasts as well as various organ systems (in Thomson and Lotze, 2003). Its principal function however, is stimulating the recruitment of mononuclear cells and neutrophils to sites of inflammation, and the activation of these cell types (in Abbas *et al.,* 2007). Consequently chemokines are secreted by

these activated macrophages and vascular epithelium expresses adhesion molecules for leukocytes resulting in leukocyte chemotaxis (in Abbas *et al.,* 2007, in Thomson and Lotze, 2003). TNF-α also stimulates the release of IL-1 by mononuclear phagocytes, which functions much like TNF-α itself, and stimulates the microbiocidal activity of neutrophils and macrophages (in Abbas *et al.,* 2007).

1.2.4 TNF receptors

TNF-α receptors are part of a large family of type I membrane glycoproteins (the TNF-R family) and are present on almost all cell types (in Thomson and Lotze, 2003). Two receptors are known, a 55kD receptor named TNF-RI (or p55) and a 75kD receptor named TNF-RII (or p75). Signalling via TNF-RI receptor is not clearly understood, as cytokine binding of TNF-RI can induce either a death signal by recruitment of an adapter protein that activates caspases and triggers apoptosis, or can activate transcription factors (in Abbas *et al.,* 2007). Cytokine binding to TNF-RII leads to the recruitment of proteins called TNF-receptor associated factors (TRAFs) to the cytoplasmic domains of the receptor, which in turn activates transcription factors such as nuclear factor κB (NF-κB) and activation protein-1 (AP-1) (in Abbas *et al.,* 2007)

1.2.5 TNF-α expression, regulation and its role in HIV infection

TNF-α is secreted mainly by mononuclear phagocytes, although antigen-stimulated T cells, natural killer (NK) cells, eosinophils, basophils, mast cells and various other non-immune cells also can be a source (in Thomson and Lotze, 2003). Release of LPS is the most potent stimulus for TNF-α production, which is why very high levels of TNF-α is seen in gram-negative sepsis. TNF-α production may also be stimulated by infection with viruses, parasites and mycobacteria (in Thomson and Lotze, 2003). TNF-α synthesis is augmented by IFN-γ which is also produced by T cells and NK cells (in Abbas *et al.,* 2007). TNF-α expression may be suppressed by selected members

of the interleukin family, including IL-10 (see *1.3.1 IL-10 Introduction*), and various other anti-inflammatory cytokines (in Thomson and Lotze, 2003).

HIV-infection causes a state of chronic immune activation, characterized by a gradual increase in pro-inflammatory cytokines with disease progression (Antoni *et al.,* 1995, Herbein and Khan 2008, Douek *et al.,* 2009). In the earlier stages of HIV-infection increased expression of membrane-bound TNF-α and membrane-bound TNF receptors on lymphocytes and monocytes are seen when compared to healthy controls (Hestdal *et al.,* 1997, Herbein and Khan 2008). In addition, HIV proteins also regulate TNF expression through targeting of the TNF receptor pathway (Herbein and Khan 2008). As the disease progresses towards AIDS, increasing levels of soluble TNF components were demonstrated in the sera of subjects (de Olivera Pinto *et al.,* 2002, Herbein and Kahn 2008).

1.2.6 TNF-α and the peripheral nervous system

Infiltration of the dorsal root ganglia by mononuclear cells and the presence of TNF-α and other cytokines therein have been demonstrated in patients with AIDS (Nagano *et al.,* 1996). In addition, increased levels of TNF-α have previously been documented in peripheral nerves from patients with HIV-DSP compared to controls (Tyor *et al.,* 1995).

TNF-α has been associated with non-HIV painful neuropathies when compared to patients with painless neuropathy and healthy controls (Uçeyler *et al.,* 2007). The increase in serum levels of TNF-α in subjects with neuropathy was not associated with a specific etiological subgroup, and was equally raised between inflammatory and non-inflammatory etiologies (Uçeyler *et al.,* 2007).

1.2.7 Expression regulated by genotypes

It has been shown that up to 60% of the variation of TNF-α serum level is genetically determined (Westendorp *et al.,* 1997). Nine single nucleotide polymorphisms (SNPs) in the *TNF-α* promoter are known, although not all appear to be functional (Bidwell *et al.,* 1999, Jeong *et al.,* 2004). The *TNF-α* –308G→A SNP (rs1800692, Entrez SNP Database) involves the substitution of guanine (G) for adenine (A), and has frequently been investigated in relation to association with inflammatory and infectious diseases (Entrez SNP Database, in Bidwell *et al.,* 1999). Alleles with the polymorphism are referred to in the literature as TNF2 alleles (–308G→A), whether heterozygous or homozygous, and are associated with a higher rate of gene and protein transcription and ultimately higher levels of circulating TNF-α (Gonzalez *et al.,* 2003, Jeong *et al.,* 2004).

The A-allelic frequency reported for African populations (6% – 8%) are lower compared to African-American (12% - 21%) and Caucasian populations (10% - 24%) (Allen, 1999, Zabaleta *et al.,* 2008, Entrez SNP Database). Interestingly, there are no reported African subjects homozygous for the TNF2 allele on the NCBI SNP database compared to about 5% amongst African-Americans (Entrez SNP Database).

1.2.8 *TNF-α* promoter SNP association studies

Associations have been reported between the TNF2 allele and inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, primary sclerosing cholangitis, pneumoconiosis, asthma and dermatitis herpetiformis, as well as infective conditions such as malaria, cerebral malaria and leprosy (in Bidwell *et al.,* 1999, Gonzalez *et al.,* 2003, Ferguson *et al.,* 2008). The TNF2 allele was also associated with severe infections in elderly Italians (Cipriano *et al.,* 2005).

The TNF2 allele was associated with a significantly increased risk of neurological complications due to malaria in Gambian children, as carriers of the allele had a seven-fold increase in risk of death or severe neurological sequelae when compared

to non-carriers of the TNF2 allele (McGuire *et al.,* 1994). In HIV-infected individuals the presence of a TNF2 allele conferred a five-fold increased risk of developing HIV-associated dementia (Quasney *et al.,* 2001).

Limited data exists on a possible association between *TNF-α* promoter genotypes or serum levels of TNF-α and the prevalence of peripheral neuropathy. A recent study failed to show a significant association between the *TNF-α* -308G→A SNP and painful neuropathies (p=0.12) in a small series of predominantly Caucasian ART-exposed patients (Cherry *et al.,* 2008b). However, in this cohort another *TNF-α* promoter SNP (-1031 T→C) was associated with a 14-fold increase (p=0.02) in odds of developing symptomatic ATN (Cherry *et al.,* 2008b). As a pilot study, we will assess whether an association exists between the presence of the TNF2 allele and painful distal sensory polyneuropathy in an HIV-infected South African clinic-based population.

1.3 Interleukin-10 background

1.3.1 Introduction

Interleukin-10 (IL-10) is an anti-inflammatory cytokine and key regulator of immune responses. Because of its function, it was originally described as cytokine synthesis inhibitory factor (CSIF) by Fiorentino *et al.* in 1989 (summarized in Thomson and Lotze, 2003). The main function of IL-10 is to inhibit monocyte- and macrophage production of a range of pro-inflammatory cytokines, including TNF-α and IL-12 (in Abbas *et al.,* 2007). IL-12 reduction is vital to the down-regulation of the inflammatory response, as it is a critical stimulus for production of interferon-γ (IFN-γ), the principal macrophage-activating cytokine (in Thomson and Lotze, 2003, in Abbas *et al.,* 2007). IL-10 also indirectly suppresses the inflammatory response by inhibiting dendritic cell stimulation of IFN-γ and by inhibition of antigen presentation and chemokine expression by mononuclear phagocytes (in Thomson and Lotze, 2003).

1.3.2 Gene and protein structure

Human IL-10 is an 18 kD non-glycosylated polypeptide, for which the encoding gene is located on chromosome 1. Structurally it is a tight non-covalent homodimer, where each monomer consists of six α-helices (termed A through F) that interact with the helices from the accompanying monomer to form two interpenetrating domains (in Thomson and Lotze, 2003).

1.3.3 Expression and regulation

IL-10 is secreted mainly by activated macrophages but can also be secreted by T-lymphocytes, B-lymphocytes, mast cells, eosinophils and NK cells (in Thomson and Lotze, 2003, in Abbas *et al.,* 2007). Stimuli required for the expression of IL-10 is cell-type specific, and represents a complex interplay between infectious agents, cytokines and the host's IL-10 genotype.

Production of IL-10 is induced by various pathogens, including HIV infection, which can directly activate monocytes and/or macrophages (Brigino *et al.,* 1997, in Thomson and Lotze, 2003). Production of IL-10 is regulated predominantly by other cytokines, but also by hormones or arachidonic acid derivatives in situations of severe stress or infection (in Thomson and Lotze, 2003). Cytokines such as IL-4, IL-13 and IFN-γ inhibit IL-10 production, whereas IL-1, IL-2, IL-3, IL-6, IL-7, IL-12 and IL-15 all induce IL-10 production in target cells (in Thomson and Lotze, 2003). TNF-α also induces IL-10 production in monocytes and appeared to show a greater influence on IL-10 production when compared to the effects of IL-1 and IL-6 (Wanidworanun and Strober, 1993).

1.3.4 IL-10 is implicated in the pathogenesis of disease

High levels of IL-10 have been documented in association with various diseases, including infective conditions such as malaria (Peyron *et al.,* 1994) and bacterial meningitis (Frei *et al.,* 1993), as well as malignancies such as cervical cancer (Stanczuk *et al.,* 2001). Westendorp *et al.* demonstrated a 20-fold increase in the risk for fatal outcome from meningococcal disease in subjects with high IL-10 production compared to those with low IL-10 production (Westendorp *et al.,* 1997).

In peripheral nerves, between two and four days after injury, IL-10 is synthesised by Schwann cells and invading macrophages as a consequence of Wallerian degeneration (Jander *et al.,* 1996). In an animal (rat) model of chronic constriction injury with neuropathic pain, a progressive increase in IL-10 levels at the injury site was associated with a resolution of hyperalgesia (Okamoto *et al.,* 2001). Further, local application of a low concentration of IL-10 to transected sciatic nerve in a murine model resulted in a reduction of scar tissue formation and increased nerve fibre regeneration (Atkins *et al.,* 2008). IL-10 may therefore have direct effects in peripheral nerves such as enhancing regeneration after injury and reducing neuropathic pain (Atkins *et al.,* 2008).

1.3.5 Expression regulated by genotypes

IL-10 production is reported to be influenced by the individual's genotype with up to 75% of the variation in IL-10 levels due to gene promoter polymorphisms (Westendorp *et al.,* 1997). The *IL-10* -1082A→G SNP (rs1800896, Entrez SNP Database) was shown to influence IL-10 transcription (Turner *et al.,* 1997), with subjects homozygous for the G-allele producing almost double the amount of IL-10 protein compared to those heterozygous or homozygous for the A-allele (Hoffmann *et al.,* 2001). This finding, however, is not consistent as other studies failed to prove an association between the G-allele and higher production of IL-10 (Lowe *et al.,* 2003).

Currently there does not seem to be consensus in the literature on whether the G- or A-allele of the *IL-10* -1082 SNP represents the ancestral allele. A number of studies reported the SNP to be a G-to-A substitution (Hoffmann *et al.,* 2001, Lowe *et al.,* 2003, Reichert *et al.,* 2007), although the NCBI SNP database (Entrez SNP Database) showed an A-allelic frequency of greater than 50% in 86% of populations (all ethnicities combined) suggesting the A-allele to be the ancestral allele. Limited data is available regarding allelic frequencies in African populations. The G-allelic frequency in a Nigerian population has been reported as 31%, whereas between 36% (Zabaleta *et al.,* 2008) and 41% of African-Americans have this allele (Entrez SNP Database, Zabaleta *et al.,* 2008).

Two additional SNPs located downstream from the *IL-10* promoter -1082 A→G SNP are the -819 C→T and the -592 C→A SNPs, which are both in linkage disequilibrium with the -1082 A→G SNP. The frequency of these alleles are marginally higher than the -1082 G-allele, with the -819 T-allele reported to have an frequency ranging from 36% to 54% in African-Americans, and the -592 A-allelic frequency ranging from 50% to 53% in the same population group (Entrez SNP Database).

Three preferential haplotypes are formed, namely GCC, ACC and ATA (Hoffmann *et al.,* 2001, Reichert *et al.,* 2008) (Table 1.1). The degree of functionality of these haplotypes seems to be solely dependent on the status of the *IL-10* -1082 SNP. Subjects homozygous for the -1082 G-allele (-1082GG) were found to be 'high

producers', subjects heterozygous for the -1082 SNP 'intermediate producers' and subjects homozygous for the -1082 A-allele 'low producers', irrespective of the status of the -819C→T and -592C→A SNPs (Table 1.1) (Hoffmann *et al.,* 2001).

Table 1.1 IL-10 production based on haplotype	
High producers	GCC/GCC
Intermediate producers	GCC/ACC GCC/ATA
Low producers	ATA/ATA ACC/ATA ACC/ACC

1.3.6 *IL-10* promoter SNP association studies

Several studies have linked 'high producer' genotypes or haplotypes with the development of disease. The 'high producer' GCC haplotype was associated with a higher risk of chronic airways infection with *pseudomonas aeruginosa* in subjects with cystic fibrosis (p=0.02) (Tesse *et al.,* 2008). The *IL-10* -1082 G-allele was also shown to be significantly associated with the development of chronic hepatitis B infection (p=0.01) when compared to subjects who recovered from hepatitis B infection (Truelove *et al.,* 2008), and subjects with cervical cancer was more likely to have the -1082 G-allele (p=0.001) when compared to controls (Stanczuk *et al.,* 2001).

The *IL-10* -1082 A-allele was significantly higher in individuals with ulcerative colitis (p<0.001) compared to healthy controls (Tedde *et al.,* 2008), and the -592 A-allele was associated with lower levels of IL-10 and higher rates of sepsis and mortality in critically ill patients (Lowe *et al.,* 2003). The abovementioned associations between the *IL-10* promoter SNP and disease do not occur in a consistent manner, suggesting that IL-10 production is not exclusively dependent on the host's genotype.

Reports of the influence of *IL-10* promoter polymorphisms on disorders of the nervous system are limited. In subjects with Guillian-Barré, the -819CC and -592CC (higher production) genotypes were more frequent than in controls, but no

association was found with disease course (Myhr *et al.,* 2003). A recent report from an HIV-infected Zimbabwean cohort found an association between homozygous and heterozygous carriers of the -1082 G-allele and reduced mortality from HIV/AIDS as well as a reduction in the CD4 cell count depletion associated with the progression of HIV/AIDS, when compared to non-carriers of the G-allele (Erikstrup *et al.,* 2007). Although HIV-associated neuropathy is likely to correlate with both these parameters of disease severity, I was unable to find any reports investigating an association between *IL-10* promoter polymorphisms and DSP.

1.3.7 Hypothesis for cytokine promoter SNP association studies

HIV infection results in a chronic state of immune activation. The magnitude and pathogenic consequences of HIV-induced host inflammation may be influenced by functional cytokine gene polymorphisms. We hypothesize that,

1) as a pro-inflammatory cytokine, *TNF-α* promoter SNP A-alleles will be increased in subjects with painful HIV-associated DSP when compared to subjects without DSP, and

2) as an anti-inflammatory cytokine, that *IL-10* promoter "high producer" G-alleles will be under-represented in those with painful HIV-associated DSP whereas the "low producer" haplotypes are more likely to occur in those with symptomatic DSP.

Chapter 2

Subjects and methods

2.1 Ethical approval

The study was approved by the Research Ethics Committee of the University of Cape Town (UCT) with references numbers UCT REC 227/2006 and UCT REC 221/2008.

2.2 Study population

This study was conducted in collaboration with UCT's Division of Endocrinology. Subjects were recruited from Crossroads Community Health Centre in Crossroads, Cape Town from August 2007 until October 2008. This institution serves a predominantly isiXhosa-speaking community and runs a dedicated clinic for an estimated 5000 HIV-positive individuals.

Study subjects were recruited from the clinic by a health care worker (myself and Dr. Joel Dave) and a trained field worker, who would also act as an interpreter between other study staff members and study participants if necessary. After being counseled by the field workers, participants received an information leaflet and signed informed consent if they wished to participate. Consent was given for study participation, blood sampling for biochemistry and storage of blood and DNA samples. Clinic folders were briefly reviewed hereafter to screen for any obvious exclusion criteria (Table 2.1). Subjects were then given an appointment date and instructed to be nil per mouth for a minimum of 8 hours prior to testing.

Table 2.1: Exclusion criteria	
1.	Age < 18 years
2.	Documented pre-existing neuropathy from a known cause
3.	Diagnosis of Diabetes Mellitus
4.	Current tuberculosis treatment for < 1 month
5.	ART for < 6 months
6.	Previously defaulting on ART
7.	Pregnancy
8.	Corticosteroid use in the past 6 months
9.	Acutely ill subjects
10.	Neurological signs not in keeping with a diagnosis of DSP (after examination)

On the day of the appointment, subjects were escorted by a field worker from Crossroads clinic to Groote Schuur Hospital by public transport. Participants were again informed about the procedure and the option to withdraw consent at any time if they were no longer interested.

2.3 Blood and data collection

Blood for a fasting lactate level was taken by finger prick without applying a tourniquet, where after IV cannulation was performed and blood taken for fasting glucose, insulin and lipid profile determinations, as well as DNA storage. An Accutrend® Lactate system (Roche, Mannheim, Germany) was used for lactate samples. A 75 gram oral glucose tolerance test (OGTT) was then performed, with blood taken for glucose and insulin measurements at 30 and 120 minutes. Impaired fasting serum glucose was defined as a fasting glucose level of least 5.6 mmol/l, and impaired glucose tolerance was defined as a two-hour glucose level of at least 7.8 mmol/l. Diabetes was defined as a fasting glucose of at least 7 mmol/l or two-hour glucose level of at least 11.1mmol/l.

All samples were collected in the appropriate Vacuette® (Greiner Bio-One, Kremsmünster, Germany) blood collection tubes, and immediately placed on ice

until the last samples were collected. Digital blood pressure readings were recorded three times with the use of an Omron® M6 Comfort BP monitor (Omron Healthcare, Kyoto, Japan). Data on weight, height and various other anthropometric measures were collected by the same investigator for all individuals, and field workers conducted interviews to complete questionnaires (Table 2.2).

A South African validated, isiXhosa version of a health-related quality of life questionnaire with a 5-dimensional profile (EQ-5D) (Jelsma *et al.,* 2004), was administered by trained field workers. The EQ-5D descriptive profile assesses health in terms of five dimensions; mobility, self care, activities of daily living (usual activities), pain or discomfort, and anxiety or depression. Each dimension has three levels; no problems, some or moderate problems or severe problems. For the purpose of analysis, responses were dichotomized as no problem vs. problems in each of the dimensions as the "severe" category was not always applicable to ambulatory subjects, and due to relatively few responses this category. Subjects were also asked to rate their current health status on a 20cm long graduated visual analogue scale (VAS) ranging from the worst imaginable (0) to the best imaginable health (100%).

Table 2.2: Information questionnaires	
1.	Sociodemographic details
2.	Family history
3.	Previous medical history
4.	Current medication
5.	Exercise
7.	Tobacco and alcohol use
8.	Pregnancy history
9.	Quality of life

2.4 Long term storage

After blood collection, tubes were spun down by technicians at 3000 rpm for ten minutes using a Beckman GS-6R centrifuge (GMI Inc., Ramsey, Minnesota, USA) and the contents of each aliquoted into 1,5 ml Eppendorf tubes, one of which was stored at -20˚C and the other stored in a -80˚C freezer for long-term storage. All investigations were performed by the same laboratory technician.

2.5 Examination for neuropathy

The Brief Peripheral Neuropathy Screen (BPNS) and reduced Total Neuropathy Score (rTNS) are validated clinical tools used to document the presence of DSP. Both clinical tools require symptoms and signs to be symmetrically present, and subjects with asymmetry were excluded from analysis. The majority of the clinical evaluations were performed by me and a minority by Prof. JM Heckmann. On several occasions both examiners performed the tests independently with 100% concordance between the two raters. A long-handled reflex hammer (Eschmann, England), 128 Hz tuning fork (RAGG, England) and solid-point pins were used for the determination of deep tendon reflexes, vibration sense and pin sensibility.

2.5.1 Brief Peripheral Neuropathy Screen (BPNS)

The BPNS (see *Appendix A*) grades symptom severity as well as vibration sense and ankle deep tendon reflexes. A visual analogue scale (VAS) scoring each symptom from 0 to 10 is used by only explaining the extremes of the scale to the patient. Relevant symptoms consisted of the following: a) pain, aching or burning; b) pins-and-needles and c) numbness experienced in the feet or lower legs. After scoring on the VAS, symptoms were graded as being grade 1 (scores 1 – 3), grade 2 (scores 4 – 6), grade 3 (scores 7 - 8) or grade 4 (scores 9 – 10). The highest individual symptom grade obtained was used as the final symptom grade.

Vibration sense is evaluated by compressing or pinching the tuning fork hard enough for the ends of the arms to touch and applying it to the interphalangeal joint on the first toe of both feet, proximal to the nail bed. Vibration loss is graded as none (> 10 seconds), mild (6 – 10 seconds), moderate (≤ 5 seconds) or severe (no vibration felt), and scored from 0 to 3 accordingly. Ankle deep tendon reflexes are tested by sitting the patient over the bedside, relaxing the lower limbs and striking the tendon in the standard manner. The reflexes and graded as being clonus, hyperactive, normal, hyporeflexia or areflexia, and scored from 0 to 4 accordingly.

2.5.2 Reduced Total Neuropathy Score (rTNS)

The TNS is a composite measure of peripheral nerve function developed by Cornblath *et al.* for the investigation of diabetic polyneuropathy (Cornblath *et al.,* 1999). A reduced version of the TNS, only incorporating clinical assessment tools, was validated to be sensitive in the diagnosis of chemotherapy-induced peripheral neuropathy against established tools (Cavaletti *et al.,* 2002, Cavaletti *et al.,* 2003). We used a reduced version of the TNS (which we will refer to as the rTNS), which consists of 5 elements, namely symptoms (A), pin sensibility (B), vibration sensibility (C), deep tendon reflexes (D) and strength (E). All 5 elements were graded from 0 to 4 (see *Appendix A*). For elements A to C, grades represented the level of involvement. Reflex determination (D) was simultaneously graded according to severity and level of involvement. Ankle- and toe plantar- and dorsiflexion (E) were only graded according to severity (Medical Research Council power grade).

2.6 Criteria for a diagnosis of DSP

As mentioned previously, no standard definition or reference standard for the investigation of DSP in epidemiological studies exists. We did not have informative special investigations like quantitative sensory threshold testing (QST) or epidermal nerve fibre density (ENFD) quantification available to aid in the diagnostic process.

Neither the BPNS nor rTNS were considered to be adequate in isolation for a rigorous definition of DSP. We used a combination of the BPNS and rTNS scoring methods as an evaluation tool to fulfill the recommended criteria for the definition as proposed by England *et al.* and to increase the sensitivity of our definition (England *et al.,* 2005). Both tools evaluate three different neuropathic symptoms, but the BPNS rates the symptoms on a VAS, and the rTNS includes the level of involvement. The BPNS requires the presence of two signs, namely impaired vibration sense and hypo- or areflexia, and records the degree of impairment for either of these signs. The rTNS grades a decrease in pin sensation in addition to the abovementioned BPNS signs, and grades the level of involvement rather than the degree of impairment. However, for deep tendon reflexes both the level and the degree of impairment is incorporated into a single severity grade.

To illustrate the difference in diagnostic sensitivity and specificity of the 2 clinical tools, Simpson *et al.* compared the original BPNS against the TNS, and subsequently revised the BPNS to require only 1 sign for the diagnosis of DSP (Simpson *et al.,* 2006). As can be seen from the Table 2.3, marked differences in sensitivity and specificity were obtained. When using only one abnormal sign for a diagnosis of DSP, the BPNS achieved a high rate of sensitivity and low rate of specificity when compared to the original TNS (which includes QST and sural- and peroneal nerve amplitudes). Specificity improved markedly when increasing the abnormal signs needed to two, at the cost of sensitivity.

Table 2.3. BPNS sensitivity and specificity compared to TNS (from Simpson *et al.,* 2006)

	Sensitivity (%)	Specificity (%)	Pos Pred Value	Neg Pred Value
BPNS (2 signs)	35	90	85	45
BPNS (1 sign)	73	68	89	42

Pos Pred Value = positive predictor value, Neg Pred Value = negative predictor value

The BPNS was also compared to a reduced version of the TNS, which omitted quantitative sensory threshold testing and nerve conduction studies (Ellis *et al.,* 2005). Using 2 signs for a diagnosis of DSP, sensitivity of the BPNS was higher in this

comparison to a reduced version TNS than it was compared to the TNS, but specificity was comparable (Table 2.4). As expected, sensitivity increased and specificity decreased when only one sign was required for a diagnosis of DSP.

Table 2.4. BPNS sensitivity and specificity compared to rTNS (from Ellis *et al.,* 2005)

	Sensitivity (%)	Specificity (%)	Pos Pred Value	Neg Pred Value
BPNS (2 signs)	49	88	53	85
BPNS (1 sign)	80	59	72	69 (calculated)

Pos Pred Value = positive predictor value, Neg Pred Value = negative predictor value

We decided to use a combination of the BPNS and rTNS, and base the diagnosis of DSP on the presence of at least 2 abnormal signs on clinical examination. This implies that any combination of hypo-/areflexia, impaired vibration sense and decreased pinprick sensibility may be considered sufficient for the diagnosis. With this definition we aimed to increase diagnostic sensitivity by examining for multiple sign, but also to increase specificity by requiring more than one to be present. Within group of subjects with DSP, symptom free subjects were classified as having asymptomatic DSP, and individuals with any grade of neuropathic symptoms were classified as having symptomatic DSP.

2.7 Clinic folder reviews

After testing, participants' clinic records were reviewed by either me, Dr. Joel Dave or Prof. Jeannine Heckmann. Descriptive data comprising the date of HIV diagnosis and WHO clinical stage on commencement of ART (if applicable) were collected. In patients on ART, previous and current drug regimens with drug doses were recorded as well as any evidence of clinical ART toxicities or virological failure. Weight measurements were collected only on ART-exposed patients, and were tracked at designated intervals from the commencement of ART.

Laboratory measures collected consisted of CD4 counts, HIV viral loads, mean corpuscular volume (MCV), alanine aminotransferase (ALT) and creatinine levels.

Due to policy in the Western Cape Health region, viral load determination pre-ART initiation was discontinued as routine practice during 2007. Consequently most individuals had their first viral load counts done after six months of ART and then at six-monthly intervals. For ART-naïve subjects, CD4 counts within six months of the time of assessment were captured from clinic folders and regarded as nadir CD4 count if no other value was available. CD4 counts at the start of ART, six months thereafter as well as the most recent values (within 6 months of assessment) were recorded for ART-exposed subjects.

2.8 Data capturing

Data capturing was done by 2 different study staff members on 2 different workstations. All datasets were entered into both Microsoft® Access databases, and compared afterwards for consistency. Applicable data fields were exported to Microsoft® Excel prior to statistical analysis.

2.9 Statistical analysis

The primary analysis compared subjects without DSP to those with DSP, jointly evaluating individuals with HIV-DSP and those with ATN. With this analysis we aimed to describe the prevalence of HIV-associated DSP and explore possible risk factors associated therewith. To investigate potential risk factors for ATN, a secondary analysis was conducted to compare individuals with ATN to those who had been exposed to ART but had not developed ATN. The cross-sectional nature of this study precluded evaluation of whether or not pre-existing HIV-DSP constitutes a risk factor for ATN. Subject characteristics were compared using unpaired t-test, Wilcoxon rank-sum test, $\chi 2$ test, or Fisher exact test, as appropriate. Risk factors for DSP in general as well as for ATN in particular were assessed using logistic regression. Variables significant in univariate analyses at a 2-sided level of 0.05 were evaluated in a multivariate model. Interaction between variables significant in univariate analysis was examined as part of the multivariate model. Basic data

manipulation was done in Microsoft® Excel 2003, and statistical analyses were done in SPSS 16 (Chicago, Illinois, USA) and StatSoft® Statistica 8 (Tulsa, Oklahoma, USA). All analyses were supervised by a biostatistician, Dr. Motasim Badri, from the Department of Medicine at UCT.

Chapter 3:

Laboratory materials and methods

3.1 Study design

We embarked on a pilot allele-association study, initially planning to genotype 30 individuals with painful HIV-DSP and 30 individuals without evidence of HIV-DSP for the *TNF-α* promoter -308 G→A SNP, as well as the *IL-10* promoter -1082 A→G SNP. The *IL-10* promoter study was expanded during the course of the experiments to include the -819 C→T and -592 C→A SNPs as well, and will be discussed in detail below. After conducting the TNF-α association study, we had excess reagents as well as eligible study participants, and decided to genotype additional subjects for the TNF-α -308 SNP. The association study did not include participants on ART, as individuals with DSP included in this group can have HIV-DSP or ATN. Samples for genotyping were numbered according to the clinical study number, and the personnel handling the samples were blinded to the clinical diagnosis of the participants.

3.2 Subject selection for genotyping

Subjects included in the *TNF-α* promoter -308 G→A genotype analysis and *IL-10* promoter haplotype analysis were selected by convenience sampling as this pilot study aimed to explore cytokine promoter gene differences in the extreme ends of the spectrum of DSP. Neuropathy free subjects were compared to subjects with symptomatic HIV-DSP, as TNF-α has been implicated in the pathogenesis of painful neuropathy.

A random selection of subjects with normal neurological examinations and without any symptoms of DSP were compared a group of subjects with symptomatic DSP, of which subjects with the highest BPNS- and rTNS scores were chosen. The selected individuals were used for both *TNF-α* promoter and *IL-10* promoter analysis,

although a larger number of subjects were included in the *TNF-α* promoter analysis based on the expected allelic frequency of the -308 G→A SNP (see *3.1 Study design*).

3.3 DNA extraction and storage

DNA extraction was performed from buffy coats prepared from Vacuette® 6ml EDTA tubes (Greiner Bio-One, Kremsmünster, Germany). As mentioned earlier, tubes were centrifuged for ten minutes at 3000rpm using a Beckman GS-6R centrifuge (GMI Inc., Ramsey, Minnesota, USA) before buffy coats containing the white cells were aliquoted into 1.5ml Eppendorf tubes and stored at -20˚C in a long-term storage facility. A Qiagen QIAamp® DNA Blood Mini Kit (Valencia, California, USA) was used for the DNA extraction, and the standard Blood and Body Fluid Spin Protocol was followed. The only alteration to the standard protocol was an increase in the incubation time after lysis from 10 minutes to an overnight incubation in an attempt to increase DNA yield. DNA was subsequently stored at -20˚C.

3.4 Primers for polymerase chain reaction (PCR)

All primers were designed using OLIGO® software (Molecular Biology Insights, Inc., Cascade, Colorado, USA) and manufactured locally by the Department of Molecular and Cell Biology, Synthetic DNA Unit, University of Cape Town. In designing the primers we aimed for a length of 21 bases with minimal possibility of primer-dimer formation. Primers used for *TNF-α* promoter and *IL-10* promoter amplification are listed in Table 3.1.

Table 3.1: *TNF-α* and *IL-10* promoter PCR primers			
Primer	Sequence	Product size (bp)	Tm (˚C)[δ]
TNF-α -308 forward primer	5' aggcaataggttttgagggccat 3'	106	58.9
TNF-α -308 reverse primer	5' cctccctgctccgattccg 3'		59
IL-10 -1082 forward primer	5' caactggctccccttaccttc 3'	134	54
IL-10 -1082 reverse primer	5' cccttactttcctcttacctatccc 3'		54

[δ] melting temperature

3.5 Determination of annealing temperatures

After DNA extraction, we established the optimal annealing temperatures for each of the primer sets by repeating PCR across a temperature gradient, using DNA from the same individual across all temperatures. Primer stock was diluted to a 50pmol/µl solution, and Promega GoTaq (Madison, Wisconsin, USA) was used in all experiments. The PCR protocol (Table 3.2), temperature gradients (Table 3.3) and PCR volumes (Table 3.4) used for both cytokine promoters are listed below.

Table 3.2: Protocol for annealing temperature gradient PCR			
Initialization		98˚C	2 minutes
35 cycles	Denaturation	94˚C	30 seconds
	Annealing	gradient	30 seconds
	Elongation	72˚C	30 seconds
Final elongation		72˚C	8 minutes

Table 3.3: Temperature gradients (˚C)					
TNF-α -308	53.3	56.7	58.4	59.9	61.8
IL-10 -1082	50.3	51.9	54.9	56.7	58.4

All gradient experiments were performed on an Eppendorf® Mastercycler Gradient PCR system (Eppendorf, Hamburg, Germany). A 2% agarose gel (Promega) was prepared, and 5µl (of a 10mg/ml solution) Bio-Rad® ethidium bromide solution

(Hercules, California, USA) was added during the preparation. During heat dissolving of the agarose, evaporation losses were replaced with distilled water. 15µl of 1kb+ DNA ladder (SolGent, Daejeon, South Korea) was added to one lane as a reference, and the gel was run for 30 minutes at 110V. Good results across most temperatures were seen for *TNF-α* promoter amplicon and a well-defined band at 58.4˚C for the *IL-10* promoter (Figure 3.1). It was decided to use an annealing temperature of 58˚C for both cytokines.

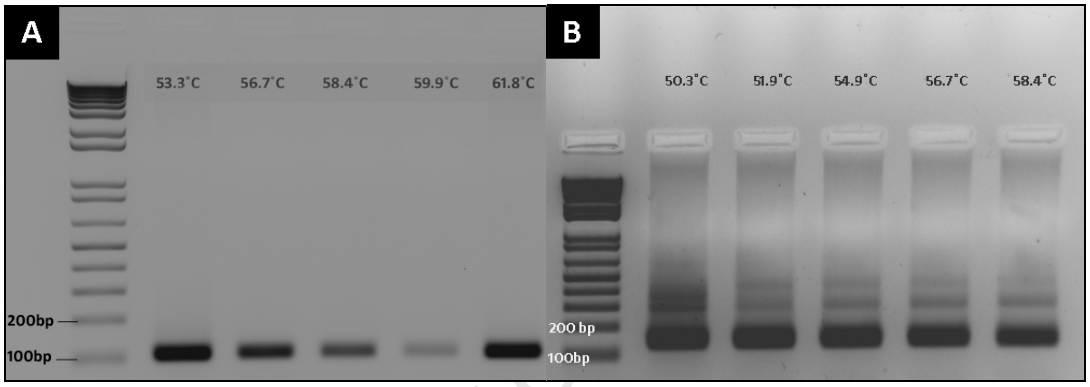


Figure 3.1: Cytokine promoter annealing temperature determination. A) TNF-α annealing temperature determination gel; **B)** IL-10 annealing temperature determination gel.

3.6 Polymerase Chain Reaction

For both cytokines, a 30µl PCR sample from each subject was prepared to minimise cost but still allow for a checking gel to be performed verifying the quality of the PCR product. Of the 30µl, 12µl of PCR product would be used for the checking gel, and 18µl for enzyme digestion. The content of each sample is listed in Table 3.4.

Table 3.4: PCR reagent volumes	
Reagent (concentration)	Volume (µl)
Forward Primer (50pmol/µl)	0.3
Reverse Primer (50pmol/µl)	0.3
dNTPs (5mM)	1.2
5X Taq Buffer	6
MgCl (25mM)	18
Distilled water	19.26
Promega GoTaq	0.14
Subject DNA	1
Total volume	**30**

The PCR protocol used was similar to that described earlier, with the annealing step set at 58˚C (Table 3.5). All reactions were performed on either an Eppendorf® Mastercycler Gradient PCR system or GeneAmp® 2700 PCR system (Applied Biosystems, Singapore). A control sample was prepared with every group of samples, with nuclease-free water replacing subject DNA. This was done to exclude any contamination with amplicons or extracted DNA. A known negative- and positive control (from a previous study by Prof JM Heckmann) were also amplified for the *TNF-α* promoter genotype analysis.

Table 3.5: Cytokine promoter PCR protocol			
Initialization		98˚C	2 minutes
35 cycles	Denaturation	94˚C	30 seconds
	Annealing	58˚C	30 seconds
	Elongation	72˚C	30 seconds
Final elongation		72˚C	8 minutes

After PCR, checking gels were run to confirm adequate amplification of the PCR product prior to restriction enzyme digestion (figure 3.2). All checking gels used were 2% (w/v) agarose gels, and underwent electrophoresis at 110V for 45 minutes. None of the checking gels showed any contamination during any of the experiments. All photographs were taken with a Kodak DC120 digital camera and

Kodak 1D Image Analysis software (Rochester, New York, USA) under UV transillumination on a Stratagene® Transilluminator 4000 (La Jolla, California, USA).

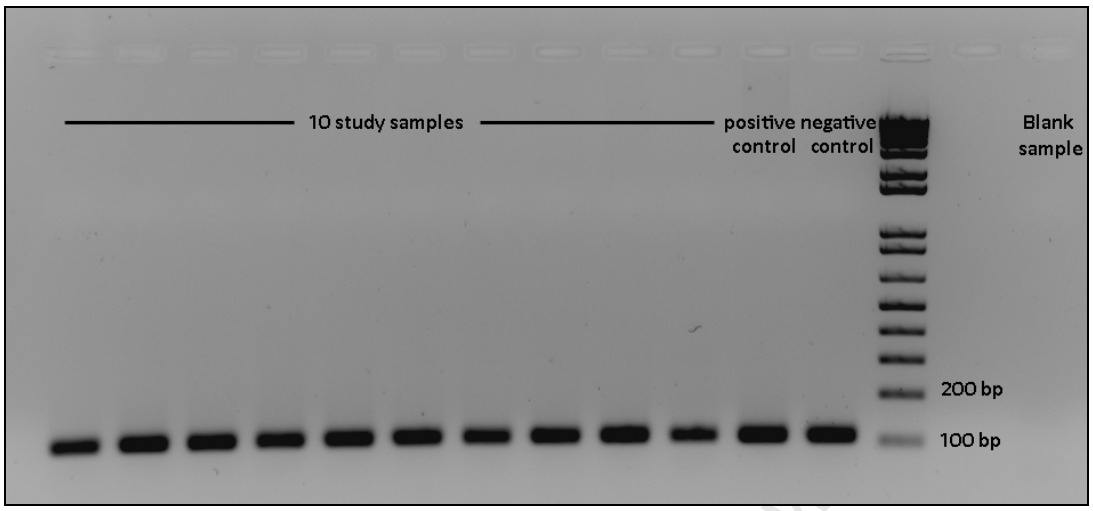


Figure 3.2: Example of a TNF-α PCR product checking gel.

3.7 *TNF-α* promoter genotyping by *NcoI* restriction

NcoI restriction enzyme digestion for *TNF-α* promoter -308 genotyping was first described in 1992 (Wilson *et al.*, 1992). The *NcoI* restriction enzyme recognises the sequence CCATGG on the forward strand, with the restriction site as shown below:

5′...C▼CATGG...3′
3′...GGTAC▲C...5′

Source: www.neb.com

A mismatch primer with a single base change at the 3' end of the primer was necessary to create an *NcoI* restriction site. As indicated in the sequence below (Figure 3.3), the enzyme digests the ancestral (GG) genotype, cleaving the 106bp amplicon in a 20bp fragment and an 86bp fragment. PCR products of subjects homozygous for the *TNF-α* promoter -308 G→A SNP will therefore not be digested and only show a 106bp strand on gel electrophoresis, whereas heterozygotes will have all three fragment sizes.

Figure 3.3: *TNF-α* promoter sequence. Highlighting denotes the forward- and reverse primer, with the red base denoting the mismatch created by the forward primer. Underlying denotes the TNF-α -308 SNP. Blue type denotes the start of the TNF-α gene.

The *NcoI* enzyme (R0193S) was supplied by New England Biolabs (Ipswitch, Massachusetts, USA), together with NEBuffer 3 that allows 100% activity of the enzyme. Final reaction volumes for enzyme digestion are shown in Table 3.6. Enzyme digestion was performed overnight (16 hours) on a GeneAmp® 2700 PCR system at 37˚C as per manufacturer's instructions. An overnight digestion was decided on to ensure complete digestion.

Table 3.6: *NcoI* enzyme digestion volumes

Reagent	Volume (µl)
PCR product	18
NcoI enzyme	0.2
NEBuffer 3	2
Total volume	**20.2**

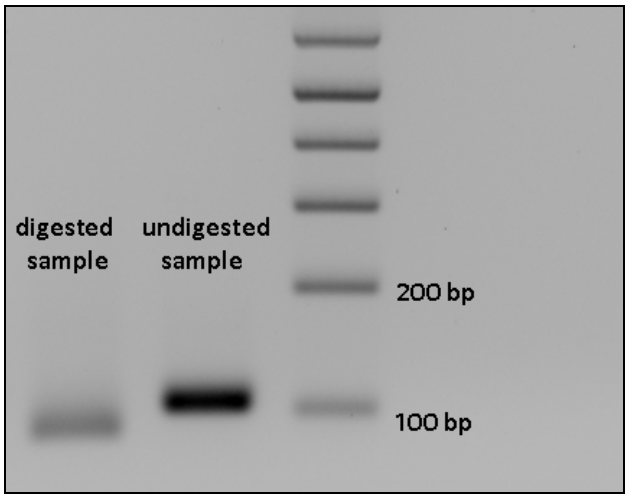
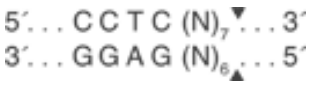


Figure 3.4: *NcoI* restriction enzyme digestion test

3.8 *IL-10* genotyping by *MnlI* restriction

MnlI restriction enzyme digestion for *IL-10* promoter -1082 genotyping was first described in 2001 (Zheng *et al.,* 2001). The *MnlI* enzyme recognises the sequence GGAG on the reverse strand of the promoter. In subjects with the ancestral (AA) genotype, the amplified DNA fragment (134bp) contains one restriction site in the 3' end of the fragment (see below). The *IL-10* promoter -1082 A→G polymorphism creates a second restriction site, and expected fragments from the different possible genotypes are tabulated in Table 3.7.



Source: www.neb.com

Table 3.7: *IL-10* promoter -1082 base pair lengths

Genotype	Number of fragments	Base pair lengths
AA	2	114, 20
GG	3	91, 23, 20
AG	4	114, 91, 23, 20

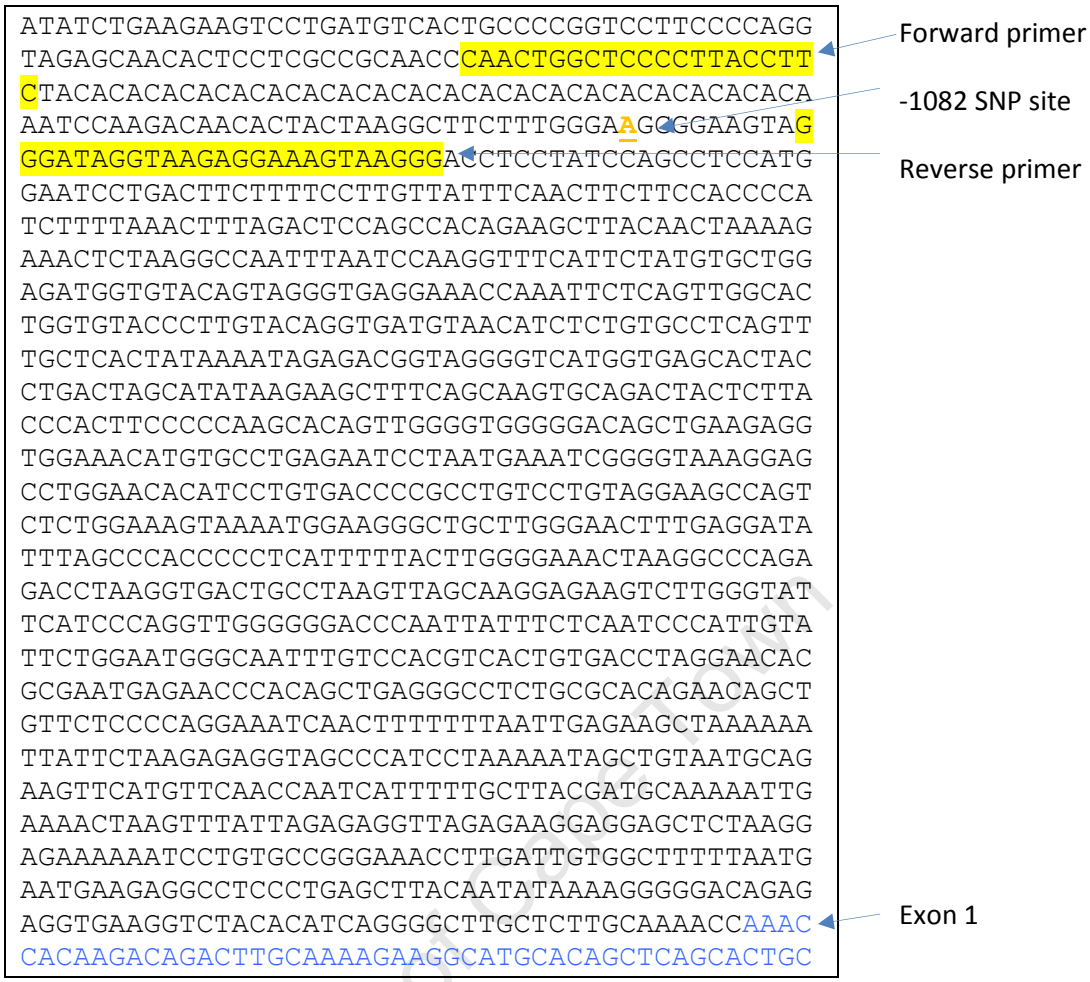


Figure 3.5: IL-10 promoter sequence. Highlighting denotes the forward- and reverse primer. Underlying denotes the polymorphism site. Blue type denotes start of the IL-10 gene.

PCR conditions, equipment and gel concentrations were the same as for *TNF-α* -308 genotyping. The *MnlI* enzyme (R0163S) was supplied by New England Biolabs, together with Bovine Serum Albumin (BSA) and NEBuffer 2 that allows 100% activity of the enzyme. Final reaction volumes for enzyme digestion are shown in Table 3.8. Enzyme digestion was also performed overnight at 37˚C, with a final enzyme-inactivation step at 65˚C for 20 minutes.

Table 3.8: *MnlI* enzyme restriction volumes	
Reagent	Volume (µl)
PCR product	18
MnlI enzyme	0.4
NEBuffer 2	2
BSA	0.2
Total volume	**20.6**

3.9 Gel electrophoresis of enzyme-restricted products

The *TNF-α* -308 genotype determination relies on the detection of either a 106 bp or an 86 bp fragment on gel electrophoresis, and *IL-10* -1082 determination on either a 114 bp or 91 bp fragment. For better separation of the similarly-sized bands, we used 4% (w/v) agarose gels with a length of 15cm (110ml). The volume of ethidium bromide (of a 10mg/ml solution) was increased to 7.5µl for larger gels. 15µl of Low Molecular Weight DNA Ladder (N3233S) (New England Biolabs, Ipswitch, Massachusetts) were used, and undigested PCR product was always added to the outermost lanes of the gel as reference points.

3.10 *IL-10* promoter sequencing

No conclusive results for the majority of subjects could be obtained by *MnlI* enzyme restriction (Figure 3.6). Bands of various sizes were seen on gel electrophoresis, and this is believed to have been caused by a CA-repeat section in the 5' end of the amplified fragment of the promoter gene. This section spans 42 bases, and misreads in this area could explain the observed minor variation in base pair length.

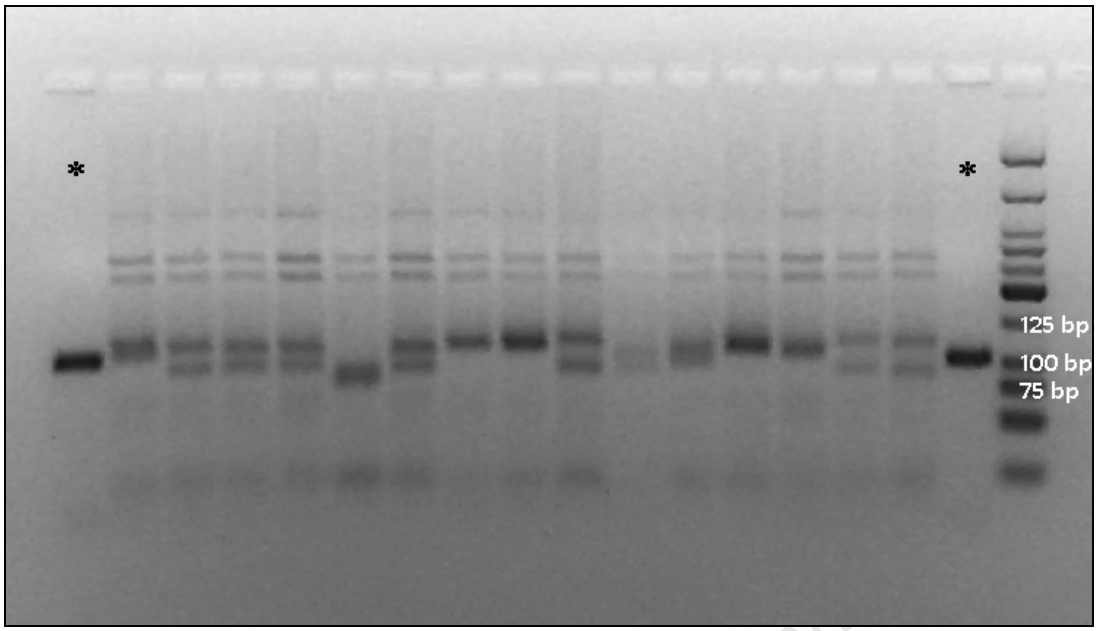


Figure 3.6: *MnlI* restriction enzyme digestion of *IL-10* promoter -1082 SNP. Various band sizes are seen apart from the expected fragment sizes mentioned in table 3.7. * denotes undigested control samples.

It was decided to sequence the *IL-10* promoter area instead. This gave rise to the opportunity to expand the amplified segment and include 2 other SNPs in the promoter gene that have been implicated in altered expression of the cytokine (see *1.3.5 Expression regulated by genotypes*).

3.10.1 Primer design

The previously designed forward primer was used for sequencing, and 2 new reverse primers were designed. Reverse sequencing was done to avoid possible problems arising from the CA-repeat section, and the 2nd reverse primer was used for internal sequencing as this resulted in better sequencing compared to the reverse primer 1 (Table 3.9, also see *Appendix C*). Primer design was done as previously described.

Table 3.9: *IL-10* promoter -1082 PCR primers for sequencing			
Primer	Sequence	Product (bp)	Tm (˚C)
IL-10 -1082 forward primer	5' caactggctccccttaccttc 3'		54
IL-10 -1082 reverse primer	5' cagtgacgtggacaaattgcc 3'	816	55
IL-10 -1082 reverse primer	5' agtaaaaatgaggggtgggc 3'	675	56

Figure 3.7: *IL-10* promoter sequence. Highlighting denotes the forward- and 2 reverse primers. Underlying denotes the polymorphism sites. Blue type denotes start of the *IL-10* gene.

3.10.2 PCR for *IL-10* promoter sequencing

PCR conditions were similar to those described in section 3.6. Annealing temperature determination by use of a gradient experiment was again done, with the only alteration to the protocol being an increase in elongation step due to the increased size of the amplified fragment (Table 3.10 and Table 3.11). An annealing temperature of 58˚C resulted in a well defined band on gel electrophoresis, and was used for all subsequent reactions. Checking gels were performed after PCR as previously described.

Table 3.10: PCR protocol for IL-10 sequencing

Initialization		98˚C	2 minutes
35 cycles	Denaturation	94˚C	30 seconds
	Annealing	gradient	30 seconds
	Elongation	72˚C	60 seconds
Final elongation		72˚C	8 minutes

Table 3.11: Temperature gradients (˚C)

IL-10 -1082	51.9	54.9	56.7	58.4	59.9

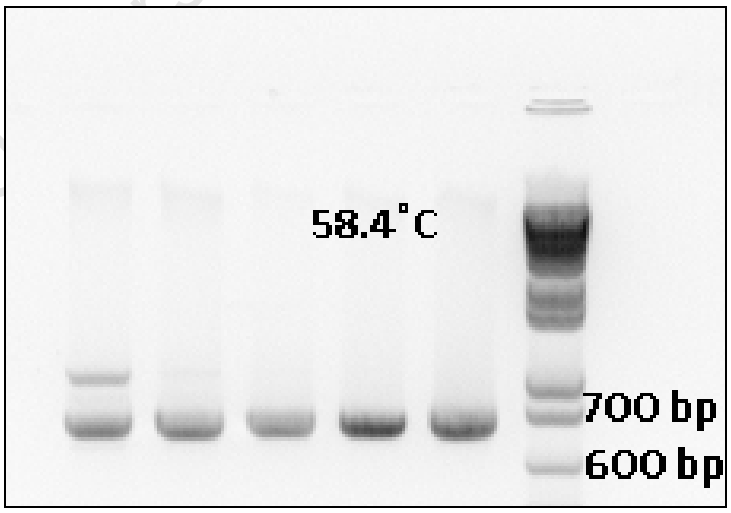


Figure 3.8: IL-10 promoter sequencing annealing temperature gel.

3.10.3 Purification of PCR product

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Valencia, California, USA) and the standard PCR Purification Spin Protocol.

3.10.4 Measuring of DNA concentrations

DNA concentration of all 60 samples were measured and was done by aliquoting 2 µl of purified DNA onto a NanoDrop® ND-1000 Spectrophotometer. Prior to measuring concentrations, the device was calibrated using a sample of nuclease free water followed by a sample of elution buffer. DNA concentrations were all satisfactory for sequencing and varied between 18.4ng/µl and 73.5ng/µl.

3.10.5 Sequencing protocol

Sequencing reactions were performed using a BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Reaction volumes and sequencing protocol are listed in Table 3.12 and Table 3.13. Samples were sequenced by the Central DNA Sequencer, University of Stellenbosch and were interpreted using Chromas® Lite software (Technelysium, Tewantin, Australia).

Table 3.12: Sequencing reagent volumes

Reagent	Volume (µl)
Big Dye	1
Buffer	0.6
H_20	6.4
Primer	1
Purified PCR product	1
Total volume	**10**

Table 3.13: Sequencing reaction protocol			
Initialization		96˚C	15 seconds
30 cycles	Denaturation	96˚C	15 seconds
	Annealing	50˚C	30 seconds
	Elongation	60˚C	4 minutes
Final elongation		60˚C	10 minutes

Chapter 4

Clinical results

4.1 Study participants

Informed consent was granted by 640 individuals, after which 42 individuals were excluded due to reasons listed in Table 4.1. The remaining 598 individuals were included in the analysis.

Table 4.1: Excluded subjects

Subjects (N)	Reason
12	Withdrawn consent
10	Unsuccessful IV cannulation after two attempts
6	Corticosteroid use in the past six months
4	Inadequate duration of ART exposure
4	Pregnancy
4	Incomplete data capturing
1	Brown-Sequard syndrome
1	Possible neuropathy due to rheumatoid arthritis
42	

4.2 DSP prevalence

The overall prevalence of DSP (as defined by our clinical definition) in this population was 49% (n=291), and approximately two-thirds (180/291) of subjects in this group suffered from symptomatic DSP (Table 4.2).

The prevalence of DSP changes significantly with a change in definition. Using a definition that requires only 1 sign for a diagnosis of DSP as used by several previously published studies (Wright *et al.,* 2008, Sithinamsuwan *et al.,* 2008) increases the DSP prevalence to 80% (477/498) ($p<0.0001$ compared to a definition including 2 signs). Participants with symptomatic DSP accounts for 42% of the cohort (253/598), and asymptomatic DSP for 38% (224/598). A significant higher

proportion (224/477, 47%) of all subjects with DSP are categorized as having asymptomatic DSP when using this definition, compared to a definition requiring 2 signs for the diagnosis of DSP (111/291, 38%) (p=0.017).

Table 4.2: DSP prevalence	
	N,%
No DSP	307 (51.3)
DSP	291 (48.7)
Asymptomatic DSP	111 (18.6)
Symptomatic DSP	180 (30.1)
Total	598 (100)

4.3 Data on symptoms and signs

Data regarding symptom and sign frequency is presented in Table 4.3. All the symptoms and signs included in the definition of DSP were significantly more prevalent in the DSP subgroup compared to the DSP-free subgroup. Within the symptomatic DSP subgroup, 69% of subjects (125/180) reported positive sensory symptoms (pain or paresthesiae) graded as being at least moderate to severe (≥4/10) on the BPNS symptom VAS. Pain was the most prevalent DSP symptom, affecting nearly half of all subjects with DSP (47%), but also present in 24% of individuals without DSP. Cramps as a neuropathic symptom were more prevalent in participants with DSP, although reported by 37% of those without DSP.

On the BPNS VAS, 278 study participants reported symptoms, although only 180 of these had DSP according to our definition. Of the remaining 98 subjects, 73 had 1 abnormal sign on examination, and 25 participants did not have any abnormalities. On retrospective recall, 17% (30/180) of individuals with symptomatic DSP reported relief from various preparations (apart from ART), most often ascribed to the use of either paracetamol or amitriptyline, but also including physical measures such as rubbing of the feet with lotions.

Hypo- or areflexia was the commonest sign in individuals with DSP, affecting 86% of the group, but also affecting 31% of individuals without DSP. The second most common sign in our study population but absent in the BPNS tool, decreased pinprick sensation, was present in 85% of subjects who were classified as having DSP and abnormal in 20% of individuals without DSP.

Table 4.3: Symptom and sign-frequency in the cohort stratified by DSP status

		No DSP N = 307	DSP N = 291	p-value
Neuropathic symptoms				
Pain	N (%)	74 (24.1)	136 (46.7)	<0.001 [ε]
Paresthesiae	N (%)	49 (16)	109 (37.5)	<0.001 [ε]
Numbness	N (%)	29 (9.4)	90 (30.9)	<0.001 [ε]
Cramps	N (%)	113 (36.8)	160 (55)	<0.001 [ε]
Neuropathic signs				
Hypo-/areflexia	N (%)	94 (30.6)	251 (86.3)	<0.001 [ε]
Vibration loss	N (%)	32 (10.4)	196 (67.4)	<0.001 [ε]
Pinprick loss	N (%)	61 (19.9)	247 (84.9)	<0.001 [ε]

[ε] χ^2 test

4.4 Risk factors for Distal Sensory Polyneuropathy

Subjects with DSP were older compared to those without DSP (p<0.001), and more likely to have been exposed to ART (p<0.001) or previously treated TB infection (p<0.001) (Table 4.4 and 4.5). Both current stavudine use (p=0.002) and stavudine use ever (p<0.001) were more prevalent in the DSP-group, but neither duration of use in currently-exposed subjects, nor duration of use in all subjects who had used stavudine was different from that of the DSP-free group (p=0.79).

With regards to laboratory markers, individuals with DSP had lower nadir CD4+ counts (p<0.001) and higher current mean corpuscular volumes (MCV) compared to those without DSP (p=0.01). The relevance of this finding is unclear, but the observed macrocytosis in the DSP-group may be related to the higher rate of ART use in this group. Total fasting cholesterol levels were significantly higher in the DSP group (p=0.007), although the values of the 75th percentile of both groups was still within the normal range. No differences in either fasting serum glucose levels, two-hour glucose levels or fasting serum lactate levels were demonstrated.

No difference was detected in smoking habits or alcohol exposure in general, although an insignificant trend towards increased alcohol use in the past 12 months was observed in DSP-free subjects (p=0.08). Significant differences were demonstrated in weight (p=0.043), waist-to-hip ratio (p<0.001) and both systolic- and diastolic blood pressure measurements (p<0.001 and p=0.019, respectively) between DSP and DSP-free groups, although the values of the 75th percentiles for systolic and diastolic measurements were within the normal range. No significant differences in height, body mass index, mid-upper arm circumference or calf skin fold thickness were demonstrated. Measured across both DSP and DSP-free groups, 3% of study participants were underweight with a BMI < 19, whereas 73% of subjects had a BMI of greater than 25. Out of 568 subjects, 141 (25%) were classified as being morbidly obese (BMI ≥ 35).

Table 4.4: Demographic, clinical and laboratory characteristics of the cohort stratified by DSP status

	¤	No DSP N = 307	DSP N = 291	p-value
Age	Median (IQR)	32 (27.5 - 37)	35 (30 - 43)	<0.001 β
Female	N (%)	239 (77.9)	223 (76.6)	0.72 ε
Systolic blood pressure	Median (IQR)	103.8 (94.7- 114.3)	109.7 (99.3 - 119.8)	<0.001 β
Diastolic blood pressure	Median (IQR)	69.8 (60.7 - 78.3)	71.5 (62.7 - 80)	0.019 β
Weight (kilograms)	Median (IQR)	64.5 (55.7 - 75.2)	67.0 (57.0- 80.3)	0.043 β
Height (meters)	Median (IQR)	1.49 (1.43 - 1.56)	1.50 (1.42 - 1.58)	0.10 β
Body Mass Index	Median (IQR)	29.1 (24.4 - 34.4)	29.7 (25 - 35.5)	0.15 β
Waist-to-Hip Ratio	Median (IQR)	0.84 (0.79 - 0.88)	0.87 (0.82 - 0.92)	<0.001 β
MUAC γ (cm)	Median (IQR)	27.5 (25 - 30.5)	28 (15 - 31)	0.37 β
Calf skin fold thickness (mm)	Median (IQR)	15.9 (9.6 - 22.6)	15.75 (8.35 - 23.1)	0.96 β
Abnormal glucose δ	N (%)	67 (29.5)	65 (30.8)	0.77 ε
CD4 nadir count	Median (IQR)	192 (122 - 378)	147 (75 - 206)	<0.001 β
MCV κ current	Median (IQR)	102.4 (92.3 - 109.8)	107.05 (99.6 - 113.8)	0.01 β
Fasting lactate	Median (IQR)	1.9 (1.3 - 2.4)	1.7 (1.25 - 2.4)	0.25 β
Fasting cholesterol	Median (IQR)	3.8 (3.2 - 4.5)	4.1 (3.4 - 4.9)	0.007 β

¤ IQR = interquartile range
β Mann-Whitney non-parametric test
ε χ^2 test
δ Abnormal glucose metabolism includes diabetic and pre-diabetic state
γ Mid-upper arm circumference
κ Mean corpuscular volume

Table 4.5: Additional risk factors for DSP and treatment use of the cohort stratified by DSP status				
	¤	No DSP N = 307	DSP N = 291	p-value
Alcohol exposure ever	N (%)	151 (49.2)	134 (46)	0.44 [ε]
Alcohol exposure in 12 months	N (%)	105 (34.3)	80 (27.6)	0.08 [ε]
Smokers currently	N (%)	46 (15)	51 (17.5)	0.40 [ε]
Daily smokers	N (%)	38 (12.4)	39 (13.4)	0.71 [ε]
Previous TB	N (%)	119 (38.8)	162 (55.7)	<0.001 [ε]
Current TB	N (%)	10 (3.3)	12 (4.1)	0.57 [ε]
Current Vit B complex use	N (%)	280 (91.2)	259 (89)	0.22 [ε]
Current ART exposure	N (%)	99 (32.2)	168 (57.7)	<0.001 [ε]
Current d4T use [δ]	N (%)	71 (23.3)	100 (36.5)	0.002 [ε]
d4T use ever	N (%)	87 (28.5)	130 (47.4)	<0.001 [ε]
d4T (ever) duration (months)	Median (IQR)	13.0 (8-20)	14.0 (8-20)	0.79 [β]
d4T current duration (months)	Median (IQR)	13 (8 – 20)	14 (8 – 20)	0.53 [β]
AZT use ever	N (%)	26 (27)	52 (34)	0.21 [ε]

[¤] IQR = interquartile range
[β] Mann-Whitney non-parametric test
[ε] χ^2 test
[δ] d4T and stavudine are used interchangeably

4.4.1 Logistic regression for the cohort, stratified by neuropathy status (DSP versus no DSP)

Individual univariate logistic regression analyses were conducted to determine risk factors associated with DSP. All variables were explored for an association with DSP, but only variables showing a significant association are presented in Table 4.6. Several potential risk factors identified by non-parametric and proportional analyses (Table 4.4 and Table 4.5), such as weight and blood pressure measurements, failed to show significance in the logistic regression analysis.

Table 4.6: Univariate logistic regression for factors associated with DSP			
	Odds Ratio	(95% Confidence	p-value
Age (per 10-year increase)	1.06	(1.04 - 1.08)	<0.001
ART use			<0.001
Yes	2.87	(2.06 - 4.01)	
No	1		
CD4 count < 200 cells/μl			<0.001
Yes	2.38	(1.62 – 3.51)	
No	1		
Previous TB infection			<0.001
Yes	2.48	(1.75 - 3.50)	
No	1		

We then proceeded to a multivariate logistic regression analysis to identify factors independently associated with a risk of developing DSP. Only variables that showed significance in the univariate analyses were included in the multivariate model, and the results presented in Table 4.7.

CD4 count failed to show an independent association with DSP, although age (per 10-year increments), ART use and previously treated TB infection remained independently associated with risk of developing DSP.

Table 4.7: Multivariate logistic regression for factors independently associated			
	Odds Ratio	(95% Confidence	p-value
Age (per 10-year increase)	1.72	(1.35 – 2.20)	<0.001
ART use			0.04
Yes	1.71	(1.03 – 2.85)	
No	1		
CD4 count < 200 cells/μl			0.31
Yes	1.32	(0.78 – 2.24)	
No	1		
Previous TB infection			0.002
Yes	1.97	(1.28 - 3.04)	
No	1		

4.5 Asymptomatic and Symptomatic DSP

An analysis was performed on the asymptomatic DSP and symptomatic DSP subgroups to determine whether any potential risk factors significantly associated with either of the groups. Individuals with symptomatic DSP were older than those with asymptomatic DSP (p=0.007) but symptomatic DSP did not associate significantly with any other risk factors, including alcohol use, previous TB infection, ART use or markers of impaired glucose metabolism (Table 4.8).

Of the 111 subjects currently with asymptomatic DSP, 30 (27%) reported having experienced HIV-related neuropathic symptoms in the past and most of these (25/30) are currently in the ART-exposed group. Of the latter 25 ART-exposed individuals currently with asymptomatic DSP, 21 (84%) reported resolution of symptoms after initiating ART, suggesting that ART may have a beneficial impact on patients with symptomatic HIV-DSP.

Table 4.8: Asymptomatic vs. Symptomatic DSP

	¤	Asymptomatic DSP N = 111	Symptomatic DSP N = 180	p-value
Age	Median (IQR)	33 (29 -39)	36 (31 – 44)	0.007 [β]
Female	N (%)	84 (75.7)	139 (77.2)	0.76 [ε]
Alcohol past 12 months	N (%)	34 (30.6)	46 (25.7)	0.36 [ε]
Previous TB	N (%)	57 (60.0)	105 (65.2)	0.40 [ε]
ART exposure	N (%)	63 (56.8)	105 (58.3)	0.79 [ε]
D4T use (current)	N (%)	37 (34.9)	63 (37.5)	0.66 [ε]
D4T use (ever)	N (%)	52 (49.1)	78 (46.4)	0.67 [ε]
D4T duration	Median (IQR)	14 (8 – 22)	14 (8-20)	0.88 [β]
AZT use	N (%)	21 (36)	31 (33)	0.72 [ε]
Height (meters)	Median (IQR)	1.52 (1.42 – 1.59)	1.48 (1.43 – 1.57)	0.31 [β]
Waist-to-Hip ratio	Median (IQR)	0.86 (0.81 – 0.92)	0.87 (0.83 – 0.92)	0.31 [β]
CD4 nadir	Median (IQR)	153 (78 – 237)	144 (73 – 203)	0.74 [β]
CD4 current	Median (IQR)	342 (212 – 499)	300 (183 – 433)	0.16 [β]
Impaired glucose	N (%)	27 (33.8)	38 (29)	0.47 [ε]
Fasting lactate	Median (IQR)	1.7 (1.2 – 2.3)	1.7 (1.3 – 2.4)	0.29 [β]
MCV current [κ]	Median (IQR)	105.5 (99.3 – 111.5)	108.1 (99.9 – 115.1)	0.37 [β]

[¤] IQR = interquartile range
[β] Mann-Whitney non-parametric test
[ε] χ^2 test
[κ] Mean corpuscular volume

4.6 Impact of ART on DSP

The prevalence of DSP in both the ART-naïve and ART-exposed subgroups are shown in Table 4.9. DSP developing in ART-naïve subjects is related to the effects of HIV-infection (HIV-DSP) whereas DSP in the ART-exposed group may be due to ART-related toxic neuropathy (ATN) as well as HIV-DSP. For the purposes of this discussion the latter group will be referred to as ATN with the understanding that in a cross-sectional analysis this definition is not entirely accurate.

Overall, DSP prevalence was significantly higher in the ART-exposed subgroup (63%) compared to the ART-naïve subgroup (37%, p<0.001). Furthermore, 23% of those with HIV-DSP had symptomatic DSP whereas 39% of the ATN group could be classified as symptomatic DSP. Similarly, asymptomatic DSP was also more prevalent amongst those with ATN (HIV-DSP 15% vs. ATN 24%). Of those with symptomatic ATN, 42% retrospectively recalled symptom onset within two to four months of initiating ART. Conversely, symptom relief (but not resolution) from ART was reported by 31% (32/105) of ART-exposed individuals.

The neuropathic symptoms of pain, paresthesiae and numbness, as expressed as a VAS-interval grading scale (0 to 4) (see *Appendix A*) showed a trend towards higher scores in the HIV-DSP group compared to those with ATN (p=0.037). However, the rTNS scores were significantly worse in the ATN group (HIV-DSP mean 3.1, SD±2.8; ATN mean 4.6, SD±3.2; p<0.001).

Table 4.9: DSP prevalence, stratified by ART exposure

	Cohort (N, %)	ART-naïve (N, %)	ART-exposed (N,
No DSP	307 (51.3)	208 (62.8)	99 (37.1)
DSP	291 (48.7)	123 (37.2)	168 (62.9)
Asymptomatic DSP	111 (18.6)	48 (14.5)	63 (23.6)
Symptomatic DSP	180 (30.1)	75 (22.7)	105 (39.3)
Total	598 (100)	331 (100)	267 (100)

Consistent with the analysis of the entire study population, age was higher ($p<0.001$) and previously treated TB infection more prevalent ($p=0.02$) amongst those with ATN compared to those without (Table 4.10). Interestingly, the median increase in MCV from ART initiation to follow-up at 6 months was also higher in subjects with ATN ($p=0.041$) compared to those on ART without ATN.

There was no observable difference in the proportion of participants who have ever been exposed to stavudine in the ATN group compared to those without ATN ($p=0.33$). There was, however, a difference between the two groups when evaluating current d4T use; a significantly lower proportion of subjects with ATN were currently (at the time of measure) exposed to stavudine, compared to those without ATN.

Table 4.10: Demographic, clinical and laboratory characteristics of ART-exposed subjects with (ATN) and without DSP				
	¤	No ATN N = 99	ATN N = 168	p-value
Age	Median (IQR)	32 (28 - 37)	36 (31 - 44)	<0.001 β
d4T exposure ever	N (%)	87 (89.7)	129 (85.4)	0.33 ε
Current d4T exposure	N (%)	71 (71.1)	100 (59.5)	0.045 ε
No d4T ever	N (%)	10 (10.3)	22 (14.6)	0.33 ε
D4T duration	Median (IQR)	13.0 (8-19)	16.5 (8-24)	0.22 β
Previous TB	N (%)	63 (63.4)	130 (77.6)	0.018 ε
Current TB	N (%)	0 (0)	2 (1.2)	0.28 ε
Current vit B Complex use	N (%)	92 (92.9)	143 (85.1)	0.06 ε
Alcohol past 12 months	N (%)	30 (30.3)	35 (20.8)	0.08 ε
Daily smokers	N (%)	7 (7.1)	20 (11.9)	0.21 ε
BMI	Median (IQR)	31.0 (24.9-35)	30.2 (24.9-35.4)	0.96 β
Weight (current)	Median (IQR)	65.3 (59.5 - 77.3)	68.0 (57.3 - 79.7)	0.37 β
Weight increase in 3 months	Median (IQR)	4.0 (0.5 - 6.6)	2.5 (0 - 5.5)	0.15 β
Weight increase in 6 months	Median (IQR)	4.1 (1.6 - 9.2)	4.9 (0.7 – 8.1)	0.64 β
Waist-to-Hip Ratio	Median (IQR)	0.86 (0.81 - 0.91)	0.87 (0.82 - 0.92)	0.38 β
MCV κ current	Median (IQR)	108.9 (103.1 - 112.3)	109.2 (103.9 - 115.7)	0.45 β
MCV κ change from baseline	Median (IQR)	18.5 (13.3 - 24.9)	21.3 (16.5 - 26.6)	0.041 β
Fasting cholesterol	Median (IQR)	4.23 (3.45 - 4.81)	4.46 (3.83 - 5.3)	0.12 β
Fasting lactate (mmol/l)	Median (IQR)	1.9 (1.3-2.4)	1.7 (1.3-2.4)	0.46 β
Impaired glucose	N (%)	35 (35.8)	57 (34.1)	0.80 ε
CD4 nadir count	Median (IQR)	125 (74 - 155)	108 (47 - 160)	0.12 β
CD4 start	Median (IQR)	132 (78 - 164)	111 (49 - 165)	0.013 β
CD4 current	Median (IQR	391 (254-503)	316 (239-467)	0.32 β
Immune reconstitution *	N (%)	64 (81.0)	95 (80.5)	0.93 ε

¤ IQR = interquartile range
β Mann-Whitney non-parametric test
ε χ^2 test
κ Mean corpuscular volume. Change from baseline is defined as the difference between pre-ART measurement and that at 6 months of ART-exposure
* Defined as an increase in CD4+ count of > 50 cells/µl between pre-ART and 6-months post-ART values

We did not show a difference in nadir CD4 count values between subjects with ATN and those without. In ART-exposed individuals the CD4 count value recorded prior to ART initiation represented "CD4 start", and was marginally higher than nadir values in both the ATN and ATN-free groups. It was also significantly higher in individuals with ATN compared to those without (p=0.013).

The definition of the immune reconstitution syndrome requires the clinical syndrome to co-occur with evidence of immune recovery on ART initiation (Dhasmana *et al.,* 2008). The relationship between ATN and immune reconstitution was evaluated by assessing the presence of ATN with the change in CD4 count from the pre-ART measurement to that at six months on ART. We arbitrarily defined immune reconstitution as an increase in CD4 count of at least 50 cells/µl as a positive result, but no association with ATN was demonstrated. Viral load counts were available for less than a quarter of ART-exposed individuals at baseline, and were therefore not used in the definition of immune reconstitution. There was also no significant association demonstrated between other measures specific to ART-exposed participants, such as an increase in body weight after three- and six-months of exposure to ART, and ATN.

Risk factors significantly associated with ATN in the previous analysis (non-parametric and proportional; see Table 4.10) were also examined by individual logistic regression to confirm significance prior to being included in the multivariate model (Table 4.11). Age (per 10-year increments), a history of previously treated TB infection and median increase in MCV (in the first 6 months) of ART, all remained independently associated with ATN in the multivariate model.

Table 4.11: Logistic regression for factors associated with ATN

	Univariate analysis		Multivariate analysis	
	OR (95% CI) [β]	p-value	OR (95% CI) [β]	p-value
Age (per 10-year	1.07 (1.03 – 1.10)	<0.001	1.92 (1.20 – 3.07)	0.006
Previous TB infection		0.018		0.015
Yes	1.99 (1.12 – 3.53)		2.99 (1.24 – 7.22)	
No	1		1	
MCV increase on ART*	1.05 (1.01 – 1.10)	0.02	1.06 (1.01 – 1.11)	0.03

[β] OR = Odds Ratio, CI = confidence Interval
* MCV = mean corpuscular volume

4.7 Adherence to ART regimens

Adherence to ART regimens as reported by the subjects was excellent; only three subjects reported inadvertently missing one to three days of dosing. Six-monthly viral load assessments post treatment initiation were available from folder reviews (N=156) and was lower than the detectable limit (<50 IU/ml) in 85% of subjects, although only 45 of these subjects had pre-ART viral load values.

4.8 Quality-Of-Life assessment

Results of the EQ-5D questionnaire are presented in Table 4.12. Within the DSP group, there was a significantly higher proportion of individuals who reported impaired mobility compared to those without neuropathy (p=0.005). No significant differences were noted in any of the other dimensions, nor in the EQ-5D VAS on current state of health (expressed as a percentage). The dimensions of pain/discomfort and anxiety/depression were most frequently reported as abnormal and often in similar proportions whether there were associated DSP or not.

Table 4.12: Proportion of affected individuals in the dimensions of the EQ-5D

	No DSP N (%)	DSP N (%)	p-value
Mobility	10 (3.3)	25 (8.6)	0.005 [ε]
Self-care	2 (0.7)	3 (1.0)	0.61 [ε]
Activities of daily living	6 (2.0)	9 (3.1)	0.37 [ε]
Pain [*]	71 (23.1)	77 (26.5)	0.35 [ε]
Behavioural symptoms [#]	42 (13.7)	42 (14.4)	0.79 [ε]
EQ-5D Health today VAS	80 (70 – 90) [¤]	80 (70 – 90) [¤]	0.81 [β]

[¤] median (interquartile range)
[β] Mann-Whitney non-parametric test
[ε] χ^2 test
[*] "pain" refers to pain or discomfort
[#] "behavioural" symptoms refer to anxiety or depression

In order to evaluate the influence of ART on the response of participants in each of the dimensions of the EQ-5D, each category was stratified by ART use and results are presented in Figure 4.1. Amongst the ART-naïve subjects, a higher proportion of subjects with HIV-DSP (blue bars) reported impaired mobility compared to those without DSP (green bars) (14% vs. 5%, p=0.004). The proportion of individuals with HIV-DSP who reported impaired mobility were also significantly higher than that of participants with ATN (14% vs. 5 %, p=0.01). In the ART-naïve subgroup those with DSP reported pain or discomfort more frequently than individuals without DSP (32% vs. 23%, p=0.07). Although not significant there was a tendency towards a lower proportion with pain or discomfort experienced by those with ATN compared to those with HIV-DSP (ATN 23% vs. HIV-DSP 32%, p=0.08). From the figure it can also be seen that subjective impairment reported by individuals with ATN was comparatively lower than that reported by participants with HIV-DSP across all 5 dimensions of the EQ-5D.

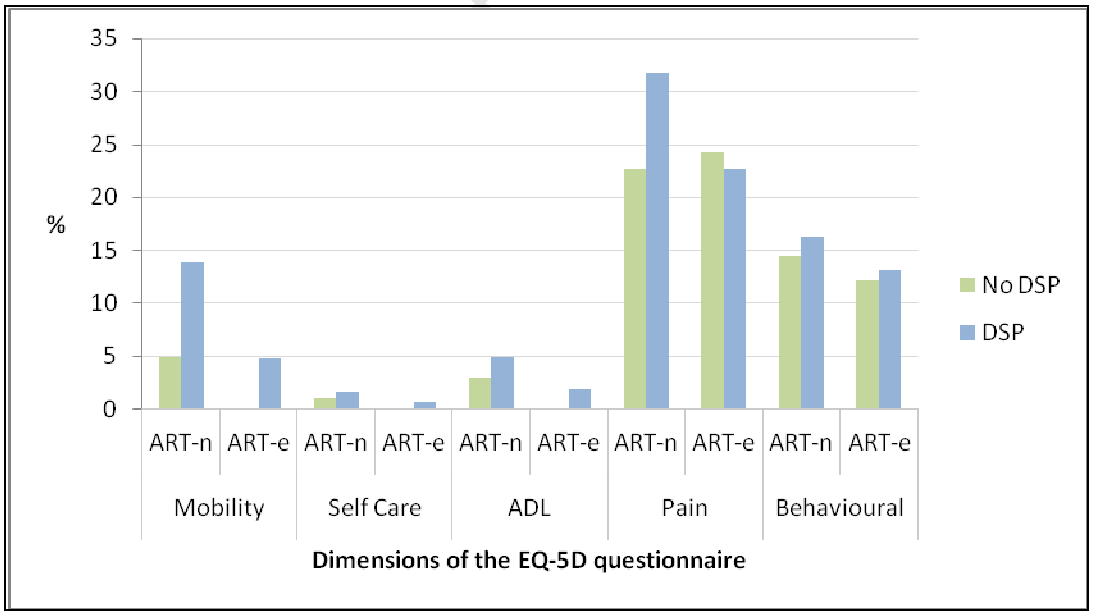


Figure 4.1: EQ-5D results, stratified by diagnostic groups and ART status. ART-n = ART-naïve, ART-e = ART-exposed. DSP (blue bars) represents both HIV-DSP and ATN, depending on ART use.

Chapter 5

Laboratory results

5.1 *TNF-α* promoter -308G→A SNP

As mentioned in earlier (see *3.9 Gel electrophoresis of enzyme-restricted products*), *NcoI* enzyme restriction cleaves the amplified 106bp PCR product in 20bp and 86bp fragments in subjects with the ancestral genotype, whereas it does not digest the PCR product of subjects with the SNP. Recognition of the genotype is based on the difference between 86bp and 106bp fragments, and a DNA ladder with markers in 25bp increments was chosen to differentiate between the expected band sizes. The gel sample (Figure 5.1) includes all three possible genotypes and a marked DNA ladder as a reference.

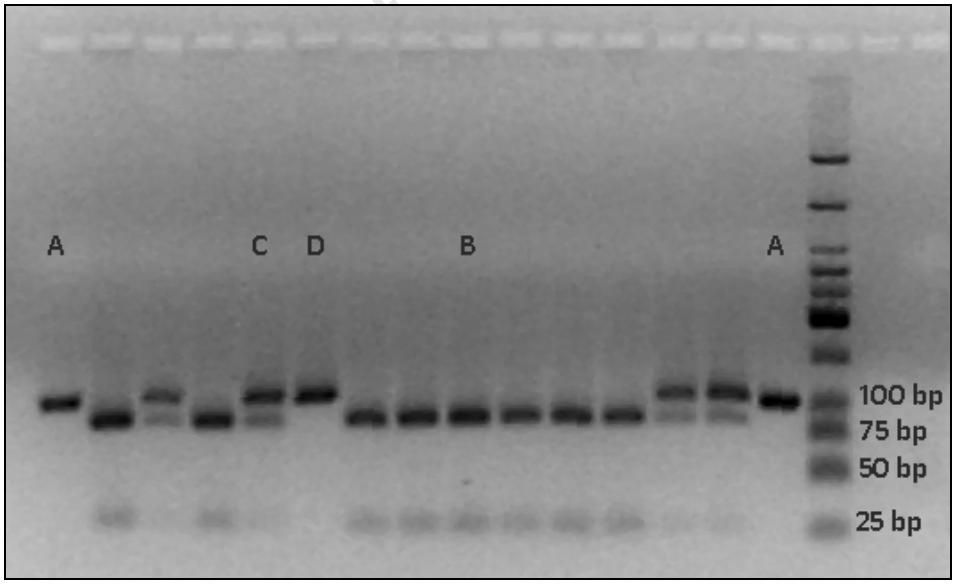


Figure 5.1: TNF-α restriction enzyme gel for -308 G→A SNP. A = uncut PCR products, B = genotype GG, C = genotype GA, D = genotype AA.

5.2 *TNF-α* promoter genotype analysis

Seventy-seven ART-naïve individuals were genotyped for the *TNF-α* promoter G→A SNP, 37 without neuropathy and 40 with symptomatic HIV-DSP. In the symptomatic group, three homozygotes (AA) and seven heterozygotes (GA) for the SNP were identified. This equals a 25% prevalence of the SNP in this group, with an A-allelic (non-ancestral) frequency of 16.25%. In the DSP-free group, two homozygotes (AA) and eight heterozygotes (GA) were identified. This equals a 27% prevalence of the SNP with an A-allelic frequency of 16.22% (Table 5.1).

Table 5.1: *TNF-α* promoter genotype results

	Symptomatic HIV-DSP	**No DSP**
Genotype	N (%)	N (%)
GG	30 (75)	27 (73)
GA	7 (17.5)	8 (21.6)
AA	3 (7.5)	2 (5.4)
	40 (100)	37 (100)
A-allelic frequency	16.25%	16.22%

TNF-α promoter -308 genotypes were further investigated for an association with symptomatic DSP by comparing the ancestral genotype (GG, referred to as TNF1) to the combined non-ancestral genotypes (GA and AA genotypes, collectively referred to as TNF2). No significant difference was demonstrated in the prevalence of the SNP between subjects with symptomatic HIV-DSP and those without neuropathy (Table 5.2).

Table 5.2: *TNF-α* promoter classification

	Symptomatic HIV-DSP	No DSP	
Genotype	N (%)	N (%)	p-value
TNF1 (ancestral)	30 (75)	27 (73)	0.84
TNF2	10 (25)	10 (27)	

5.3 *IL-10* promoter -1082 A→G, -819 C→T and -592 C→A SNPs sequencing

A total of 60 subjects' DNA was sequenced, comprising 30 subjects in each of the symptomatic HIV-DSP and DSP-free groups. Figure 5.2 represents the three possible genotypes for each of the SNPs.

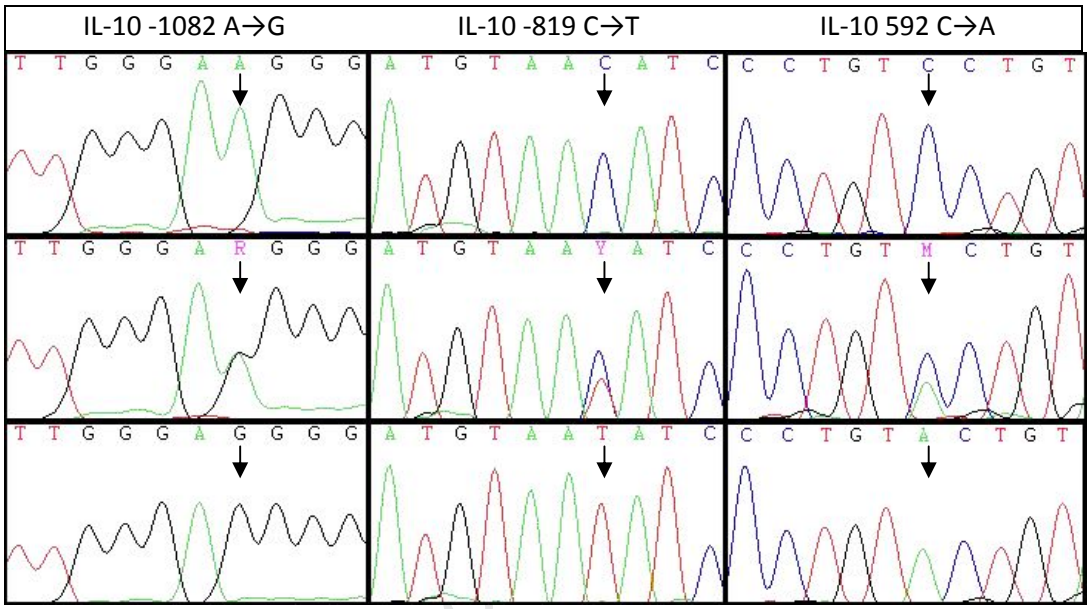


Figure 5.2 *IL-10* promoter sequence samples. Columns from left to right represent the -1082, -819 and -592 SNPs. Rows from top to bottom represent homozygotes (ancestral), heterozygotes and homozygotes for the respective polymorphisms.

The results of the three *IL-10* promoter genotypes and the allelic frequency of each of the non-ancestral alleles are summarized in Table 5.3. Each of the *IL-10* promoter polymorphisms was further categorized according to ancestral- and non-ancestral genotypes (similar to TNF-α classification, Table 5.4). No significant difference in any of the *IL-10* promoter SNPs were demonstrated between the neuropathy-free and symptomatic HIV-DSP groups.

Table 5.3: *IL-10* promoter genotyping results		
	Symptomatic HIV-DSP	**No DSP**
-1082 Genotype	N (%)	N (%)
AA	14 (46.7)	10 (33.3)
AG	13 (43.3)	17 (56.7)
GG	3 (10)	3 (10)
	30 (100)	30 (100)
G-allelic frequency	31.7%	38.3%
-819 genotype	N (%)	N (%)
CC	13 (43.3)	16 (53.3)
CT	17 (56.7)	10 (33.3)
TT	0 (0)	4 (13.3)
	30 (100)	30 (100)
T-allelic frequency	28.3%	30%
-592 genotype	N (%)	N (%)
CC	14 (46.7)	16 (53.3)
CA	16 (53.3)	10 (33.3)
AA	0 (0)	4 (13.3)
	30 (100)	30 (100)
A-allelic frequency	26.7%	30%

Table 5.4: *IL-10* promoter classification			
Genotype	Symptomatic HIV-DSP N (%)	No DSP N (%)	p-value
1082-1 (ancestral)	14	10	0.29
1082-2	16	20	
819-1 (ancestral)	13	16	0.44
819-2	17	14	
592-1 (ancestral)	14	16	0.61
592-2	16	14	

5.4 *IL-10* promoter haplotype classification

IL-10 promoter haplotypes in relation to IL-10 production have been defined and described previously (see *1.3.5 Expression regulated by genotypes* and Table 1.1). We explored the frequency of each of these haplotype groups in neuropathy-free subjects and in those with symptomatic HIV-DSP, but did show any significant differences (p=0.64, Table 5.5).

Table 5.5: *IL-10* production by haplotype

	Symptomatic HIV-DSP N (%)	No DSP N (%)	p-value
High producers	3 (10)	3 (10)	
Intermediate producers	13 (43.3)	17 (56.7)	
Low producers	14 (46.7)	10 (33.3)	
	30 (100)	30 (100)	0.64

Chapter 6:

Discussion

6.1 Prevalence

Data from this cross-sectional, observational study in South African urban ambulatory subjects confirms that DSP is a common problem in our HIV-infected population. Using a rigorous clinical definition of DSP that requires the presence of at least two neuropathic signs, we found an overall DSP prevalence of 49%. Moreover, the prevalence of symptomatic DSP almost doubles from 23% in ART-naïve subjects to 39% in ART-exposed subjects.

Prevalence estimates of DSP vary substantially depending on the population studied and the definition employed to determine neuropathy status. Prevalence figures from selected studies together with the definition of neuropathy used are summarized in Table 6.1, and the corresponding DSP prevalence from our study using the respective definitions are tabulated for comparison. By using our definition of DSP, the prevalence in this community clinic-based population is higher than those of recently published studies from developed and developing populations, some of which had similar-sized cohorts to our study (Wright *et al.,* 2008, Sithinamsuwan *et al.,* 2008, Affandi *et al.,* 2008, Hung *et al.,* 2008).

Table 6.1: Comparative prevalences	SDSP* ≥2 signs	SDSP any 1 sign	AAN-SDSP** ≥2 signs	BPNS-SDSP 1 sign#	BPNS-SDSP 2 signs
SA study (N=598)	**30%**	**43%**	**26%**	**37%**	**18%**
USA (N=76) (Cherry *et al.,* 2006)			49%		
Australian (N=71) (Cherry *et al.,* 2006)			55%		
Asia-Pacific (N=640) (Wright *et al.,* 2008)				20%	6%
Thailand (N=100) (Sithinamsuwan *et al.,* 2008)		38%	10%		
Indonesia (N=99) (Affandi *et al.,* 2008)				34%	
American ART only (N=502) (Hung *et al.,* 2008)	26%				

* SDSP as defined in this study, symptoms + ≥ any 2 signs (decreased pin ± vibration ± reduced/absent ankle reflexes).
**American Academy of Neurology-SDSP clinical definition recommended for field studies includes symptoms + reduced/absent ankle reflexes + decreased sensation (pin or vibration)
Cherry *et al.*, 2005.

From the developed countries, an American study with a similar sample size to our cohort had a comparable DSP prevalence, although all of their study participants were exposed to ART. Of these subjects, 23% of those exposed to dNRTI therapy had DSP compared to 63% of this community-based SA population (Table 6.1) (Hung *et al.,* 2008). Smaller samples from the USA and Australia (Cherry *et al.,* 2006) showed higher DSP prevalence figures, but included notably older subjects, which possibly accounts for the higher prevalence rates; these study populations also did not exclude subjects with known glucose intolerance.

From developing countries, a multi-centre study spanning the Asia-Pacific region found a lower DSP prevalence (20%) amongst a study population comparable to our cohort in size and dNRTI use (Wright *et al.,* 2008). On the other hand, smaller studies from the same region (Thailand and Indonesia) reported DSP prevalence

figures comparable to ours (Sithinamsuwan *et al.,* 2008, Affandi *et al.,* 2008). It is clear that prevalence figures vary depending on the criteria used for defining neuropathy. To illustrate this point, prevalence figures from this South African cohort vary from 18% to 43% according to the different clinical definitions, as presented in Table 6.1 (also see *6.6 Small fibre sensory neuropathy study tools*).

6.2 Clinical symptoms and signs of DSP

All the neuropathic signs and symptoms included in the definition of DSP were significantly more prevalent in subjects with DSP compared to those without. However, symptoms were also reported frequently by individuals who were not categorized as having DSP i.e. without at least two neuropathic signs. Pain was the most prevalent DSP symptom affecting nearly half of all individuals with DSP, but also reported by a quarter of subjects without DSP. It is unclear why such a large proportion of study participants without a diagnosis of DSP experiences symptoms, although it is recognized that our definition excluded subjects who only had 1 abnormal sign on examination. Prospective evaluation of ENFD in subjects with pain in the absence of symptoms might be useful to define this phenotype, and to evaluate the possibility that it may represent a transitional state to developing clinically evident neuropathy in susceptible individuals.

Decreased or absent deep tendon reflexes at the ankle was the most common sign in the DSP group, followed by decreased pinprick sensation. This may be important for the use of the BPNS as a screening tool as decreased pinprick sensation is not included in the BPNS.

6.3 Risk factors for DSP

Risk factors in our population not only include advancing age and ART exposure, factors previously identified in developed countries (Cherry *et al.,* 2006, Smyth *et al.,* 2007), but also a frequent history of previously treated TB infection. Contrary to our expectations, impaired glucose metabolism, a history of alcohol exposure and

gross markers of nutritional state such as weight and body mass index, were not significantly associated with DSP in this population. On anthropometric measures, waist-to-hip ratio was significantly higher in those with DSP compared to those without, but the significance of this is unclear in the absence of association with other metabolic markers.

Although studies have reported that nutritional indices such as decreased serum albumin and weight loss are associated with neuropathy (Tagliati *et al.,* 1999; Snider *et al.,* 1983, So *et al.,* 1988), the impact of nutritional status on DSP has not been systematically studied. While our study showed no association between DSP and macronutritional status as reflected by body weight, BMI, mid-upper arm circumference or calf skin fold thickness, micronutrient status may play a role in preventing DSP and needs further investigation.

The indirect consequences of TB infection or its therapy was shown to be independently associated with DSP, irrespective of ART treatment status. TB is a common problem in the Western Cape, and this Cape Town-based population's prevalence of previous TB infections is also similar to other cohorts from South Africa (Lawn *et al.,* 2006). A possible reason for an increased association of DSP with a history of previous TB may come from the results of a double-blind placebo-controlled micronutrient supplement study in Tanzanians on TB therapy. They showed a reduced incidence of peripheral neuropathy in those who received supplements, irrespective of HIV status (Villamor *et al.,* 2008). The supplements used consisted of vitamins A, B (1, 2, 3, 6, and 12), C, E, folic acid and selenium. In South African primary health care clinics, patients on TB therapy will occasionally receive pyridoxine supplementation as isoniazid influences pyrodixine metabolism (Standard Treatment Guidelines and Essential Drugs List For South Africa, 2006), and two tablets of vitamin B complex per day are usually prescribed for HIV-infected individuals. One of the currently-used vitamin B complex tablets (in the Western Cape state healthcare sector) provide less than a tenth of the dose of some of the vitamin B subgroups compared to that used in the abovementioned study, and also do not contain vitamins A, C, E, folic acid or selenium (from the South African Medicines Formulary). In addition to this problem, a report from 2004 on

the progress made in the first year of ART-rollout in South Africa showed that 13% of ART-clinics experienced stock outs of antiretrovirals, and 32% of clinics experienced stock outs of nutritional and vitamin supplements during that year. A further 9% of ART-clinics do not provide nutritional or vitamin supplements at all (Stewart and Loveday, 2005). Our data also suggested a trend towards lower vitamin B complex use in subjects with ATN compared to ART-exposed subjects without DSP.

We could not confirm subject height as a risk factor for DSP, as recently found by two studies from Australia and Indonesia (Cherry *et al.,* 2008b, Affandi *et al.,* 2008). These findings were based on study populations consisting of 13% and 3% females respectively, whereas our study had a female preponderance.

Hepatitis C was previously thought to be a confounder in the diagnosis of HIV-associated neuropathies, including DSP (Estanislao *et al.,* 2005) but not all studies have shown an association with DSP (Cherry *et al.,* 2006, Wright *et al.,* 2008). In our setting (Cape Town), hepatitis C virus infection has very low background prevalence ranging from <0.1% in blood donors to 1.6% amongst high risk HIV-infected white males who have sex with males (Tucker *et al.,* 1997, Amin *et al.,* 2004). Although we did not record the sexual habits of our study population, hepatitis C infection should have a limited impact on our prevalence rate as our study population were predominantly female (77%) as generally found in South African cohorts (Lawn *et al.,* 2006, Boulle *et al.,* 2007).

No risk factor with the exception of age differentiated between asymptomatic and symptomatic DSP; subjects with symptomatic HIV-associated DSP were significantly older than those who were asymptomatic. Obvious causes of symptomatic neuropathy such as alcohol and measure of glucose intolerance were equally distributed amongst participants with symptomatic and asymptomatic DSP.

6.4 Impact of ART and associated ATN

Antiretroviral therapy is often associated with the risk of developing ATN (Cherry *et al.,* 2006, Smyth *et al.,* 2007). Although overall DSP is associated with ART use in this population, the proportions of individuals who are currently using d4T as well as those who have ever used d4T previously, were lower in subjects with ATN compared to neuropathy-free individuals. This was consistent with a recently published report of a significantly lower ATN rate in dNRTI-exposed subjects compared to those not taking dNRTIs (Hung *et al.,* 2008). This observation may reflect judicious avoidance of stavudine-containing regimens in subjects with neuropathic symptoms prior to ART initiation, or may suggest an as yet, unaccountable factor(s) and requires further longitudinal study.

We found no evidence of cumulative toxicity of stavudine use based on a similar duration of exposure to the drug in the ATN and ATN-free groups, whether you consider those on the drug currently or previously. This observation is also consistent with previous reports by several groups in which they conclude that, despite an increased risk of developing DSP within the first few months of starting on a dNRTI regimen, most subjects tolerated dideoxynucleoside therapy and without a late increase in the risk of worsening neuropathy (Hung *et al.,* 2008, Cherry *et al.,* 2006, Arenas-Pinto *et al.,* 2008, Schifitto *et al.,* 2005, Simpson *et al.,* 2006). Data has shown that the risk of developing DSP peaks at three months of exposure to stavudine, where after it decreased (Arenas-Pinto *et al.,* 2008). Also, subjects from our study who had never used stavudine, although comparatively few, were as likely to develop DSP as those on stavudine containing regimens.

The increased risk of developing DSP after initiation of ART raised a question as to whether recovery of the host's immune system is at play. The immune reconstitution syndrome generally manifests in the first three months after commencing ART in a spectrum of diseases (Dhasmana *et al.,* 2008). Attempts have been made to define criteria for quantifying immune recovery, but this is frequently not informative as the syndrome can manifest before quantitative changes in immune status are demonstrable, and even more so in developing

countries where viral load counts and CD4 counts are frequently missing (in Dhasmana *et al.,* 2008). To explore the possibility of host immune reconstitution as a contributory factor in the early development of NRTI-induced DSP (ATN), we used an arbitrary definition of immune recovery as a rise in CD4 count of 50 cells/µl within the first 6 months after ART commencement, but found no association.

The risk factors for ATN were similar to DSP overall with increasing age and previously treated TB infection identified as independent risk factors for ATN. As an additional risk factor, MCV increase from the pre-ART baseline until follow-up at six months associated independently with ATN. The relevance of this finding is still unclear and warrants further study.

Lactate was previously found to associate with ATN although based on a small study (Brew *et al.,* 2003), but did not associate with ATN in this study. A substantial risk of hyperlactataemia was previously demonstrated amongst black South African women weighing over 75kg on stavudine-based regimens (Boulle *et al.,* 2007), but this finding could not be replicated in our study despite the fact that nearly a third of female subjects on stavudine weighed more than 75kg. A notable difference however exists in the stavudine dosage as the previously mentioned study was conducted on a population using a 40mg twice-daily dose compared to our population where the majority of d4T-exposed subjects used 30mg twice a day.

6.5 Quality of life and DSP

The majority of subjects with symptomatic DSP experienced neuropathic symptoms of moderate to severe intensity as measured by the BPNS VAS, indicating its importance as a primary health care burden. Neuropathy has an adverse effect on quality of life and pain in the HIV population has been shown to impact upon other clinically important parameters such as utilization of health services, affective disorders, and functional status (Rosenfeld *et al.,* 1996; Pandya *et al.,* 2005; Rusch *et al.,* 2004, Hays *et al.,* 2000).

Our data show that in all dimensions of the EQ-5D there was a tendency for ART to show a positive impact on the lives of participants, irrespective of neuropathy status. Although individuals with ATN consistently reported less impairment compared to those with HIV-DSP, the difference was only significant in the dimension of mobility impairment. This is largely comparable to the profile of a prospectively-studied ART-exposed cohort from the same geographical location and culture, which found that health-related quality of life improved over the 12-month period following ART initiation (Jelsma *et al.,* 2005).

The EQ-5D's dimension of pain/discomfort did not discriminate between subjects with or without DSP. Importantly, poor pain control is estimated to occur in over half of the HIV-population being treated for pain (Breitbart *et al.,* 1998). The low use of amitriptyline by our population suggests either poor efficacy in neuropathic pain management (Kieburtz *et al.,* 1998) or under-recognition of depression and a more generalised perception of discomfort or pain in HIV/AIDS subjects irrespective of neuropathy. Both these scenarios have been encountered in other developing nations (Wright *et al.,* 2008) where the burden of disease in conjunction with limited health budgets highlights the need for cheap but effective strategies to manage patients' pain.

We did not show an association between neuropathy and mood, though cultural variables may be at play; the EQ-5D has been validated in isiXhosa across all five domains, although the concept behind the word "depression" differ in Xhosa culture (Jelsma *et al.,* 2004). To a Xhosa speaker, "depression" is regarded as temporary in nature and necessarily associated with a cause, and reported rates in the validation study were lower than expected (Jelsma *et al.,* 2004). Compared to the previously mentioned cohort from Cape Town (Jelsma *et al.* 2005), our study population's response to both the pain/discomfort and depression/anxiety dimensions were similar in the ART-exposed subgroups, but was lower in our ART-naïve subjects compared to those from the reference study. Another South African study found subjects on ART to report less impairment than those awaiting ART, although this study was conducted at the start of the governmental ART roll-out

programme and possibly comprised subjects with more advanced HIV-infection and AIDS (Louwagie *et al.,* 2007).

In summary, the results of the quality of life assessment did not differentiate between individuals with or without DSP. The EQ-5D is generally used as a tool to assess the impact of interventions on health-related quality of life, and confirmed that ART had a beneficial impact on most of our HIV-infected study population.

6.6 Small fiber sensory neuropathy study tools

To our knowledge there are no previous reports of observational, systematic examination for DSP on HIV-infected African subjects using validated tools. Although selected studies from Africa mention peripheral neuropathy prevalence in the context of adverse effects of ART, it is often without reference to the diagnostic criteria for DSP that were used (Jamisse *et al.,* 2007, Hawkins *et al.,* 2007). A recent publication mentioned their criteria which included either subjective reporting of neuropathic symptoms or the presence of a single neuropathic sign on physician examination (Hoffmann *et al.,* 2008). These cases would therefore include many of our current subjects not categorized as DSP.

The variation in the DSP prevalence figures in this cohort (Table 6.1) depending on the clinical definition employed highlights the importance of establishing a stringent clinical definition that can be used in field or epidemiological studies, but that has been validated against a gold standard reference test such as ENFD determination on skin biopsy. The validation study should be conducted by examiners blinded to the result of the reference test, and should be conducted in the population or setting where it is intended for use (Benatar, 2006). Preferably, this definition should also be applicable in resource-constraint populations, where even the availability of basic instruments such as tuning forks, may be limited.

A definition for DSP was proposed by England *et al.* (see *1.1.7.1 Proposed definitions*) based on a systematic analysis of peer-reviewed literature and consensus among experts in the field (England *et al.,* 2005, in Benatar, 2006). The

various definitions for clinical research studies and epidemiologic studies were ranked by estimated ordinal likelihood of disease, as different reference standards were used in the literature. However, as pointed out by Benatar (2006) the proposed definition was based on diagnostic modalities that overlapped with the reference tests used in the studies thereby artificially inflating sensitivity and specificity of the tests.

Little data is available correlating clinical diagnostic tests for DSP in HIV-infected populations with a gold standard such as ENFD estimation from skin biopsy. An adapted version of the original BPNS (requiring only 1 sign in the presence of symptoms for the diagnosis of SDSP) correlated well with nerve fibre loss in ENFD measurements at the distal leg in HIV-associated DSP (Cherry *et al.,* 2005), but did not perform well against the TNS as a diagnostic tool (Table 2.3 and Table 2.4). Further study is needed to determine which clinical test provides an adequate screening tool for the small fibre neuropathy associated with HIV-infection and that of ATN.

6.7 Cytokine promoter polymorphism genotyping

***TNF-α* promoter -308 G→A SNP**: In this pilot study, we found a background frequency of the *TNF-α* -308 A-allele of 16% in our control (DSP-free) subjects, which was similar to the A-allelic frequency of the individuals with symptomatic HIV-DSP (16%). Therefore, no association was found between the presence of the TNF2 allele and symptomatic HIV-DSP. Although the number of subjects in each group was relatively small (n=30), it would appear unlikely that a larger cohort would find a different result as there were almost no difference in SNP frequency between the DSP-free and symptomatic HIV-DSP groups.

Unexpectedly, the A-allelic frequency was higher in this Xhosa-speaking South African cohort than that previously reported in subjects from sub-Saharan Africa, comprising mostly Nigerians (Entrez SNP Database). We also found 6% of our population to be homozygous for the A-allele which had not been previously listed

on the NCBI SNP database for African populations, but the homozygous individuals were evenly distributed between symptomatic HIV-DSP and DSP-free groups.

Recently published studies from Australia and Indonesia found the frequency of the *TNF-α* promoter -308 A-allele in subjects with DSP to be 13% (Cherry *et al.,* 2008b [calculated]) and 10% (Affandi *et al.,* 2008). These reports were based on small study populations (N=55 and 96 respectively), and one study found an insignificant increase in representation of the -308 A-allele in subjects with ATN (p=0.12, Cherry *et al.,* 2008b). However, both studies identified the TNF-α -1301 C-allele (non-ancestral) to be associated with ATN. A recent study from India found this allele to be associated with higher serum levels of TNF-α in malaria-related fever (Sohail *et al.,* 2008). They also showed the TNF-α -308 G→A and -1031 T→C SNPs to be in linkage disequilibrium in patients with malaria, but not in healthy controls (Sohail *et al.,* 2008), and this finding did not seen apparent from the Indonesian study (Affandi *et al.,* 2008).

We did not investigate the *TNF-α* promoter -308 SNP in association with ATN and can therefore not confirm the abovementioned studies' findings. We also did not investigate the TNF-α -1031 T→C SNP in association with HIV-DSP, as the data regarding its significance was only published after we conducted the association pilot study. Haplotype associations varying with ethnicity may be responsible for the inconsistent observed responses to genetic polymorphisms (Lu *et al.,* 2008, Cherry *et al.,* 2008b, Zabaleta *et al.,* 2008), and warrants further study in African populations.

IL-10 promoter SNPs: Neither of the three IL-10 promoter SNPs, nor the IL-10 haplotypes as classified according to the previously reported rate of IL-10 production (Hoffmann *et al.,* 2001), associated with DSP status in this pilot study. This South African population's IL-10 -1082 G-allelic frequency of 35% correlates well studies from Zimbabwe (30%, Erikstrup *et al.,* 2007), as well as that reported for African-Americans (36% -41%) on the NCBI SNP database. The frequencies of both the IL-10 -819 T-allele (29%) and -592 A-allele (28%) were lower than those

previously reported in African-American individuals (36% to 54% and 50% to 53% respectively) (Entrez SNP Database).

Our aim with this small pilot study was to ascertain whether a larger study would be feasible, but our results suggest against this and the pilot study was therefore not expanded.

6.8 Study limitations

Our study is also limited insofar as we did not evaluate all subjects for other possible causes of a distal polyneuropathy, such as a vitamin B12 deficiency. Poor clinic record keeping and poor compliance to governmental protocol with regards to blood sampling limited the usefulness of some of the parameters such as viral load measurements.

Our definition of DSP consisted only of clinical measures, without electrophysiological or histological confirmation of the diagnosis as these are very difficult to perform in field studies. Although electrophysiological tests are not always needed for the diagnosis of DSP, it is useful in the diagnosis of a pure small fibre neuropathy. The cross-sectional nature of this study precluded evaluation of whether or not pre-existing HIV-DSP constitutes a risk factor for ATN. It was also not possible to accurately establish the prevalence of ATN, as ART-exposed individuals could have either ATN or HIV-DSP, and the temporal relationship between ART initiation and possible symptom development in our population warrants longitudinal investigation.

6.9 Conclusions

In conclusion, we found that DSP is a frequent and clinically significant problem in a HIV-infected African community clinic-based population, where its frequency increases with antiretroviral therapy, age and previously treated TB infection, and is commonly associated with moderate-severe pain and/or paresthesiae. Previous

associations of HIV-DSP in populations from the developed countries such as male gender (Morgello *et al.,* 2004) and subject height (Affandi *et al.,* 2008, Cherry *et al.,* 2008), were not replicable in this African population. This study present novel results showing that neuropathy status was not associated with duration of stavudine exposure in Africans, and with careful avoidance of stavudine-containing regimens in older subjects, especially with a history of prior TB infection, the prevalence of DSP may be reduced.

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Appendix A:

Clinical evaluation tool

SA BRIEF PERIPHERAL NEUROPATHY SCREENING/EXAM

Patient Number: _____ Date of Visit (DD/MM/YYYY) ____/____/20____

We are going to ask you a few questions about sensation in your legs. We will also briefly examine the nerves in your arms and legs. We may advise the clinic doctor on treatment.

BPNS : INSTRUCTIONS FOR RECORDING SYMPTOMS: *Ask subject to rate the severity of each symptom in 1a to 1c on a scale of 0 (absent) to 10 (most severe) for right and left feet, legs- worst in last week.* Enter the score for each symptom in the block marked Severity. Enter extent of symptoms eg Soles of feet/ toes (TNS=1); up to ankle (TNS=2); up to knee (TNS= 3) or above (TNS=4) on the TNS score overleaf.

1a. Pain, aching, burning in feet or legs. Ingaba iinyawo zakho zibuhlungu, ziyaqagamba, ziyatshisa kangangeeyeki ezimbini?

Normal	Mild	–	–	–	–	–	–	–	–	Severe
0	1	2	3	4	5	6	7	8	9	10
Andinantlungu! –		–		–		Ndineentlungu ezigqithisileyo!				

Nn1 Score 1a

1b. "Pins-and-Needles" in feet or legs. Ingaba iinhawo zakho zineenaliti noonotaka kangangeeyeki ezimbini?

Normal	Mild	–	–	–	–	–	–	–	–	Severe
0	1	2	3	4	5	6	7	8	9	10
Andinantlungu! –		–		–		Ndineentlungu ezigqithisileyo!				

Nn2 score 1b

1c. Numbness (lack of feeling) in feet or legs. Ingaba iinyawo zakho zinobundindisholo kangangeeyeki ezinibini?

Abukho ubundindisholo		–	–	–	–	–	–	–	–	Andiva nto
0	1	2	3	4	5	6	7	8	9	10

Nn3 Score 1c

TOTAL SENSORY PRESENCE/SEVERITY SCORE: Obtain the single highest severity score from 1-10 in 1(a - c) above:

0 = Grade 0 1-3 = Grade 1
4- 6 = Grade 2 7-8 = Grade 3
9-10= Grade 4 **Total sensory severity GRADE** ___/4 nn4

1d. If a symptom was present in the past, but not now i.e "Currently Absent" Yes (1) N/A (0) nn5

1e. Do you have any other unpleasant symptoms in you legs such as cramps? Yes (1) No (0) nn6

1f. Has anything helped for the pain (medicine or other) ? _____ Yes (1) No (0) nn7
1g. Do you think the ARV treatment helped your symptoms? Yes (1) No (0) Unknwn (2) nn8

1h. Did the sensory symptoms in your feet start or get worse within 2-4 months of starting ARV Rx?
Yes (1) No (0) Unknwn (2) nn9

1i. Did the sensory symptoms in your feet start or get worse within 2-4 months of changing the dose of ARV Rx?
Yes (1) No (0) Unknwn (2) nn10

2. INSTRUCTIONS FOR EVALUATING PERCEPTION OF VIBRATION:
Press the 2 ends together of a 128 Hz tuning fork, and release suddenly; place the vibrating tuning fork on the subject's clavicle; can they recognise the vibration or "buzzing" (ngcungcazela) of the tuning fork? Repeat and immediately place the vibrating tuning fork firmly on the interphalangeal bone (not nail) of one great toe and begin counting the seconds. Subject to tell you when the "buzzing" stops. Repeat on the other side.

Vibration Perception
(Take highest score but both R & L must be abnormal)

0- Vibration felt for >10 seconds (normal)
1- Vibration felt for 6-10 seconds (mild loss)
2- Vibration felt for 5 seconds or less (moderate loss)
3- No feeling of vibration (severe loss)

	Right	Left		
Great toe interphalangeal bone	____	____	(2) use highest value_____/3	nn11

3. INSTRUCTIONS FOR EVALUATING DEEP TENDON REFLEXES:
With the subject seated, the examiner uses one hand to press upward on the ball of the foot, dorsiflexing the subject's ankle to 90 degrees. Using a reflex hammer (long-handled), the examiner strikes the Achilles tendon.

Reflexes4- Absent
3- Reduced (difficult to elicit)
2- Normal deep tendon reflexes
1- Hyperactive deep tendon reflexes
0- Clonus

	Right	Left		
Ankle Reflexes: (Take highest score but both R & L must be abnormal)	_____	_____	(3) use highest value____/4	nn12

Final score for BPNS (1+2+3) ____/11 nn13

Reduced TNS score: tick in the box :

	0	1	2	3	4	
a. Sensory symptoms from 1a,b,c: Pain, burning pins or numbness	*none*	*Only in toes or soles of feet*	*Symptoms extend to ankle or wrist*	*Symptoms extend to knee or elbow*	*Symptoms knee or elbow or functionally disabling*	nn14
b. Pin sensibility	*normal*	*Reduced in fingers /toes*	*Reduced up to wrist/ ankles*	*Reduced up to elbow/ knee*	*Reduced above elbow/ knee*	nn15
c. Vibration sensibility (use normal as for BPNS)	*normal*	*Reduced in fingers /toes*	*Reduced up to wrist/ ankles*	*Reduced up to elbow/ knee*	*Reduced above elbow/ knee*	nn16
d. Deep tendon jerks	normal	Ankle reflexes reduced	Ankle reflexes absent	Ankle reflexes absent, other reduced	All reflexes absent	nn17
e. Strength- ankle and toes plantar & dorsi-flexion	normal	Mild weakness (MRC 4)	Moderate weakness (MRC 3)	Severe weakness (MRC2)	Paralysis (MRC 0-1)	nn18

Final score for rTNS (a+b+c+d+e) _____/20 nn19

Appendix B

EuroQol 5D Questionnaire

EQ - 5D

Health Questionnaire

South African English adapted version

By placing a tick in one box in each group below, please indicate which statements best describe your own state of health TODAY.

Mobility

I have no problems in walking about	❑
I have some problems in walking about	❑
I am confined to bed	❑

Self-Care

I have no problems with self-care	❑
I have some problems washing or dressing myself	❑
I am unable to wash or dress myself	❑

Usual Activities (*e.g. work, study, housework, family or leisure activities)*

I have no problems with performing my usual activities	❑
I have some problems with performing my usual activities	❑
I am unable to perform my usual activities	❑

Pain/Discomfort

I have no pain or discomfort	❑
I have moderate pain or discomfort	❑
I have extreme pain or discomfort	❑

Anxiety/Depression

I am not anxious or depressed	❑
I am moderately anxious or depressed	❑
I am extremely anxious or depressed	❑

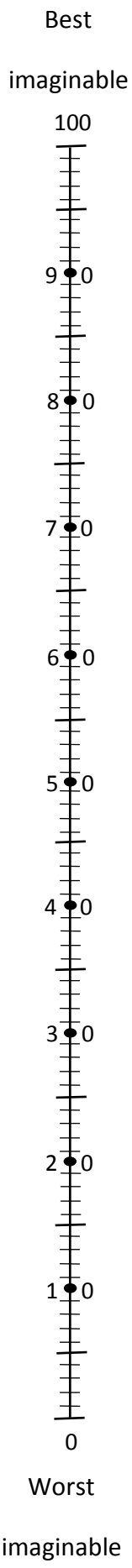
Compared with my general
level of health over the past 12 months,
my state of health today is:

Better	❑	PLEASE TICK
Much the same	❑	ONE
Worse	❑	BOX

To help people say how good or bad their state of health is, we have drawn a scale on which the best state you can imagine is marked 100 and the worst state you can imagine is marked 0.

We would like you to indicate on this scale, in your opinion, how good or bad your own health is today. Please do this by drawing a line from the box below to whichever point on the scale indicates how good or bad your state of health is today.

Your own state of health



Appendix C

Laboratory troubleshooting

IL-10 promoter sequencing: comparing 2 reverse primers

The 2 reverse primers designed for the IL-10 sequencing reaction were compared by using both primers on PCR product amplified from the same subject's DNA using reverse primer 1 (see *3.10.1 Primer design*). Both primers produced readable sequences, but sequencing with reverse primer 1 resulted in more background noise and occasional misreads. It was therefore decided to do internal sequencing with reverse primer 2 for all sequencing reactions.

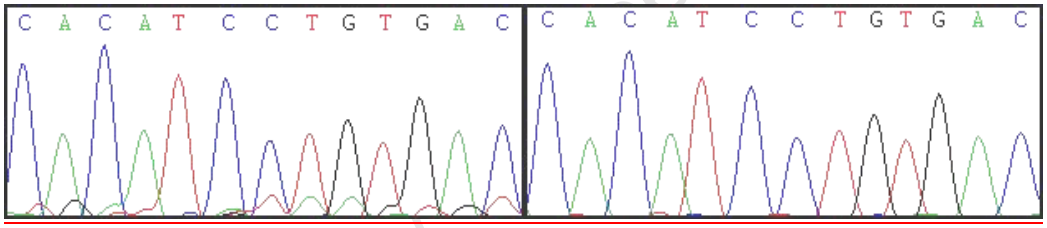


Figure C.1: Sequences of reverse primer 1 (left) and reverse primer 2 (right)