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Measurement of Intraepidermal Nerve Fibre Density in Individuals with Antiretroviral Toxic Neuropathy

Imraan Goolam Patel



Degree of Master of Science in Medicine by research only

Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2011

DECLARATION

I, Imraan Goolam Patel, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

…….. (Signature of candidate)

……. day of ……., 2011

DEDICATION

In the name of the Almighty

The most merciful, the most beneficent

PRESENTATIONS

Patel IG, Kamerman P. Measurement of Intraepidermal Nerve fibre density in individuals with HIV associated toxic neuropathy. (Oral presentation at the 4th Pan African Pain Congress, Cape Town, South Africa, 11-13 March 2011)

Abstract

HIV-associated sensory neuropathy (HIV-SN) is a common complication of HIV infection and its treatment with dideoxynucleoside drugs such as stavudine. Pain is a symptom in about 75% of cases of HIV-SN. The aim of this study was to set up the intraepidermal nerve fibre density (IENFD) quantification technique in a South African Laboratory and then to use this technique to investigate whether the presence of pain in individuals with HIV-associated sensory neuropathy was associated with the dying back of epidermal nerve fibres at the site at which pain was experienced.

The Intraepidermal nerve fibre density (IENFD) quantification technique was set up using biopsies taken from healthy subjects. The staining technique as well as the quantification technique was carried out until relative proficiency was achieved. Thereafter, a small study was set up to evaluate our second aim. For this study, 15 participants were recruited and divided into two groups, a 'Pain' Group (n= 9) (participants that experienced pain at the lower leg biopsy site) and a 'No-Pain' group (n= 6) (participants that did not experience pain at the lower leg biopsy site). Six 3mm skin biopsies were taken from each participant. Samples were fixed, cryoprotected and then 50 micron sections of each biopsy were analysed immunohistochemically for the presence of intraepidermal nerve fibres using the antibody for protein gene product 9.5 (PGP 9.5).

There was a significant reduction in IENFD at the lower leg biopsy site of the ‘Pain’ group compared to the ‘No-Pain’ group (6 ± 2 fibres/mm vs 13 ± 4 fibres/mm, $p < 0.05$). However, there was no difference in IENFD between the groups at the thigh biopsy site (16 ± 1 Fibres/mm vs 16 ± 3). There was also no significant difference between the IENFD of the ‘No-Pain’ group lower leg biopsies and the IENFD of their thigh biopsies (13 ± 4 Fibres/mm vs 15 ± 2 Fibre/mm).

The IENFD quantification technique was successfully demonstrated in a South African Laboratory. Using this technique, it was found that the presence of pain in individuals with HIV-SN is associated with reduced IENFD at the site of the pain.

Acknowledgements

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I would like to express my sincere gratitude to the National Research Foundation (NRF) for their financial support during this project as well as the Belgian Technical Cooperation for granting me the BTC student fellowship.

My eternal gratitude goes to my father, for all his assistance and support; to my mother, for her constant good advice and encouragement; to my wife, for her tireless patience and perseverance during the most trying of times and- last but not least- to my son, for always finding a way to turn my frown upside down.

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LIST OF ABREVIATIONS AND ACCRONYMS

ACTG:	AIDS Clinical Trials Group
AIDS:	Acquired Immunodeficiency Syndrome
ART:	Antiretroviral Therapy
ATN:	Antiretroviral Toxic Neuropathy
CART:	Combination Antiretroviral Therapy
CHBH:	Chris Hani Baragwanath Hospital
CNS:	Central Nervous System
DDx:	Dideoxy Nucleoside Analogue
DNA:	Deoxyribonucleic Acid
DRG:	Dorsal Root Ganglion
DSP:	Distal Sensory Polyneuropathy
EFNS:	European Federation of Neurological Societies
HAART:	Highly Active Antiretroviral Therapy
HERC:	Human Ethics Research Council
HIV:	Human Immunodeficiency Virus
HIV-SN:	Human Immunodeficiency Virus- Sensory Neuropathy
IENFD:	Intraepidermal Nerve Fibre Density
mtDNA:	Mitochondrial Deoxyribonucleic Acid
NCS:	Nerve Conduction Studies
NPS:	Neuropathic Pain Scale
NRTI:	Nucleoside Reverse Transcriptase Inhibitors

PAb:	Primary Antibody
PBS:	Phosphate Buffered Saline
PGP 9.5:	Protein Gene Product 9.5
TBS:	Tris Buffered Saline
WHO:	World Health Organisation

CHAPTER 1 : LITERATURE REVIEW

1.1 Introduction

Prior to the introduction of highly active antiretroviral therapy (HAART), infection with human immunodeficiency virus (HIV) was terminal. The infection progressed from a relatively asymptomatic period, of indefinite duration, to the acquired immune deficiency syndrome (AIDS), which was marked by severe complications associated with severe immune suppression. Once at the advanced AIDS stage of the infection, progression to death was rapid, typically occurring within one or two years thereafter (Gelman 2007). During this time when HAART was not available it was found that pathological changes in the central nervous system (CNS) were very common especially during stages of advanced immunosuppression (Power *et al.* 2009; Heaton *et al.* 2011). Approximately 35% of the autopsies done on individuals with AIDS found that the cause of death was related to underlying CNS pathology (Klatt 1988 cited in Gelman 2007). Although peripheral neuropathy was not a contributor to the high mortality rate, it did produce a painful disability which, in most cases impaired mobility to a large extent. With the introduction of HAART, in 1996 and 1997, the HIV-1 virus was generally transformed from a lethal, progressive disease into a chronic infection that could be adequately managed through medicine which resulted in

prolonged survival (Gelman 2007). However, the burden of nervous system complications of HIV infection did not decrease appreciably, or may have actually increased (Power *et al.* 2009; Heaton *et al.* 2011). Indeed, the prevalence of the major peripheral nervous system complications of HIV-infection, like HIV-SN, increased upon introduction of highly active antiretroviral viral therapy possibly as a result of the toxic effects of the didioxy-nucleoside analogues (DDx) present in HAART (Bacellar *et al.* 1994; Smyth *et al.* 2007). This toxic neuropathy is clinically indistinguishably from the neuropathy that develops in the absence of HAART (Skopelitis *et al.* 2007).

1.2 Incidence and Prevalence of Antiretroviral Toxic Neuropathy

Antiretroviral toxic neuropathy (ATN) is a complication associated with HIV resulting from the use of particular DDx drugs such as zalcitabine, didanosine and stavudine (Ellis *et al.* 2010). The most common of these drugs still in use being stavudine, which, until very recently, was used in first line treatment regimens in developing countries such as South Africa (WHO *et al.* 2010). There is a shortage of accurate data regarding reported prevalence and incidence for ATN as most epidemiological studies do not clearly distinguish between HIV-SN that develops in the treatment naïve patients (HIV-associated distal sensory neuropathy; HIV-DSP) and ATN (Gonzalez-Duarte *et al.* 2008). However, a paper by Sacktor (2002) showed that the overall prevalence of HIV-DSP and ATN

rose in the years after the introduction of HAART while, during that same time, the incidence of HIV-DSP decreased by approximately 50% and the incidence of ATN steadily increased. This was most probably due to the toxic effects caused by the introduction of antiretroviral therapy (ART) coupled with prolonged survival due to the same drugs (Sacktor 2002) (Figure 1.1).

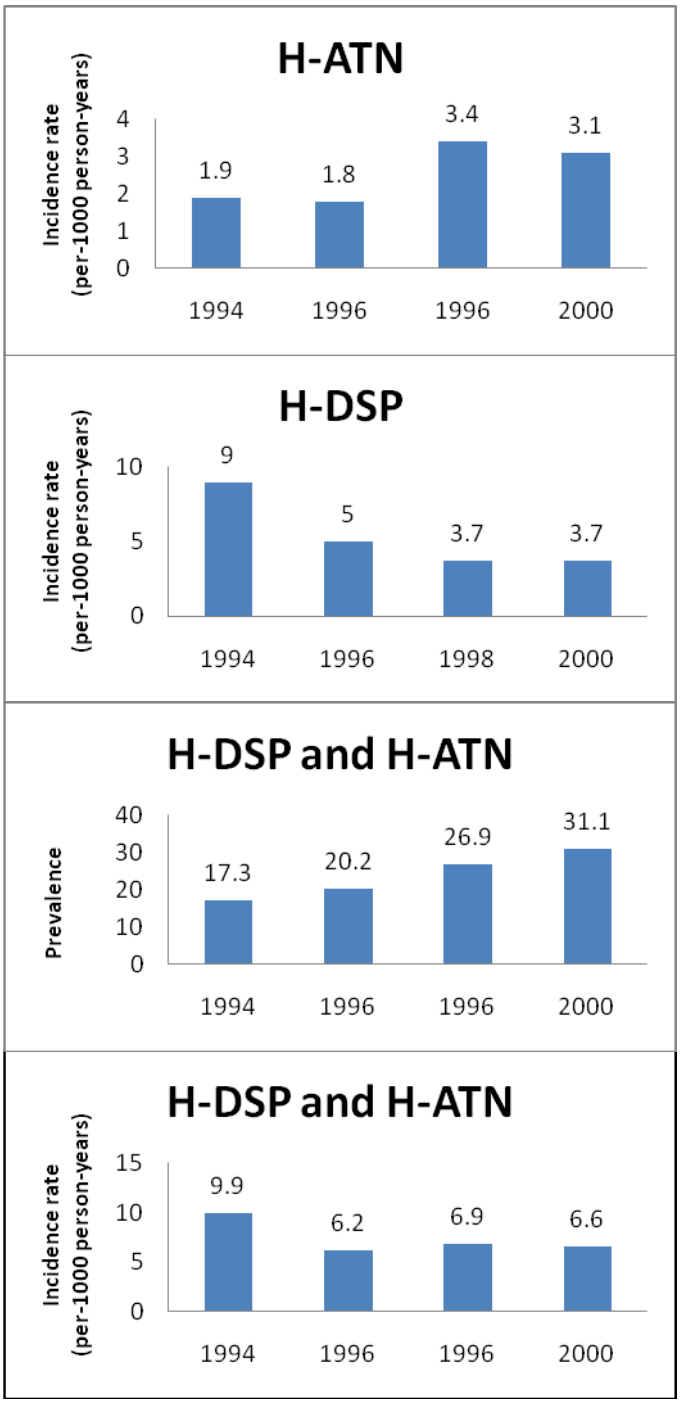


Figure 1.1 Data presented by Sacktor (2002) looking at the incidence and prevalence of HIV-SN before and after the introduction of HAART

The toxic effects of the dideoxynucleoside analogues in HAART have been confirmed in a more recent study where it was shown that after exposure to stavudine (6 months or longer), 18 out of 44 patients, with HIV, who previously had no clinical signs of a neuropathy developed a neuropathy (ATN) (Pillay 2011). In their studies on South African cohorts Wadley *et al.* (2010) found that 57% of their participants had a clinical diagnosis of HIV-SN after being exposed to stavudine while Maritz *et al.* (2010) showed that HIV-SN was significantly related to Stavudine use with 58% of their sample population presenting with a clinical diagnoses of HIV-SN after exposure to stavudine. Maritz *et al.* (2010) further showed that the frequency of symptomatic HIV-SN increased significantly after exposure to stavudine (from 23% to 36%). The high prevalence rates reported in these South African cohorts- the frequency data presented by Maritz *et al.* (2010) reflects prevalence in their study cohort- suggest that HIV-SN is a common problem in HIV patients exposed to stavudine in South Africa and possibly the rest of the developing world (Cherry *et al.* 2010). However, it must be noted that at the time of writing this dissertation, the first line treatment regimen for HIV in South Africa was changed to exclude stavudine in favour of tenofovir, a nucleotide reverse transcriptase inhibitor with no reported clinical neurotoxicity.

1.3 Clinical Features

The clinical features of HIV-DSP and ATN are indistinguishable except for the timing of the onset of the signs and symptoms in relation to the patients exposure to ART containing one or more of the toxic nucleoside reverse transcriptase inhibitors (NRTIs) like stavudine (D4T), zalcitabine (ddC) or didanosine (ddI) (Gonzalez-Duarte *et al.* 2008). Generally a patient who experiences signs and symptoms of the disorder within six months or longer after beginning ART will be diagnosed as having ATN and in some cases symptoms may be reduced after stopping therapy (Petersen *et al.* 1995; Blum *et al.* 1996). Those patients who experience signs and symptoms of the disorder prior to initiating ART and who possess no co-morbid risk for distal sensory polyneuropathy (DSP) (e.g. diabetes mellitus) will be diagnosed as having HIV-DSP. In the latter case the condition may be exacerbated after the initiation of ART or improve (Sacktor *et al.* 2009).

HIV-SN is characterised by symptoms and signs which appear in a typical stocking and glove distribution with the distal leg being the worst and generally the first part of the body affected (Gonzalez-Duarte *et al.* 2008). Pain is the predominant symptom (Smyth *et al.* 2007; Maritz *et al.* 2010; Wadley *et al.* 2011). However, other uncomfortable symptoms like pins and needles sensations and numbness may be present together with the painful sensations or without (Smyth *et al.* 2007; Ellis *et al.* 2010; Maritz *et al.* 2010; Wadley *et al.* 2011) (tale 1) . The symptoms are reportedly more

severe during the night while sleeping and after walking (McArthur *et al.* 2005).

The signs that are typically associated with peripheral sensory neuropathy include reduced vibratory sense, numbness and absent ankle jerk reflexes (Table 1.1). Apart from pain, the most common sign and symptom generally associated with HIV-SN are reduced ankle jerk reflexes and numbness respectively (Tagliati *et al.* 1999). These measures were shown to be strongly related to increased sensory threshold (warming, cooling and vibration sensations as well as reduced intraepidermal nerve fibre density (IENFD) (Cherry *et al.* 2005). The signs mentioned above typically present themselves bilaterally and may be asymptomatic during the onset of the disorder.

Table 1.1 Prevalence of Signs and Symptoms in HIV-SN

	Signs and Symptoms n (%)						
	N	Pain	Pins & Needles	Numbness	↓ Vibration	↓ Ankle Reflexes	↓ Pin Prick
Wadley *et al.* 2011	226 (57)	172 (76)	105 (46)	108 (48)	99 (44)	191 (85)	n/a
Maritz *et al.* 2010	291	136 (47)	109 (38)	90 (31)	196 (67)	251 (86)	247 (85)
Tagliati *et al.* 1999	63	45 (71)	45 (71)		41 (65)	42 (66)	

There are no pathognomonic features of HIV-SN that allow a definite diagnosis, but rather the diagnosis is based on clinical examination often informed by simple, reliable and valid screening tools such as the Aids Clinical Trial Group Brief Peripheral Neuropathy Screen (ACTG BPNS) and the Neuropathic Pain Scale (NPS) (Galer and Jensen 1997; Cherry *et al.* 2005). In order to rule out other forms of neuropathy such as entrapment neuropathies or neuropathies that involve the motor system (muscles of the fool and distal leg), other test such as nerve conduction studies (NCS) and needle electromyography can also be performed. However, for a verified diagnosis of a small fibre neuropathy like ATN, measurement of intraepidermal nerve fibre density (IENFD) can be employed. The reason for NCS not being as good a test as IENFD quantification for the diagnosis of peripheral neuropathy is that NCS typically assesses large myelinated nerve fibres and would require invasive procedures to accurately assess intraepidermal nerve fibres. On the other hand, IENFD quantification is a safe technique that is able to effectively assess intraepidermal nerve fibres, thus assisting in confirming a neuropathy diagnosis (Lauria and Lombardi 2007). Gonzalez–Duarte *et al.* (2008) described this technique as being '…valuable for the diagnosis of DSP, for monitoring its progression over time and for predicting the likelihood of the condition developing in asymptomatic patients'.

I shall describe the use of IENFD in more detail in Section 1.7 of this dissertation.

1.4 Risk Factors

Included among the risk factors for the development of HIV-SN are increased age, nutritional deficiencies and alcohol exposure (McArthur *et al.*, 2005). A study by Childs *et al.* (1999) looked at the relationship between plasma viral load and CD4 T-cell counts and the hazard for developing neuropathy. While they did not separate data gathered from patients with ATN and those with HIV-DSP, their initial findings suggest that patients with high plasma viral loads and low CD4 T-cell counts were at highest risk for developing SN. However, after adjusting for the presence of AIDS-defining illnesses and antiretroviral drug use, this association was lost. Wadley *et al.* (2011) have shown that age and height are the only factors that may be able to identify possible progression into symptomatic ATN after exposure to Stavudine in a South African cohort. This finding is supported by a previous study which showed that patients who receive HAART were at higher risk of developing symptomatic ATN due to prolonged survival (increased age) (Schifitto *et al.* 2005). Cherry *et al.* (2006, 2009) and Smyth *et al.* (2007) also found that increased age as well as previous exposure to ddI or d4T was associated with the development of symptomatic HIV-SN. Also in a South African cohort, Maritz *et al.* (2010) found that increased age was a risk factor, but unique to their study, they also found that higher mean systolic blood pressure, higher waist to hip ratio and elevated serum triglyceride levels were all significantly associated with HIV-SN. Ellis *et al.* (2010) found that increased age, lower CD4 count, current combination antiretroviral therapy

(CART) use as well as previous use of certain toxic NRTIs were predictors of HIV-SN. In the same way, a number of studies have looked at risk factors associated with HIV-SN, these findings have been summarised in table 1.2.

Table 1.2 Risk Factors Associated with HIV-SN

Risk Factor	Reference
↑ Age	Childs *et al.* 1999; Tagliati *et al.* 1999; Morgello *et al.* 2004; Lichtenstein *et al.* 2005; McArthur *et al.* 2005; Schifitto *et al.* 2005; Cherry *et al.* 2006; Pettersen *et al.* 2006; Smyth *et al.* 2007; Cherry *et al.* 2009; Ellis *et al.* 2010; Maritz *et al.* 2010; Wadley *et al.* 2011
↑ Height	Cherry *et al.* 2009; Wadley *et al.* 2011
↑ Viral Load	Childs *et al.* 1999; Lichtenstein *et al.* 2005; Pettersen *et al.* 2006
↓ CD4 Count	Tagliati *et al.* 1999; Schifitto *et al.* 2002; Pettersen *et al.* 2006; Ellis *et al.* 2010
Toxic NRTI Use (d4T, ddl or ddC)	Lichtenstein *et al.* 2005; Cherry *et al.* 2006; Pettersen *et al.* 2006; Smyth *et al.* 2007; Cherry *et al.* 2009; Wadley *et al.* 2011
Alcohol Consumption	McArthur *et al.* 2005; Pettersen *et al.* 2006
Nutritional deficiencies	McArthur *et al.* 2005

1.5 Pathology of HIV-SN

It is well established that some NRTI's used in HAART are neurotoxic and therefore contribute to the development of peripheral neuropathy (Riddler *et al.* 1995; Cherry *et al.* 2003; Gelman 2007; Cherry *et al.* 2009). However most neuropathological characterizations of HIV-SN are from patient cohorts recruited in the pre-HAART era, therefore the pathology is more representative of what happens in DSP rather than ATN. Moreover, animal studies indicate that neuropathy in models of viral and antiretroviral neuropathy produce similar neuropathology (Wallace *et al.* 2007). In general, HIV-SN is characterised by distal axonal degeneration, affecting mostly the small unmyelinated C-fibres (Gonzalez-Duarte *et al.* 2008). In more severe cases axonal degeneration of myelinated fibres may also occur (Cherry *et al.* 2003; McArthur *et al.* 2005; Gonzalez-Duarte *et al.* 2008). There is very little evidence to support axonal regeneration (Hahn *et al.* 2007). HIV-SN fits the profile of a dying back neuropathy well, with epidermal nerve fibre density loss evident at the affected site (predominantly the distal leg) as well as degeneration of the rostral gracile tract which is the CNS equivalent of the peripheral degeneration (Rance *et al.* 1988). The pathophysiology of the neuropathy, whether drug or virus-induced, remains obscure, but is thought to involve mitochondrial dysfunction and infiltration of activated immune cells into the dorsal root ganglia and nerve trunks (for review: Kamerman *et al*., 2012).

The cause of the mitochondrial dysfunction and dysimmune response in DSP and ATN may be different, but they share a common endpoint, that being intraepidermal nerve fibre die back. Since the introduction of the panaxonal marker PGP 9.5, numerous studies have been carried out showing that patients with DSP and ATN have a reduction in the number of intraepidermal nerve fibres, especially at the site of the distal leg (McArthur *et al.* 1998; Polydefkis *et al.* 2002; Smith *et al.* 2005; Lauria *et al.* 2007; Sommer and Lauria 2007; Zhou *et al.* 2007).

1.6 Pathogenesis of HIV-SN

The pathogenesis of sensory neuropathy (SN) is not completely understood. While DSP and ATN are clinically similar, the pathogenesis of the disorders are distinct (Gonzalez-Duarte *et al.* 2008). Brannagan *et al.* (1997) showed, using polymerase chain reaction (PCR) amplification, *in situ* hybridisation and reverse transcription (RT) *in situ* PCR, that HIV DNA was present in dorsal root ganglion (DRG) neurons in all the participants who had a peripheral neuropathy, but was not present in those participants who did not possess clinical manifestations of DSP. On the other hand, a study by An *et al.* (1999) showed that HIV-1 infection occurs predominantly in the mononuclear cells of the nervous system (microglia and macrophages). They also found that neuronal support cells (astrocytes and oligodendrocytes) were HIV-1 DNA positive suggesting another reservoir of the HIV-1 virus in the peripheral nervous system. However, they did not find any convincing evidence suggesting direct

infection of neurons by the HIV-1 virus. The more accepted view falls in favour of infection of non-neuronal cells in the peripheral nervous system by the HIV-1 virus rather than a direct infection of neurons (An *et al.* 1999).

As stated previously, the most common pathophysiological feature associated with ATN is mitochondrial dysfunction (Chen and Cheng 1989; Dalakas *et al.* 2001; Lehmann *et al.* 2011). Many studies have shown that ARV toxicity occurs as a result of mitochondrial DNA (mtDNA) depletion followed by a disturbance in mitochondrial oxidative processes (Chen *et al.* 1991; Brinkman *et al.* 1998). Indeed, a study by Brew *et al.* (2001) showed that plasma lactate concentrations, a marker of mitochondrial dysfunction, significantly differentiated between stavudine induce ATN and HIV-DSP where 90% of the participants with stavudine induced toxic neuropathy had a plasma lactate concentration above normal compared to just 10% of the participants with HIV-DSP (Brew *et al.* 2003). And, a study by Famularo *et al.* (1997) showed that patients on treatment with the NRTIs didanosine, zalcitabine or stavudine had serum levels of acetyl-L-carnitine, a regulator of metabolic function in peripheral nerves, which was less than the lowest levels measured in HIV-positive patients who did not present with a neuropathy. They also showed that patients who did not have any clinical evidence of a neuropathy had serum levels of acetyl-L-carnitine which were comparable to levels found in healthy individuals. These findings are supported by another study by Valcour *et al.* (2009), who showed that most of the subjective measures associated with HIV-SN

improved during the course of the treatment with acetyl-L-carnitine. They also reported a modest improvement in vibration sensation in their study participants. However, since these findings were secondary to their actual objectives and since they did not incorporate a placebo group into the study, the results should be interpreted with caution. On the other hand, it must be noted that, more recent research has cast some doubt on these findings. In their study Cherry *et al.* (2006) showed that there was no association between plasma lactate and the development of ATN ($p=0.327$). Also, in their randomized multicentre placebo-controlled study, Youle *et al.* (2007) showed that, while total symptom score improvement was greater among patients treated with Acetyle-L-Carnitine (ALC) compare to the placebo group, this difference was not statistically significant. More research is thus needed to confirm the effects of lactate on the development of ATN as well as the efficacy of ALC on reducing the symptoms associated with this disorder.

While many hypotheses have been brought forward to explain the possible mechanisms underlying mitochondrial dysfunction in ATN, the DNA polymerase-γ hypothesis described by Lewis and Dalakas (1995) seems to be the most accepted. The γ-isoform is a necessary component of mtDNA replication (Lewis and Dalakas 1995). However, the toxic NRTIs (d4T, ddC, ddI) found in ART inhibit the γ- isoform hence disrupting mitochondrial protein synthesis eventually disrupting oxidative phosphorylation- which is needed to provide energy in neurons- (Lewis *et*

al. 1995; Youle 2007), and increase mitochondrial membrane permeability, which can initiate neuronal apoptosis or peripheral axonal die back due to lack of sufficient energy production (Youle 2007).

Thus far I have briefly reviewed the epidemiology and pathogenesis of HIV-SN, and now I will look more closely at the diagnostic technique which has been employed in Europe and the United States, for some time now, to help confirm the diagnosis of a peripheral neuropathy. Our Lab is the first to employ this technique in South Africa.

1.7 Skin Biopsy and Quantification of Intra-epidermal Nerve Fibre Density

The basic neurologic screening tools such as the ACTG BPNS are important tools for the rapid assessment of HIV-positive patients, to determine the presence of HIV-SN, in the clinic and for epidemiological studies, but confirmation of the diagnosis requires additional supportive data (Cherry *et al.* 2005). Indeed, diagnostic criteria for peripheral neuropathy require the detection of a nerve lesion to confirm the diagnosis of a neuropathy (Treede *et al.* 2008). The skinpunch biopsy and intraepidermal nerve fibre density quantification technique, which will be discussed shortly, is a quantitative measure that has been shown to be useful in confirming a neuropathy diagnosis especially in atypical cases which are difficult to diagnose solely on clinically presentation and history. In the ensuing discussion it will be made clear how valuable this tool is.

1.7.1 The Punch Skin Biopsy Technique

As a consequence of the types of diagnostic tests previously used to verify peripheral neuropathy, it was thought that neuropathies affected large nerve fibres only as the tests used to confirm the diagnosis, such as nerve conduction studies, typically assessed large nerve fibres (Smith *et al.* 2005; Lauria *et al.* 2007). However, with the introduction of the punch skin biopsy technique just over a decade ago, it has been shown that several types of peripheral neuropathy may preferentially affect unmyelinated small diameter C-fibres and the finely myelinated Aδ fibres (Lauria *et al.* 2005; Lauria *et al.* 2007). As such, the technique can confirm nerve lesions not detected by standard nerve conduction studies. The technique is inexpensive as well as painless, if carried out correctly, and has been given an A-recommendation by the European Federation of Neurological Societies (EFNS) for its reliability and safety (Lauria *et al.* 2007; Lauria *et al.* 2010). The punch biopsy is well suited for clinical use as it requires no specialist training and can be done in an ordinary examination room. The procedure does not require much time to administer; the patient is conscious throughout the procedure and is able to function normally immediately after the biopsy is taken. Histopathology results from multiple biopsies can be obtained within a few days of taking the biopsies.

Unlike other diagnostic techniques such as the sural nerve (the major nerve bundle that supplies nerve branches to the back of the leg and forms the lateral dorsal cutaneous nerve) biopsy technique, the punch skin

biopsy technique is useful in that it allows for repeated sampling around a specific site (in the vicinity of a specific nerve innervation area) (Lauria *et al.* 2007). This is valuable as it provides investigators with the opportunity of assessing, over a period of time, degeneration or regeneration of epidermal nerve fibres as a result of changes in management interventions (Lauria *et al.* 2007). Another useful feature of the punch skin biopsy technique is that it allows the investigator to retrieve information regarding dermal and sweat gland innervation as well, this could provide the clinician with more accurate information of the disorder underlying epidermal nerve fibre die back (i.e. whether there are underlying autonomic disorders also present with sensory neuropathy) (Lauria *et al.* 2007). Due to the advantages of the punch skin biopsy technique and the limitations for clinical use found in other diagnostic tools (Table 1.3) , skin biopsy has now become the established means of verifying the diagnosis of HIV-SN (Smith *et al.* 2005).

Table 1.3 Diagnostic tests used to evaluate peripheral neuropathy: Advantages and Disadvantages

Test	Advantages	Disadvantages	Reference
Nerve Conduction Studies	• Effective in diagnosing subclinical neurologic disorders such as entrapment neuropathies and neuropathies that involve the motor system	• Evaluates large myelinated fibres which are typically normal in small fibre neuropathy	• Martin *et al.* 2003
Quantitative Sensory Testing	• Non invasive • Shown to be effective in assessing small fibre impairment	• The results of the test place a lot of weight on the participant's input, thus the test cannot be used as an exclusive criteria for diagnosing neuropathy	• Lauria *et al.* 2005 • Lauria *et al.* 2007 • Gonzalez-Duarte *et al.* 2008
Sural Nerve Biopsy	• Invasive technique • Permanent sensory loss may result • Multiple biopsies cannot be taken	• Can give indications as to the severity of length dependant neuropathies such as length dependant diabetic neuropathy and alcoholic neuropathy	

1.7.2 Reproducibility of Intraepidermal Nerve Fibre Density as a Measure in Clinical Trials

With the introduction of antibodies against the panaxonal marker protein gene product 9.5 (PGP9.5), it has become possible for peripheral nerve fibres to be analysed using immunohistochemical staining (Ridley 1968; Kennedy and Wendelschafer-Crabb 1993; McArthur *et al.* 1998). In this way, intraepidermal nerve fibres may be evaluated either qualitatively or quantitatively to determine disease progression or abatement (Smith *et al.* 2005; Lauria *et al.* 2010). To date, a number of studies have been conducted to show the efficacy of using IENFD as an endpoint measure for clinical trials looking at disorders such as diabetic neuropathy and HIV-SN. One such study by Smith *et. al.* (2004) assessed the reliability of using IENFD as an endpoint measure in clinical trials by looking at the inter and intra-observer variability of counting intraepidermal nerve fibres as well as the variability of IENFD assessment from skin sections that were processed at two different laboratories. Their results showed that the intraclass correlation coefficient for each of the measures was 98%, signifying a high level of reproducibility. They also found that there was no significant difference between measurements of IENFD when the sections were processed at two different laboratories and counted by one observer. In the same study they also investigated the number of sections per biopsy that needed to be counted in order to attain the highest level of reproducibility. They found that four sections per biopsy produced the best results, with intra-observer variability being 6.4% ± 7.9. However, the

EFNS recommendation suggests that at least three sections should be counted for accurate results to be obtained (Lauria *et al.* 2010).

1.7.3 IENFD Normative Reference Range

In order for IENFD to be used as an endpoint measure in clinical trials, there need to be a standard reference range of epidermal nerve fibre density obtained from healthy individuals. This reference range should be reliable and should have a high degree of specificity and efficacy. Three studies in particular have looked at the IENFD in normal healthy subjects (McArthur *et al.* 1998; Umapathi *et al.* 2006; Bakkers *et al.* 2009), of which two were used to determine the IENFD range recommended by the EFNS guidelines on IENFD (Lauria *et al.* 2010).

McArthur *et al.* (1998) set out to determine a normative reference range in humans and to establish diagnostic effectiveness for SN. They found that the IENFD in normal controls was 21 ± 10 fibres /mm at the thigh and 14 ± 7 fibres/mm at the distal leg with the fifth percentiles being 5 fibres/mm and 4 fibres/mm respectively. They also showed that the fifth percentile thigh to distal leg ratio was 1:0.72 fibres/mm and noted that the there was no significant effect of age on IENFD except in the youngest age decile (10- 19 years) where IENFD was significantly higher (20 ± 7 at the distal leg and 32 ± 13 at the thigh) (Table 1.4).

Table 1.4 Intraepidermal Nerve Fibre Density by Age Decile

Skin Biopsy Site	No. of fibres per millimetre by age, y						
	10 - 19 (n = 8)	20 – 29 (n = 18)	30 – 39 (n = 20)	40 – 49 (n = 19)	50 – 59 (n = 12)	60 – 69 (n = 9)	70 – 79 (n = 12)
Distal part of leg							
Mean ± SD	20 ± 7	11 ± 5	12 ± 7	15 ± 6	14 ± 6	13 ± 7	15 ± 8
Range	13 - 37	4 - 23	4 – 31	3 - 25	7 - 25	1 - 20	2 - 32
Median	19	10	11	15	13	16	12
80th percentile interval	14 - 33	6 - 21	4 – 22	9 - 23	8 - 24	3 - 20	6 - 27
Thigh							
Mean ± SD	32 ± 13	16 ± 6	18 ± 9	22 ± 7	21 ± 14	24 ± 8	23 ± 8
Range	15 - 51	3 - 27	4 – 41	3 - 33	10 - 58	16 - 38	11 - 40
Median	27	16	17	24	17	20	23
80th percentile interval	17 - 49	8 - 24	6 – 30	15 - 32	10 - 46	16 - 38	12 - 36
Thigh/distal part of leg							
Mean ± SD	2 ± 1	2 ± 1	2 ± 1	2 ± 1	2 ± 1	5 ± 10	2 ± 2
Range	1 - 3	0 - 3	0 – 5	1 – 3	1 - 3	1 - 32	1 - 9
Median	1	2	2	1	1	2	2
80th percentile interval	1 - 2	1 - 2	1 – 4	1 – 3	1 - 3	1 - 20	1 - 5
P (Mann- Whitney test), distal part of leg vs thigh	0.06	0.02	0.03	<0.001	0.13	0.01	0.02

Adapted from (McArthur *et al.* 1998)

Inter- and intra- observer reliability was assessed using blinded comparisons of IENFD by one observer counting twice as well as between three observers. The correlation coefficients for the different counts ranged from 0.74 to 0.86 ($p<0.001$), while inter-observer reliability correlation coefficients ranged from 0.86 to 0.94 ($p<0.001$), thus signifying a high level of reliability.

As stated previously, one advantage of the punch skin biopsy and IENFD quantification technique is that it allows for multiple testing around a specific nerve innervation territory. Sections from adjacent biopsies taken from a specific site (distal leg or thigh) were compared to assess intrasite variation between 2 biopsies processed at the same time. The investigators obtained correlation coefficients of $r=0.87$ and $r=0.86$ for the distal leg and thigh respectively signifying a high level of association between two biopsies extracted from the same site. The investigators also achieved the highest specificity, efficiency and positive and negative predictive values by using the fifth percentile cut off at the distal leg (table 1.5).

Table 1.5 Performance characteristics of intraepidermal nerve fibre linear density measure

	Sensitivity%		Specificity%		Efficacy %		Positive Predictive Value, %		Negative Predictive Value, %	
Percentile	Thigh	Distal leg	Thigh	Distal leg	Thigh	Distal leg	Thigh	Distal leg	Thigh	Distal leg
10th	45	60	88	90	82	86	45	55	89	75
5th	25	45	96	97	84	88	56	92	86	90

Adapted from (McArthur *et al.* 1998)

In the largest normative study to date, Bakkers *et al.* (2009) assessed normative values for IENFD to establish the reliability and validity of IENFD in patients with sarcoidosis. With a sample population of 188 healthy controls, they found a significant age dependant decrease of IENFD with lower IENFD at the distal leg found in men compared with women (table 1.6). This is in contrast with what McArthur *et al.* (1998) showed, and could be due to them using age deciles instead of age of individual subjects when comparing with IENFD (Umapathi *et al.* 2006).

Table 1.6 Intraepidermal nerve fibre density normative values for clinical use

Age, y	Women (n = 97)		Men (n = 91)	
	5th quantile IENFG values per age span	Median IENFD values per age span	5th quantile IENFG values per age span	Median IENFD values per age span
20-29	7	11	5	9
30-39	6	11	5	8
40-49	5	10	4	8
50-59	4	9	3	7
60-69	3	8	2	6
≥70	3	7	2	6

Adapted from (Bakkers *et al.* 2009)

1.7.4 IENFD and HIV-SN: Disease Progression

In their study, Zhou *et al.* (2007) demonstrated the relationship between IENFD and the characteristics of HIV-SN. They looked at 100 HIV-positive (HIV+) individuals who either had symptomatic neuropathy, asymptomatic neuropathy, or who were neuropathy-free and grouped them according to the severity of the epidermal nerve fibre die back found at the distal leg and proximal thigh. They found that there was no relationship between age, CD4 T-cell count or log HIV-1 RNA and IENFD. Also, of the participants whose epidermal nerve fibre density (ENFD) was normal, it was found that 31% had abnormal morphological changes in the epidermal nerves of the distal leg, which indicates that subclinical neuropathology also occurs in the HIV-positive individuals without neuropathy even though IENFD remains within the normal range. Finally, they showed that participants with symptomatic neuropathy had a lower IENFD at the lower leg compared to the neuropathy free group ($p = 0.01$). However, this was not the case with regard to the asymptomatic group ($p = 0.20$). Thus, indicating that there was no consistent pattern between IENFD loss and neuropathy symptoms. In an earlier study, Polydefkis *et al.* (2002) looked at the relationship between IENFD in HIV-SN and measurements of neuropathy severity and progression of HIV disease. They found that there was a significant difference between IENFD and baseline neuropathic pain when pain was assessed using a doctor-assessed and patient-assessed global classification of pain. They also observed a statistical trend between viral load and IENFD where patients

with lower viral loads tended to have slightly higher IENFD than patients who had detectable levels of HIV RNA ($0.05 < p < 0.01$). With regard to CD4 T-cell counts and IENFD, they found that increased CD4 T-cell counts were strongly related to increased IENFD at the distal leg ($r_s = 0.29$, $p = 0.030$). This is in conflict with the results obtained by Zhou *et al.* (2007). One possible reason for this might be that Polydefkis *et al.* (2002) used a sample population with a CD4 T-cell count range of 25-1156 cell/ml, while Zhou *et al.* (2007) used a sample population with a CD4 T-cell count range no more that 300 cell/ml. Thus, the tighter CD4 T-cell count range of patients in the Zhou cohort might have reduced variation in the CD4 T-cell counts compared to the Polydefkis cohort, reducing the ability to detect differences.

In their study, Skopelitis *et al.* (2007) found that ENFD was lower in both the asymptomatic and the symptomatic HIV-SN participants compared to controls. Further, Skopelitis *et al.* (2007) also found that one third of their cohort had a silent neuropathy (no signs or symptoms) that was not detected by nerve conduction tests or vibratory threshold tests and could only be detected by obtaining IENFD measurement. This supports the notion that IENFD quantification has become an established means of confirming a neuropathy diagnosis **especially in atypical cases which are difficult to diagnose solely on clinically presentation and history**. This IENFD quantification technique has been employed for the first time as a therapeutic outcome measure by McArthur *et al.* (2000). They

reported satisfactory reproducibility of the technique between baseline testing and at 18 weeks after treatment, where baseline epidermal nerve fibre densities were closely correlated to densities after 18 weeks. Although, they did not observe any significant treatment effects after 18 weeks, their study demonstrated the important use of IENFD quantification as an end point measure in clinical trials.

In the same context, Cherry *et al.* (2005) used the IENFD quantification technique to validate a peripheral neuropathy screening tool. They found that the technique was most well suited to be used in a clinical setting compared to other techniques like sensory threshold testing due to its cost effectiveness and relative simplicity. From their findings it can also be seen that **IENFD quantification is a useful test, with good specificity, that can differentiate HIV-positive patients with and without peripheral neuropathy.**

1.7.5 Final Note

HIV is very common in South Africa with approximately 5.6 million people living with HIV/AIDS (UNAIDS 2010). The prevalence of HIV-SN in South Africa is between 49 and 57% with the prevalence of symptomatic (painful) neuropathy ranging between 47 and 76% of those with HIV-SN (Maritz *et al.* 2010; Wadley *et al.* 2011). Many patients with HIV-SN go undiagnosed or misdiagnosed due to the lack of appropriated tools in the clinical setting. We have just described how useful the skin punch biopsy and

intraepidermal nerve fibre density quantification technique is in confirming a HIV-SN diagnosis especially in atypical cases which are difficult to diagnose solely on clinically presentation and history. It is therefore necessary that this tool be evaluated in a South African population in order to evaluate its efficacy and practicality in the South African clinical setting. While IENFD staining and quantification has been around for quite some time now, to my knowledge, the technique is not available in South Africa. It is therefore necessary for the procedure to be set up and tested for use in order to assess HIV patients for small fibre neuropathies such as ATN, which at times is very difficult to confirm using standard clinical evaluation.

On a broader note, the relationship between HIV-SN and epidermal nerve fibre die back has received a lot of attention since the introduction of the PGP9.5 antibody. While most studies only looked at the relationship between epidermal nerve fibre density and the assessment of HIV-SN, some studies showed a relationship between symptomatic and asymptomatic neuropathy with regard to their peripheral nerve fibre density severity (Zhou *et al.* 2007). However, no studies to date have looked at the relationship between IENFD and co-localised with pain at the distal leg in patients with antiretroviral toxic neuropathy. This relationship is a very important one as it would allow for further investigation into the painful symptoms of HIV-SN, which are resistant to treatment (Phillips *et al.* 2010).

1.8 Objectives

1.8.1 Primary objective

To establish the IENFD staining technique in a South African Laboratory.

1.8.2 Secondary objective

To undertake a pilot study, using the newly established technique, to determine whether IENFD differs between individuals with HIV-SN that experience pain at the site of the biopsy compared those who do not have pain at the site of the biopsy.

CHAPTER 2 : Materials and Methods

2.1 Ethical clearance

The study was approved by the University of the Witwatersrand Human Research Ethics Committee (HERC protocol number M090670).

2.2 Participant recruitment

HIV-infected individuals from the Green House Pharmacy at Chris-Hani Baragwanath Hospital (CHBH), Johannesburg, South Africa were screened for peripheral neuropathy. Informed consent and explanations of the screening procedure were done in the participant's mother tongue via a translator. Thirty-five individuals consulted and were screened. Fifteen of these individuals were eligible for inclusion in the study. Participants were included only if they were on ARV therapy for a minimum of six months, reported experiencing neuropathic symptoms after the initiation of HAART, had a CD4 T-cell count greater than 350cells/mm^3, and showed bilateral signs and symptoms of a peripheral neuropathy. Participants with diabetes mellitus or who had any skin lesions on their lower legs (e.g., Kaposi's sarcomas) were excluded from the study. Via a translator, all the participants gave written, informed consent to participate.

2.3 Peripheral neuropathy assessment

Participants were assessed for peripheral sensory neuropathy using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen (ACTG-BPNS). This screening tool is very practical, especially in the clinical setting where our participant recruitment took place, as it is simple and quick to administer, and does not require extensive training or specialised equipment (Cherry *et al.* 2005). The presence and severity of the symptoms were assessed by self-report. Participants were asked to identify the presence or absence, and intensity, of the following three symptoms: Pain, pins and needles, and numbness. Symptom intensity was rated on an 11-point numerical pain rating scale anchored at 0 (currently no pain) and 10 (severe pain). Patients were scored 11 if they had never experienced the symptom (always been normal), and 00 of they had previously had the symptom, but they didn't currently have the symptom. The participant was also asked to indicate the location of the symptoms experienced, bilaterally. The signs assessed were ankle jerk reflexes and vibration sense in the great toe. Vibration sense was assessed using a 128 Hz tuning fork, which was placed on the participant's distal interphalangeal joint of the large toe, and the time taken for the participant to stop feeling the vibration stimulus was measured. Perception times of 10 seconds or less were regarded as a sign of reduced function (Cherry *et al.* 2005). The ankle jerk reflex (deep tendon reflex) was assessed using a reflex hammer struck against the Achilles

tendon. If a reflex could not be elicited, the participants were asked to clench their fists to reinforce the reflex. If still no reflex could be elicited, the reflex was recorded as absent. Symptoms and signs were assessed bilaterally with a primary diagnosis of HIV-SN being made if the participant possessed at least one sign and one symptom bilaterally.

After the assessment, participants were placed into either the “No Pain” group (n=9) or the “Pain” group (n=6). The No Pain group consisted of participants who showed signs (absent ankle jerk reflex, reduced vibration sense) and at least one symptom (numbness or pins and needles) of the neuropathy, but did not report experiencing pain at the skin biopsy site of the ankle or thigh. Four participants who experienced pain in their feet but not at the site of the ankle, where skin biopsies were taken from, were also included in the No Pain group. The Pain group consisted of participants who showed signs of a peripheral neuropathy and at least one symptom, which had to include pain. This pain had to extend proximally to the site of the ankle biopsy, but not past the knee.

2.4 Skin punch biopsy

Under sterile conditions, six 3mm skin biopsies were taken using the punch skin biopsy method described by the European Federation of Neurological Societies (Lauria *et al.* 2010). Three biopsies were taken from the distal calf (about 10cm above the lateral malleolus) and three from the upper thigh. The thigh biopsies were regarded as control biopsies

as this site is only affected very late in the progression of ATN (Wulff *et al.* 2000). The area where the biopsy was taken from was cleaned with alcohol, and then anaesthetised with the local anaesthetic lignocaine (Fresenius 2%) without adrenalin. The anaesthetic was administered subdermaly using a 30G needle. Enough anaesthetic was administered for a bleb to form just under the skin (subdermally) which was greater than 3mm in diameter. The anaesthetic was left to work for approximately 5 minutes. Thereafter a syringe needle was used to test anaesthesia of the area, by lightly pricking the bleb on its periphery, while asking the patient if any pain or sharp sensations were felt. On confirmation that no pain was felt a 3mm skin punch biopsy tool (Paramount Surgimed: India) was used to biopsy the anaesthetised skin. The doctor taking the biopsy placed the punch in the centre of the bleb and applied a downward pressure while turning the punch clockwise and counter-clockwise until approximately only one third of the punch blade was still visible. Thereafter, the punch was removed and using sharp-nose forceps the biopsied skin was lifted from its base and detached using a rounded scalpel blade. In this way three biopsies were removed in a triangular formation per site (ankle and thigh). Using sterile gauze swabs, pressure was applied to the area from where the biopsies were removed until the bleeding had stopped. The biopsy sites were then dressed with waterproof adhesive bandage (Kendon Medical, South Africa) placed over some sterile gauze swabs, and the participant was given detailed information regarding care for the

wound as well as the contact details of the investigator should they have queries about wound care and wound healing.

2.5 Fixation and Cryoprotection

The biopsies that were removed were stored in a Paraformaldehyde/Lysine/Periodate (PLP) fixative (Appendix 1B) for a minimum of 12 hours but not longer than 24 hours. Thereafter, the biopsies were placed in glycerol based cryoprotectant overnight, and then immersed in 150µl of cryoprotectant and stored at -70ºC until the immunohistochemistry was performed.

2.6 Sectioning

We used a Microm HM 450 (freezing) sliding microtome (Thermo Scientific, South Africa) to section the biopsies (Figure 2.1). The stage of the microtome was frozen using crushed dry ice. Chunks of dry ice were placed in the area surrounding the stage to keep the stage and the biopsy frozen throughout the sectioning. A platform of frozen sucrose solution, approximately 5mm high, was made on the stage using a dropper. Thereafter, an additional drop or two of sucrose solution was place on top of the frozen platform, and the biopsy sample placed in this drop and correctly orientated before it froze. The biopsy and sucrose were allowed to freeze (Figure 2.2). Some crushed dry ice was sprinkled on the biopsy

to ensure that the biopsy and mounting fluid were frozen. The microtome was set to section at 100 microns. Approximately 5 sections were made at this thickness. These sections were discarded. The microtome was then set to section at 50 microns and four sections were made. The sections were placed in 96 well filter plates (Millipore, Ireland) filled with glycol based antifreeze (Appendix 1B). Twelve additional sections per biopsy were collected in a second plate. This second plate was stored at -70°C and used as a backup in the event that immunostaining failed on the initial sections.



Figure 2.1 Freezing sliding microtome. Fibre optic lights were used to avoid melting the sucrose stage and dry ice during sectioning



Figure 2.2 Frozen biopsy mounted onto sucrose stage

2.7 Immunochemical staining

Immunocytochemical staining was performed one day after the biopsies were sectioned. Day 1 of the immune-staining of the skin sections included bleaching of melanin from the sections, blocking and treating the sections with Triton X-100 and treating with primary antibody. All the procedures were carried out at room temperature and I ensured that the sections were fully submerged in every solution placed in the wells, not allowing the sections to float or dry out. The wells were emptied using a Millipore Multiscreen Vacuum Manifold (MSVMHTS00, Millipore, South Africa) which was connected to a chemical duty pump (WP6122050, Millipore, South Africa). The sections were bleached by placing them in

0.25% Potassium Permanganate for 15min on the shaker table. After washing the sections with Tris Buffered Saline (TBS) the sections were then placed in 5% oxalic acid for 5min. Thereafter the sections were washed twice for 10min in TBS. The sections were then place in blocking solution (Appendix 1B) for 4 hours on the shaker table at room temperature. After blocking for non specific binding the sections were placed in a solution containing primary antibody (Appendix 1B). The primary antibody (BML-PG9500-0100- Polyclonal PGP9.5- Enzo Life Sciences, USA) was added at a 1:900 – 1:2000 dilution (see Chapter 4 for details). The sections were left in the primary antibody solution overnight at room temperature on the shaker table at 125 rpm.

On day 2 of the immune-staining, the sections were washed in TBS. Thereafter, the sections were placed in the secondary antibody solution (Appendix 1B). The secondary antibody (BA 1000, Biotinylated Goat-Anti-Rabbit, Vector Laboratories, USA) was present in this solution at a dilution of 1:100. The sections were left in the secondary antibody for 1 hour at room temperature on the shaker table at 125rpm. Thereafter, the sections were place in a 30% methanol per phosphate buffered saline (PBS) solution with 1% Hydrogen Peroxide for 30min at room temperature on the shaker table. At the same time, the avidin biotin (Vector ABC Kit, Vector Laboratories, USA) solution was prepared, 30min prior to being used. The solution was made up in PBS at a dilution of 1:100. After washing the sections in PBS they were placed in the previously prepared avidin biotin

solution for 1 hour at room temperature on the shaker table at 125rpm. Finally, the sections were placed in the blue chromogen peroxidase substrate (Vector SG Substrate kit, Vector Laboratories, USA) (Appendix 1B). This reaction is light sensitive and the solution had to be prepared just before it was used. Four drops of the solution were added to each well of the 96-well plate via a 0.45 micron syringe filter. The sections were allowed to incubate in the solution for 6-8 minutes. If time permitted the sections were mounted and cover slipped the same day. Otherwise, the sections were submerged in PBS and kept in the refrigerator overnight.

2.8 Staining Procedure

2.8.1 Establishing appropriate primary antibody concentration

The appropriate primary antibody (PAb) concentration was established using 3 sections from a thigh biopsy of one of the participants. We tested PAb dilution of 1:900, 1:1400 and 1:2000.

2.8.2 Testing for nonspecific binding of the secondary antibody

Using one thigh biopsy section from the same participant, we completed the immunostaining procedure without adding any PAb to rule out non-specific binding of the secondary antibody.

2.8.3 Positive control and validation of IENFD quantification technique

We conducted positive control immunohistochemical runs as well as validated our IENFD counting technique using one thigh and one ankle biopsy from a participant with painful HIV-SN. After these runs, slight amendments were made to the protocol which will be described under section 3.1.

2.9 Mounting and counterstaining

The sections were mounted four at a time on gelatine subbed slides (The sections for each biopsy were mounted on a separate slide). A weighing boat was filled with a solution of PBS and 0.01% Triton X-100. The section to be mounted was submerged in the solution and dragged to the wet/dry interface of the slide using a syringe needle. The section was orientated correctly and the slide was lifted out of the solution. This was done for all 4 sections individually. Before the section dried out, the slide was placed under a dissecting microscope and checked to make sure the tissue was not folded. The slide was then left for 30 minutes to allow the tissue to air dry.

When the tissue was sufficiently dry it was placed under tap water to remove any dried salt residue. The slides were then placed in eosin for 6-8 seconds. Thereafter, the slides were dehydrated in 95% Ethanol (3 x 1 min) and 100% Ethanol (3 x 1 min). They were then placed in Xylene (3 x 1 min). The slides were cover slipped by placing a drop of Xylene on the cover slip with Permount. Each slide was then placed on top of the cover slip and left to set in a slide box until nerve fibre quantification could take place.

2.10 Intraepidermal nerve fibre density (IENFD) quantification

IENFD quantification was carried out using a Nikon LABOPHOT light microscope. Attached to the microscope was a Moticam 2300 3 mega pixel camera. The Moticam Images Advanced 3.2 software was used to acquire data regarding the skin section. Using the software supplied with the camera, 1mm of the skin section was demarcated. Counting was done manually following standardised rules for IENFD quantification (Lauria *et al.* 2010). These rules include 1) fibres that crossed the dermal/epidermal junction were only counted if they could be traced to a root fibre 2) fibres that branched before the dermal/epidermal junction were counted as 2 fibres and 3) fibres that branched after the dermal/epidermal junction were counted as only one fibre. In this way the IENFD of three sections per biopsy were quantified. Epidermal nerve fibres were counted under the light microscope at 40X magnification. The average count of the three

sections was used as the IENFD of that participant. The only information that was available to the investigator during the IENFD quantification was the number of the biopsy from where the skin sections were acquired (ankle or thigh). In this way some observer blindness was achieved.

2.11 Statistical Analysis

Mann-Whitney U-tests were performed to compare the groups. The confidence intervals were set at 95%.

CHAPTER 3 : Staining Technique Results

3.1 Immunohistochemical staining technique validation

Sectioning, immunohistochemical staining and IENFD quantification Techniques were established using a thigh and ankle biopsy from one of the participants. Non-specific binding of the secondary antibody was assessed using a thigh biopsy skin section as describe in chapter 2, section 2.8.2. The blocking step proved effective as the secondary antibody did not bind to any non specific sites in the absence of the primary antibody (Figure 3.1). The dermal/epidermal junction, while present, was not clearly differentiated.

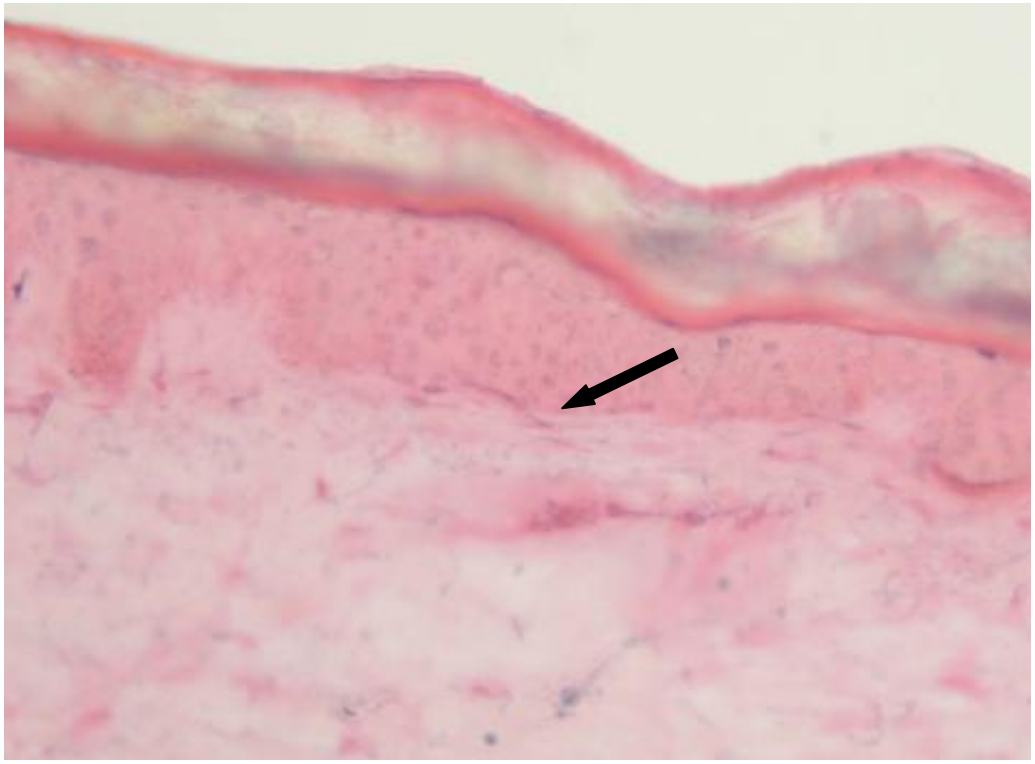


Figure 3.1 50µm skin section showing no prominent signs of non specicifc binding by the secondary antibody. The dermal/epidermal junction which is not clearly differentiated, is depicted by the black arrow. (20X magnification)

I assessed the appropriate primary antibody concentration using 3 thigh skin sections as described in chapter 2, section 2.8.1. The highest titre of primary antibody I used (1:900) produced a very dark stain (Figure 3.2). I opted against using the primary antibody at this concentration in later stains as the lack of contrast between the background and the nerve fibres made quantification difficult. The nerve fibres stained very lightly when using the primary antibody titre of 1:2000 (Figure 3.3), which made quantification difficult because the nerve fibres could not be traced effectively, and this, I thought, would lead to inaccurate quantification. Staining with a primary antibody titre of 1:1400 produced a good compromise; with good nerve fibre staining that was not diminished by background noise. Therefore, I used this dilution in all further staining. (Figure 3.4).

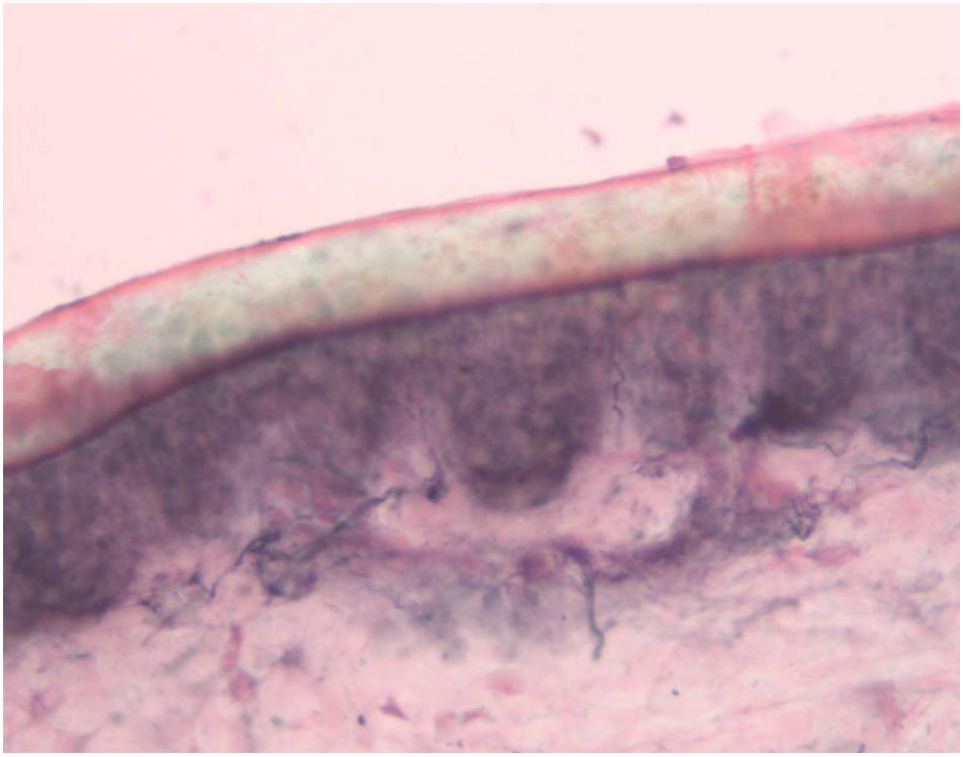


Figure 3.2 50µm skin section showing primary antibody staining at a dilution of 1:900. Nerve fibres are not easily discernable due to the lack of contrast between the background and the nerve fibres. (20X magnification)

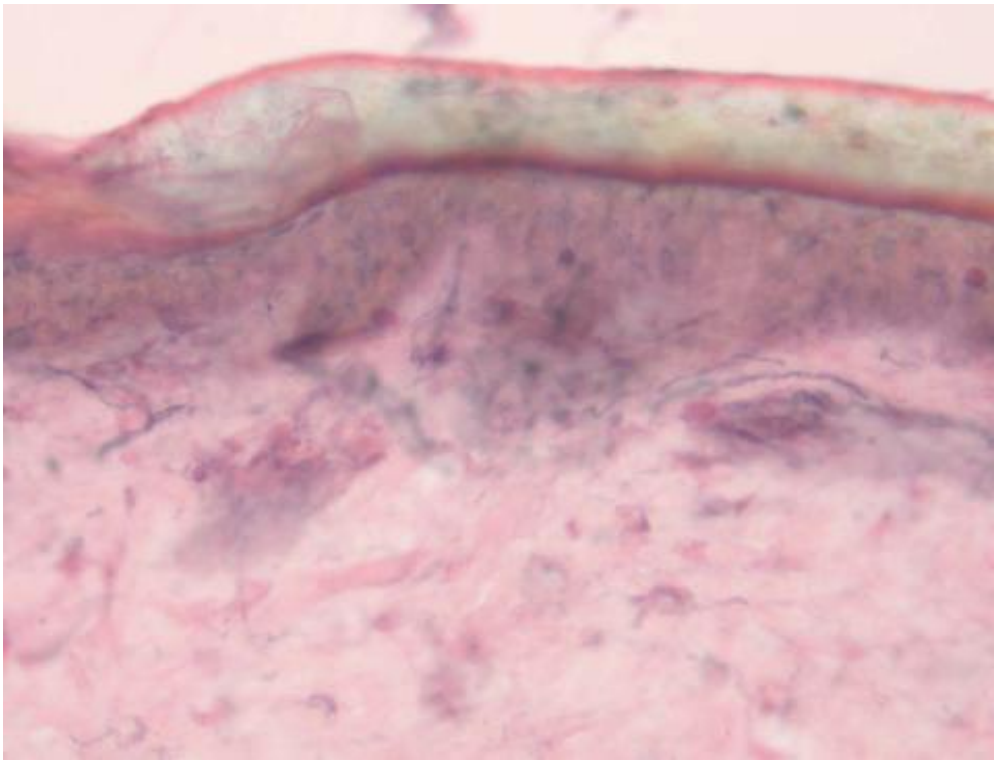


Figure 3.3 50µm skin section showing primary antibody staining at a dilution of 1:2000. At this dilution nerve fibres were stained fairly light which was not adequate for accurate quantification. (20X magnification)

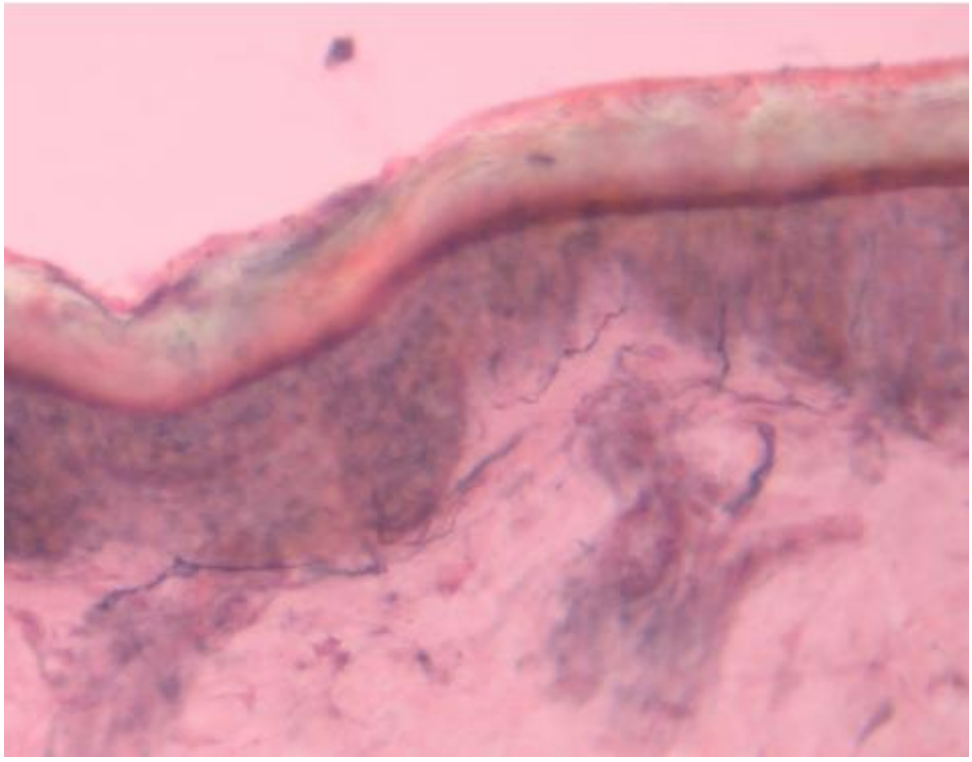


Figure 3.4 50µm skin section showing primary antibody staining at a dilution of 1:1400. The contrast between the background and the nerve fibres is adequate for making quantification possible without much difficulty. (20X magnification)

Positive control tests were done as described in chapter 2, section 2.8.3. We used these test to trouble shoot our immunohistochemical staining technique as well as to validate our IENFD counting technique. Figure 3.5 shows a thigh biopsy skin section, the black arrows depict intraepidermal nerve fibres. There are a number of nerve fibres present in this section as this area (the thigh) is only affected quite late in the progression of peripheral neuropathy. Hence the IENFD appears relatively normal. Figure 3.6 shows an ankle biopsy skin section of a participant with pain, the black arrows depict intraepidermal nerve fibres. There are much fewer nerve fibres present in this section as this area (the ankle) is the first and most severely affected area in the progression of peripheral neuropathy (McArthur *et al.* 2005; Gonzalez-Duarte *et al.* 2008). Figure 3.7 shows an ankle biopsy skin section of a participant without pain at the site from where the biopsy was taken; the black arrows depict intraepidermal nerve fibres. The density of intraepidermal nerve fibres is comparable to the density found in the thigh skin sections. Figure 3.8 shows the magnification at which IENFD quantification was carried out.

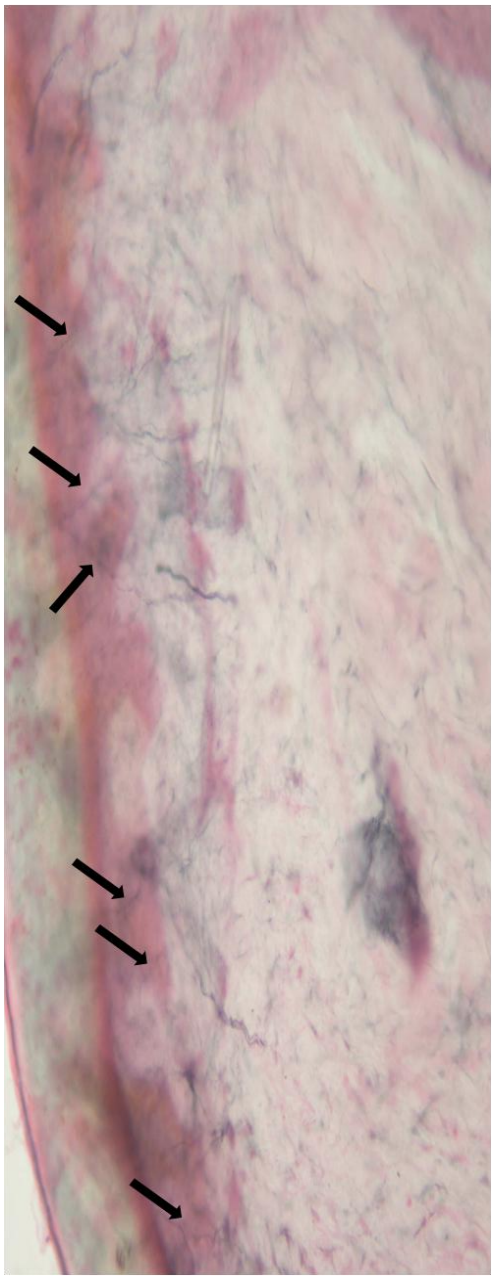


Figure 3.5 thigh biopsy skin section, the black arrows depict intraepidermal nerve fibres (20X magnification)



Figure 3.6 Ankle biopsy skin section of a participant with pain at the site of the biopsy, the black arrows depict intraepidermal nerve fibres (20X magnification)

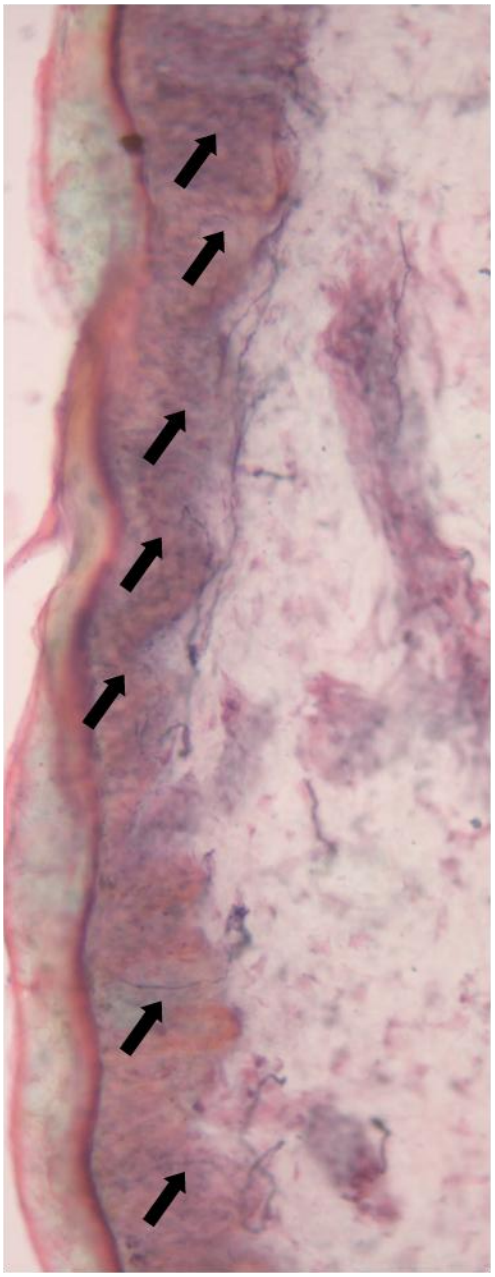


Figure 3.7 Ankle biopsy skin section of a participant without pain at the site of the biopsy, the black arrows depict intraepidermal nerve fibres (20X magnification)

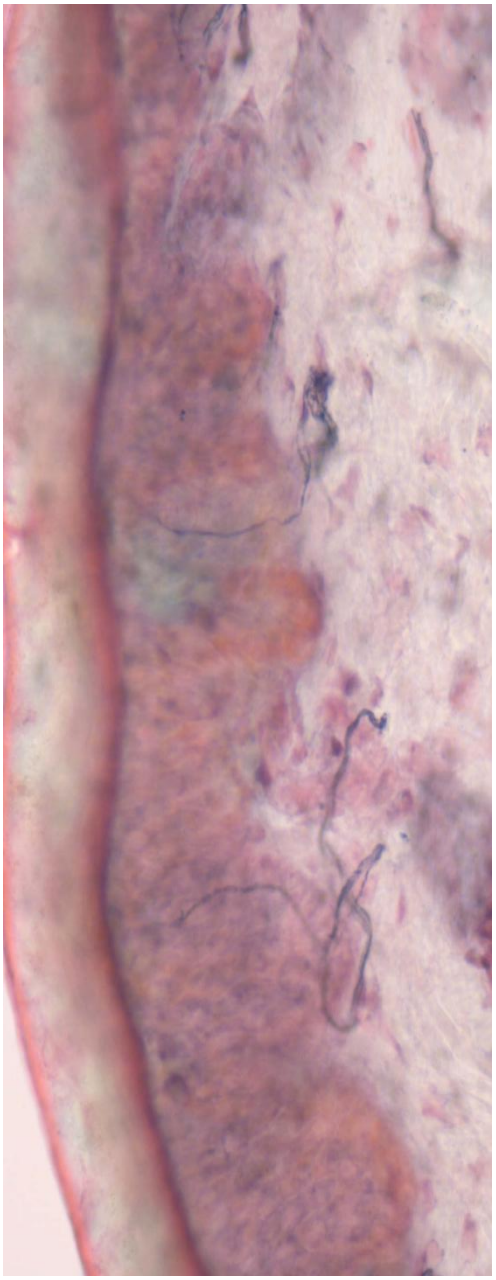


Figure 3.8 Magnification at which IENFD quantification was carried out (40X magnification)

CHAPTER 4 : IENFD Study Results

4.1 Demographic Data

15 participants were recruited that met all of the inclusion criteria and were willing to have skin biopsies taken. Table 4.1 shows the demographic data for the sample population. 10 Females and 5 males took part in the study. The mean age among our participants was 42 (SD 8.1). All the participants had CD4 T-cell counts greater than 350 cell/mm^3 with the majority (80%) being greater than 400 cell/mm^3. The median CD4 T-cell count was 492 cells/mm^3 (353-770). Six of the participants had signs of neuropathy, but did not have pain at the site of the lower leg biopsy. Of these six participants, four experienced pain in their feet, but this pain did not extend to the ankles. These participants were placed in the 'No Pain' group. Nine participants had a symptomatic neuropathy which extended up to the ankles, but not further than the knee. These participants were placed in the 'Pain' group. Pain intensity was measured on a scale from 0-10, 0 being no pain and 10 being severe. The median pain intensity experienced by the 'Pain' group was 10 (IQR: 7-10), while the median pain intensity for those participants who experienced pain in their feet from the 'No Pain' group was 5 (IQR: 1-10).

Table 4.1 Participant Demographic, Pain Intensity Score and IENFD

Sample size	15
% Female	66
Age (years) *	42 (8.1)
CD4 cell count (cell/mm^3) ǂ	510 (353- 770)
Number of participants without pain at the ankle	6 (40%)
Number of participants with pain at the ankle	9 (60%)
Number of participants with pain in their feet, but not at the ankle	4

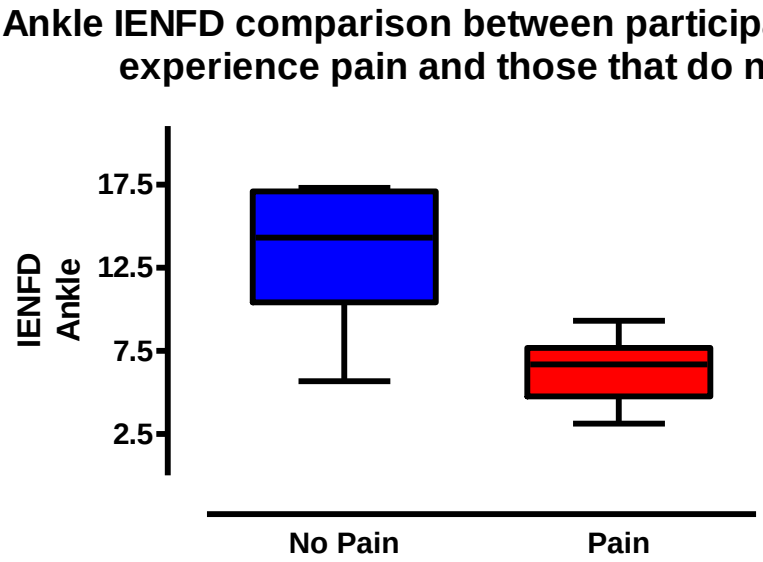
*mean (SD)
ǂmedian (interquartile range)

4.2 Significance Testing

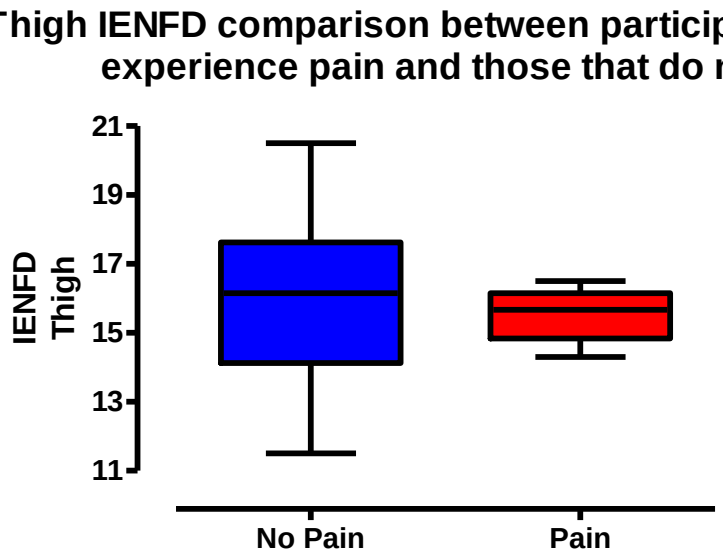
IENFD was assessed using the guidelines set out by the EFNS as described in chapter 2, section 2.10. Biopsy sections were labelled with the biopsy number from which the sections were obtained. In this way IENFD quantification was carried out under blinded conditions. The results from statistical comparisons of ankle and thigh IENFD between the 'Pain' group and the 'No Pain' group have been tabulated in table 4.2 and depicted in figures 4.1 a) and b). We found that ankle IENFD between the 'Pain' and 'No Pain' groups were significantly different (p= 0.016) (fig. 4.1a). However, there was no significant difference in thigh IENDF between the 'Pain' and 'No Pain' groups (p=0.408) (fig. 4.1b). We also found that there was no significant difference in IENFD of the ankle in the 'No Pain' group compared to the thigh IENFD in the 'No Pain' group (p=0.485). The ankle to thigh IENFD ratio for the 'No Pain' group and the 'Pain' group was 0.81 and 0.43 respectively (Figure 4.2a and 4.2b)

Table 4.2 Ankle and thigh IENFD comparisons between the pain and non pain groups

	IENFD (fibres/mm) Pain group	IENFD (fibres/mm) No Pain group
Ankle vs Ankle	6 ± 2	13 ± 4
Thigh vs Thigh	16 ± 3	16 ± 1



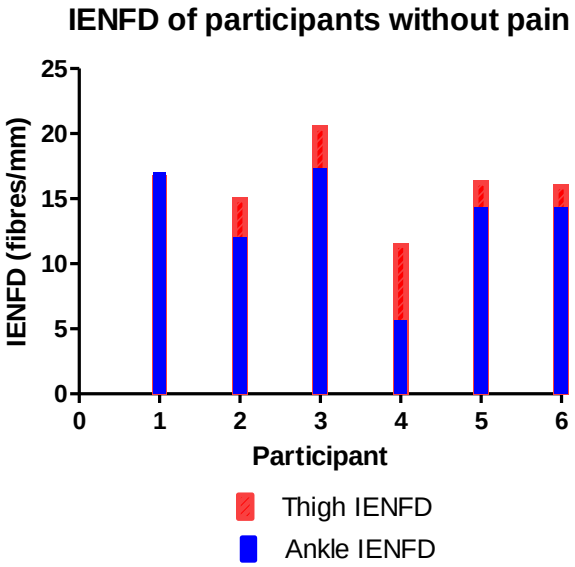
a)



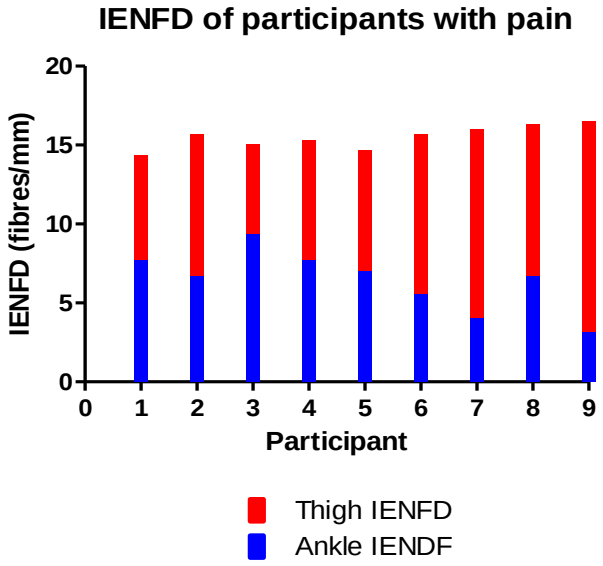
b)

Figure 4.1a) Box and Whisker plots comparing the range of ankle IENFD between the Pain and No Pain groups

Figure 4.1b) Box and Whisker plots comparing the range of thigh IENFD Between the pain and no pain groups



a)



b)

Figure 4.2a) Graph showing thigh and ankle IENFD for participants who did not experience pain at the ankle. The thigh to ankle IENFD ratio is 0.81.

Figure 4.2b) Graph showing thigh and ankle IENFD for participants who experienced pain at the ankle. The thigh to ankle IENFD ratio is 0.43.

CHAPTER 5 : Discussion

Antiretroviral Toxic Neuropathy affects 49-57% of South Africans living with HIV/AIDS who have started HAART (Maritz *et al.* 2010; Wadley *et al.* 2011). While the pathophysiology of this disorder has been documented, it is still under much debate (Brinkman *et al.* 1998; Cherry *et al.* 2002; Brew *et al.* 2003; Kamerman *et al.* 2012). A recent study by Evans *et al.* (2011) has shown that most cases of neuropathy found in the clinic tend to be asymptomatic. This finding provides a useful starting point to understand why most cases of HIV-SN go undiagnosed in clinical settings, especially in developing countries such as South Africa- where public health facilities are overcrowded- affording patients only a few minutes with their doctors, which is insufficient time to conduct a thorough neurological examination. As stated in Chapter 1, in complex clinical cases, basic neurologic screening tools are insufficient in confirming a neuropathy diagnosis, and while these tools provide a useful diagnostic starting point, nerve conduction tests as well as epidermal nerve fibre density quantification provide much more reliable measures to confirm a neuropathy diagnosis (Hermann *et al.* 1999; Treede *et al.* 2008). In recent years, IENFD quantification has received considerable attention, with many studies looking at the efficacy, reliability and validity of the technique (McArthur *et al.* 1998; Polydefkis *et al.* 2002; Smith *et al.* 2005; Lauria *et al.* 2007; Obermann *et al.* 2007; Skopelitis *et al.* 2007; Sommer *et al.* 2007; Zhou *et al.* 2007; Lauria *et al.* 2010). However, to date, the IENFD quantification

technique has not been demonstrated in South Africa. This dissertation described how I successfully established the staining technique and IENFD quantification procedure in a South African laboratory. With the technique set up, I undertook a pilot study that looked at whether IENFD at the distal leg differed between participants that experienced pain at that site and those that did not. My findings indicate that there is a negative relationship between the presence of pain at the biopsy site and IENFD, with the IENFD of participants with pain at the biopsy site being significantly less than the IENFD of participants without pain at the site. At the same time, I found that there was no significant difference in IENFD at the distal leg biopsy site, in participants who did not experience pain at that site, compared to the IENFD at the thigh biopsy site for our entire sample population. This is significant as it indicates that, individuals with a neuropathy but no pain at the distal leg (the site where biopsies are taken from to verify a neuropathy diagnosis) may have IENFD that is within the normal range. Thus, by assessing nerve fibre density at the distal leg alone, it is possible that an incorrect assessment will be made. I will now discuss some points related to the establishment of the technique as well as some problems that were faced during the pilot study.

5.1 Sample Selection

Our participant recruitment took place at a busy outpatient pharmacy at Chris Hani Baragwanath hospital. I was able to obtain a sample size of 15 participants. This was largely due to the high CD4 T-cell count that was

set as one of our inclusion criteria. As this was the first time that skin punch biopsies were taken for IENFD quantification in any population group in South Africa, I erred on the side of caution in this HIV-positive population because I was unsure of how well tolerated the procedure would be, and the ability of the participants to care for the wound in an outpatient setting. Thus, to minimise the risk of participants developing a secondary infection from the biopsy site, I decided to set the CD4 T-cell count to a minimum of 350 cells/mm^3. Many of the participants who were screened and who had bilateral signs and symptoms of a neuropathy had a CD4 T-cell count bellow 350cell/mm^3. Hence, once I was comfortable that the patients could care for the wound, I requested permission from the Human Research Ethics Committee of the University of the Witwatersrand, to have the CD4 T-cell count inclusion criteria reduced to a minimum of 200 cells/mm^3, which is still above international practice where no lower limit is set (Skopelitis *et al.* 2007; Zhou *et al.* 2007). However, this request was denied owing to the reason stated previously. In addition to strict exclusion criteria, participants were reluctant to take part in the study as they felt uncomfortable about having a biopsy taken and the fact that no remuneration was offered for participation as an inducement. In the end we were only able to screen approximately 35 patients, of which 15 were recruited. This was regarded as a sufficient number of participants for our purposes, namely to run a pilot study using a technique that has just been established in South Africa.

5.2 Establishing the IENFD quantification technique

I used the guidelines set out by the European Federation of Neurological Sciences for scoring the stained biopsies (Lauria *et al.* 2010) and the procedure drawn up by the Cutaneous Nerve Research Laboratory at John Hopkins University to complete the biopsy collection, processing and staining[1]. This procedure has been described in Chapter 2. We initially did the immunohistochemical staining using biopsies obtained from healthy subjects. This was done to obtain a feel for the procedure and to iron out any problems we might come across. I obtained sufficient biopsy samples from each participant to complete immunohistochemical staining a number of times if the need arose. The procedure progressed well in the laboratory with a marked difference in staining quality between the initial stages of the staining procedure compared to the staining of the latter skin sections. Problems that I came across initially included:

1. Making sense of the procedure that we were following, namely the procedure drawn up by the John Hopkins Cutaneous Nerve Research Laboratory. The guide assumed some experience in the field of histology. We were able to overcome this hurdle by consulting a histologist (Glenda Norman) who was able to give more details as to how the different procedural steps should be carried out.

[1](http://www.hopkinsmedicine.org/neurology_neurosurgery/specialty_areas/cutaneous_nerve_lab/multimedia/)

2. Initially, it was found that the sections would not slice uniformly. This caused a problem when mounting the sections as well as during quantification of the epidermal nerve fibres due to the section tearing or shredding. In order to counter this problem I cooled the room as well as the microtome blades so that the frozen sections did not melt as fast during the sectioning. This worked very well and made handling of the sections easy thereafter.
3. One important factor that had to be kept in mind during the entire immunohistochemical staining procedure was not to allow the sections to dry out while in the wells. In the beginning I found that some of the sections were destroyed during the staining procedure due to this very reason. I therefore adjusted the suctioning system to suction at a lower rate allowing the solution to drain out without placing strain on the section. I also adjusted my pippetting technique by reducing the amount of solution pipetted into each well so that spillage was minimised if a well did not drain completely during a previous step. In this way adjustments were made a number of times to ensure that the sections were well preserved throughout the staining procedure.
4. Difficulty with mounting of the skin sections onto the slides was also experienced in the beginning. The sections were very thin and mounting 3 sections per slide proved to be quite challenging. However, with practice and a practical demonstration by a

histologist (Glenda Norman) this problem was overcome quite quickly.

5.3 Assay optimization

Three titres of primary anti-body were tested to establish the optimal titre to get clear staining of the intraepidermal nerve fibres. When the primary anti-body was diluted to 1:2000 it produced very little contrast and made nerve fibre quantification difficult. Using a dilution of 1:900 produced too much background staining, making the nerve fibres very difficult to differentiate from the background. We found that a primary anti-body titre of 1:1400 produced the best results allowing clear differentiation of the fibres from the background, thus making quantification relatively easy. The dermal-epidermal junction was also well differentiated at this concentration. This is very important as the IENFD quantification guidelines use this junction as a reference point for determining whether a branched epidermal nerve fibre should be counted as two fibres or one (Lauria *et al.* 2010).

I completed one immuno-staining run without using any primary antibody in order to rule out non-specific binding of the secondary antibody. The secondary antibody did not bind to any other sites and thus secondary antibody staining of epidermal nerve fibres was ascertained.

5.4 The Pilot Study

As soon as the immunohistochemical staining as well as the IENFD quantification technique was well established in the laboratory, staining of the study participant's skin sections were carried out. Over the years, many studies looked at the association between IENFD and the presence of HIV-SN or ATN (McArthur *et al.* 1998; Hermann *et al.* 1999; Cherry *et al.* 2002; Polydefkis *et al.* 2002; Smith *et al.* 2005; Umapathi *et al.* 2006; Obermann *et al.* 2007; Zhou *et al.* 2007) and while it has been shown that reduced IENFD does confirm the diagnosis of peripheral neuropathy (Skopelitis *et al.* 2007), no studies have looked at the association between the site at which pain is experienced and the IENFD. In this pilot study we looked at this association, comparing the IENFD of participants who experienced pain at the distal leg site, where biopsies are routinely taken from, and those participants that did not experience pain at this site.

The majority of our participants (n=12) had CD4 T-cell counts above 400cell/mm^3. This was relatively high compared to previous studies which assessed participants with CD4 T-cell counts ranging from 25cell/mm^3 to greater than a thousand cells/mm^3 (Polydefkis *et al.* 2002; Obermann *et al.* 2007; Zhou *et al.* 2007). However, this was not surprising given our exclusion criterion of participants having a CD4 T-cell count greater that 350 cells/mm^3.

Our participants mainly were female (n=10). Females make up the majority of patients in the clinic, and were more willing to take part than males in order to assist their condition in any way possible.

We assessed participants for peripheral neuropathy with a focus on whether the participant experienced pain at the distal leg biopsy site or not. Of the participants that did not experience pain at the distal leg biopsy site (n=6), four experienced pain to some extent under their feet. None of the participants with symptomatic ATN were receiving any pain management treatment. At the same time, none of the participants were diagnosed as having peripheral neuropathy in their medical records. This points to the common problem that peripheral neuropathy is by and large under-diagnosed in hospitals in South Africa, which is consistent with international studies on diagnosis and treatment of neuropathy (Beilke *et al.* 2004; Verma *et al.* 2004). However, I was requested by the head of the HIV clinic at Chris Hani Baragwanath Hospital to place a note into the files of the patients that we screened, informing the clinic doctors of the outcome of the screening.

The nominative reference range for IENFD for healthy individuals and those with neuropathy have been established and the guidelines published by the EFNS (Lauria *et al.* 2010). We used the reference range set by McArthur *et al.* (1998) as they gave a normal range for the proximal and distal leg regions (thigh and ankle). I found that our participants without

pain at the distal leg biopsy site had an IENFD within the normal range (range: 12 – 17 fibres/mm) with only one participant, who experienced severe pain underfoot but not at the site of the biopsy, showing similar patterns of IENFD as that found in patients with pain at the distal leg (6 fibres/mm). This difference could have been due to an error in self-report of pain distribution by the participant due to the pain at the distal leg biopsy site being negligible compared to the severe pain on the soles of his feet. On the other hand, it could mean in cases where pain is severe that peripheral nerve die back does extend more proximally than if the pain is less severe, but without significant pain at the more proximal sites. This can only be accurately evaluated with a larger sample size and additional biopsies taken from different sites around where the pain is experienced.

The IENFD of the participants with pain at the distal leg biopsy site was largely found to be below normal (range: 3 – 9 fibres/mm). However, three of the nine participants in this group had IENFD scores at the lower end of the normal range, which may indicate that even low-normal IENFD may be associated with pain, suggesting that absolute normal values for IENFD are uninformative. On the other hand, relative change in IENFD may be more informative, however, this is difficult to assess in cross sectional studies and will have to be evaluated further in a larger study. Never the less, when comparing the two groups, IENFD at the distal leg biopsy site was negatively associated with pain. On the other hand there was no

significant difference between the two groups IENFD at the thigh, a site where neither group reported pain, and the thigh IENFD for both groups fell within the normal range for healthy individuals. Finally, when examining the IENFD thigh to ankle ratio, participants that did not experience pain at the distal leg biopsy site had a ratio closer to one (normal), while the group that experienced pain had a ratio closer to zero (abnormal).

While pain is the most common symptom associated with ATN in this population (Maritz *et al.* 2010; Wadley *et al.* 2011), Evans *et al.* (2011) showed that most cases of neuropathy in the clinic tend to be asymptomatic. Screening tools alone cannot provide a definitive diagnosis for ATN. Indeed, the sensitivity of neuropathy screening tools means that as many as 20% of patients with the condition will be incorrectly diagnosed as not having a neuropathy. While the results of our prospective study shed some light as to the complexity of the pathology of ATN, a broader study would provide more detail in this regard. Such a study should include comparisons of the known risk factors for developing ATN and include biopsies from under the foot of those patients who experience pain in this region only. This is important, since we have shown that pain at the site of the biopsy and IENFD are associated. Thus, the diagnostic efficacy of IENFD would be limited if the biopsy is taken from a standard site, in this case the ankle, instead of from the site where the patient is feeling pain. Indeed, some studies have found a relatively

low diagnostic efficacy of IENFD quantification (McArthur *et al.* 1998; Polydefkis *et al.* 2002; Zhou *et al.* 2007). This low efficacy may purely be related to the biopsy being taken from an inappropriate level for the extent of a painful disease. Therefore, any future study should evaluate biopsies from multiple regions in order to verify the diagnostic efficacy of IENFD quantification. A study of this nature would provide much needed insights into the pathology of ATN in respect of the painful symptoms experienced by those suffering with this disorder.

CHAPTER 6 : Conclusion

In keeping with the aim of this study, I established IENFD quantification in a South African laboratory. The value of this technique, in confirming a neuropathy diagnosis, has been demonstrated in Western countries. However, this technique is not only limited to assessing a HIV-SN diagnosis, but its use can be extended to other small fibre neuropathies, such as diabetic neuropathy, which is a serious problem in developing countries.

Our second aim was to see whether IENFD differs between individuals, with HIV-SN, that experience pain at the distal leg biopsy site compared to those who do not have pain at the same site. In this regard I have also shown, in a small study, that IENFD at the distal leg is negatively related to pain in individuals who experience pain (at the site where the distal leg biopsy is taken), however, this relationship does not hold true for individuals who did not experience pain at the same site, even though they might have experienced pain in the soles of their feet. This small study may not be enough to infer any solid conclusions; however, it does provide a good base for any further studies that look at the efficacy of IENFD quantification in assessing ATN. For this purpose it is necessary that biopsies, which are taken to confirm the presence of a neuropathy, be taken from the site at which a patient experiences pain and not necessarily

from the distal leg site where nerve fibre die back is thought to first develop.

APPENDIX 1A: List of Chemicals/Reagents

List of Chemicals/Reagents and Their Suppliers		
Chemical	**Ref. Number**	**Supplier**
Triton X-100	T8532-500ML	Sigma-Aldrich, South Africa
Oxalic Acid Dihydrate	00376-500G	
Phosphate Buffered Saline	P5368-10PAK	
Ethylene Glycol (99%)	102466-2.5L	
Potassium Permanganate	13206-500G	
Methyl Alcohol (99.8%)	322415-2L	
Xylenes	534056-4L	
Tris Buffered Saline	T6664-10PAK	
Absolute Ethanol	32221-2.5L	
VECTASTAIN Elite ABC Kit	PK-6100	Vector Laboratories, USA
Biotinylated Anti-Rabbit IgG	BA-1000	
Normal Goat Serum	S-1000	
UCH-L1 (PGP9.5), rabbit pAb	PG9500-0100	Enzo Life Sciences, USA

APPENDIX 1B: List of Solutions

List of Solutions and Their Ingredients	
Solution	**Ingredients**
Lysine Stock	L-Lysine 0.1M Sorrensons Phosphate Buffer
Phosphate Buffer	33.65g Sodium Phosphate-Monobasic 7.7g Sodium Hydroxide
0.4M Sorrensons Phosphate Buffer	49.4g Sodium Phosphate Dibasic Anhydrous 800ml dH_2O
PLP	30ml Lysine stock 8% Paraformaldehyde 0.085g Sodium m-Periodate
Glycerol Based Cryoprotectant	20% Glycerol 20% 0.4M Sorrensons Phosphate Buffer 60% dH_2O
Glycol Based Antifreeze	300ml Glycerol 300ml Ethylene Glycol 300ml dH_2O 100ml Phosphate Buffer
Blocking Solution	Tris Buffered Saline (TBS) 0.5% Powdered Milk 1% Triton X-100 5% Normal Serum
Primary Antibody Solution	TBS 0.5% Triton X-100 2.5% Normal Serum 0.5% Powdered Milk Primary Ab @ 1:1400
Secondary Antibody Solution	TBS 0.1% Triton X-100 2.5% Normal Serum Secondary Ab @ 1:100

APPENDIX 2A: Participant Information Sheet

INFORMATION SHEET

Pathological Changes in Epidermal Nerve Fibres of People with Antiretroviral Toxic Neuropathy

Good day. I am Imraan Patel, a researcher in the School of Physiology at Wits University, and I research the causes and treatment of pain experienced by people infected with HIV. I am undertaking this research in conjunction with Professor Peter Kamerman of the School of Physiology at Wits University and Dr Natalia Dinat of the Palliative Care Unit at Chris Hani Baragwanath Hospital.

Some people being treated for infection with HIV develop nerve damage in their feet and lower legs, which can be painful, but we do not know what the exact cause of this pain is. In this research project, we want to find out what changes occur in the nerve endings of people being treated for infection by HIV who develop pain in their feet, compared to people who do not develop this pain. You are hereby invited to take part in this research project. By participating in this study you will help us better understand why some HIV-infected individuals with treatment-related nerve damage feel pain in their feet while others do not. This information ultimately may help us improve the overall care of people infected with HIV.

If you choose to take part in this study we shall ask you a series of questions about yourself, such as your name, age, contact details, and when you were first diagnosed with HIV. We also will need to know your CD4 count and what medications you are on, and how long you have been on these medications. If you are not sure of this information, we shall, with your permission, obtain this information from your medical records. We also shall perform two quick, non-

invasive and normally painless tests of the nerve function in your lower legs. The tests, which we can do while you are waiting to see your doctor, involve placing a vibrating tuning fork on the big toe of your foot and asking you to say when you can no longer feel the vibration, and testing your reflexes by softly tapping the back of your lower leg.

If you have damage to your nerve in your legs, we also would like to perform an invasive procedure which requires us to remove 8 small samples of skin from your leg; 4 samples from your ankle, and 4 samples from your hip. The size of each skin sample is " ● ", and the areas of skin we shall take the samples from will be anaesthetised before hand. The procedure, which will be done by a doctor, may cause some discomfort. This discomfort typically presents itself as a ticklish, cooling sensation which occurs during the administration of the anaesthetic and abates as soon as the procedure is complete. However, sometimes a mild, and short-lived, stinging sensation may be experienced. The biopsy is not usually painful, and the wound typically heals completely within a month, with little or no scarring. You will need to keep the wound clean and dry for at least three days after the samples are taken, and we shall supply you with the necessary plasters. If you are unsure of how to care for the wounds, we shall help you change the plasters on a daily basis. We will phone you on a daily basis to check whether you are having any problems with keeping the wound clean and dry, and whether you have any concerns about the wound healing. If you have any problems with the wound, or caring for the wound, we shall arrange, with your permission to visits you and address your concerns. We need to take these skin samples to check for changes in the nerve endings in your skin that may result in some people developing pain in their legs and others not.

Participation in this study is completely voluntary and you are free to choose not to participate or to withdraw from the study at any stage without disadvantage to

yourself. All the information we collect will be kept confidential and will be available only to the researchers and yourself. We do not need, and will not ask for, any other information from your records. All records of your results will be identified only by a reference number, which we shall assign to you should you choose to participate in the study, so that you remain anonymous. You will not directly benefit in any way by participating in the study. The outcomes of this project as a whole, but not your individual information, will be made available to the scientific and medical community. This project has been approved by the Wits University Human Ethics Research Committee.

Thank you for considering participating in this study. Please read this information sheet carefully before signing the consent form. If you have any queries, please contact myself, Imraan on 082 504 0504, or visit me at my office, room 7M16, 7th floor, Wits Medical School, 7 York Road, Parktown.

Yours sincerely
Imraan Patel

APPENDIX 2B: Participant Consent Form

CONSENT FORM

Pathological Changes in Epidermal Nerve Fibres of People with Antiretroviral Toxic Neuropathy

You are invited to take part in the study looking at the pathological changes that occur in the epidermal nerve fibres of people with antiretroviral toxic neuropathy. Please read the information sheet carefully. Once you are satisfied that you understand all the information, and you wish to take part in this study, please complete the form bellow.

I, _____, have read the information sheet regarding the research project entitled “Pathological changes in epidermal nerve fibres of people with antiretroviral toxic neuropathy”. I understand that I shall undergo a neurological examination to test nerve function in my legs and I shall have skin biopsies taken from my lower and upper leg.

I hereby give my full consent to participate in this study knowing the risks and discomfort that I may experience. I am aware that participation in this study is completely voluntary and that I may withdraw from the study at any time without prejudice to me.

_____ _____

Signed (subject) Date

_____ _____

Signed (researcher) Date

APPENDIX 3: Ethical Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Imraan G Patel

CLEARANCE CERTIFICATE	**M090670**
PROJECT	Pathological Changes in Epidermal Nerve Fibres of People with Antiretroviral Toxic Neuropathy
INVESTIGATORS	Mr Imraan G Patel.
DEPARTMENT	School of Physiology
DATE CONSIDERED	09.06.26
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 09.08.07 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof P Kamerman

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

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