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Factors associated with developing symptomatic HIV-associated sensory neuropathy

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FACTORS ASSOCIATED WITH DEVELOPING SYMPTOMATIC HIV-ASSOCIATED SENSORY NEUROPATHY

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, South Africa, 2013





DECLARATION

I, Antonia Louise Wadley, declare that this thesis is my own work, except where otherwise specified. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree of examination at this or any other University.

_____ day of _____ 2013

RESEARCH OUTPUTS

1. Peer reviewed original research publications

Wadley, A.L., Lombard, Z., Cherry, C.L., Price, P., Kamerman, P.R. “Polymorphisms in uncoupling proteins genes UCP2 and UCP3 are not associated with HIV-associated sensory neuropathy in African individuals”. Journal of the Peripheral Nervous System, in press

Chew, C.S.N., Wadley, A.L., Lombard, Z., Kamerman, P.R., Price, P. (2013) “Characterization of TNF haplotypes in a Southern African population”. Genes and Immunity, in press

Wadley, A.L., Lombard, Z., Cherry, C.L., Price, P., Kamerman, P.R. (2012) “Analysis of a previously identified ‘pain protective’ haplotype and individual polymorphisms in the *GCH1* gene in Africans with HIV-associated sensory neuropathy: a genetic association study.” JAIDS **60**: 20-23, 2012

Wadley, A.L., Cherry, C.L., Price, P., Kamerman, P.R. (2011) “HIV neuropathy risk factors and symptom characterization in stavudine-exposed South Africans.” Journal of Pain and Symptom Management, **41**: 700-706

Cherry, C.L., Affandi, J.S., Brew, B.J., Creighton, J., Hooker D.J., Imran, D., Kamarulzaman, A., Kamerman, P., McArthur, J.C., Price, P., Smyth, K., Tan, IL., Vaneer, S., Wadley, A., Wesselingh, S., Yunihastuti, E. (2010) “Hepatitis C seropositivity is not a risk factor for sensory neuropathy among HIV patients.” Neurology, **74**: 1538-42

2. Reviews (both were peer reviewed)

Kamerman, P.R., Wadley, A.L., Cherry, C.L. (2012) “HIV-associated sensory neuropathy: risk factors and genetics” Current Pain Headache Reports **16**: 226-36

Cherry, C.L., Wadley, A.L., Kamerman, P.R. (2012) “Painful HIV-associated sensory neuropathy” Pain Management **2**: 543-552

3. Published abstracts

Wadley, A.L., Cherry, C.L., Price, P., Kamerman, P,R. (2010) “Pain associated with HIV-associated sensory neuropathy in a Black South African cohort.” European Journal of Pain, Supplements 4: 90

4. Abstracts

Wadley, A.L., Lombard, Z,. Cherry, C.L., Price, P., Kamerman, P,R. UCP3 but not UCP2 associates with pain intensity in HIV-associated sensory neuropathy in black Africans 14th World Congress on Pain, Milan, Italy 2012

Wadley, A.L., Lombard, Z,. Cherry, C.L., Price, P., Kamerman, P,R. A Previously identified polymorphism in TNFA does not associate with presence of symptomatic neuropathy in black Africans with HIV-associated sensory neuropathy 14th World Congress on Pain, Milan, Italy 2012

P Price, C Chew, A Wadley, Z Lombard, CL Cherry, PR Kamerman. Genetic studies illuminate the complex role of TNF in sensory neuropathy in HIV patients. Australasian Society for HIV Medicine Annual Meeting, Melbourne, 2012

CL Cherry, JS Affandi, BJ Brew, P Kamerman, JC McArthur, P Price, IL Tan, S Vanar, A Wadley, E Yunihastuti. Ethnicity is an independent predictor of neuropathy risk in HIV patients. 18th Conference on Retroviruses and Opportunistic Infections, Boston, 2011

Wadley, A.L., Lombard, Z,. Cherry, C.L., Price, P., Kamerman, P,R. The role of *GCH1* polymorphisms on pain perception in black Africans with HIV-associated sensory neuropathy. 13th World Congress on Pain, Montreal, Canada. September 2010

Wadley, A.L., Lombard, Z,. Cherry, C.L., Price, P., Kamerman, P,R. Pain associated with HIV-associated sensory neuropathy in a black South African cohort. Third International Conference on Neuropathic Pain, Athens, Greece. May 2010.

Wadley, A.L., Lombard, Z,. Cherry, C.L., Price, P., Kamerman, P,R. The role of *GCH1* polymorphisms on pain perception in black Africans with HIV-associated sensory neuropathy. 4th PainSA congress, Durban, South Africa. May 2010
Winner of best oral research presentation

Cherry, C.L., Affandi, J.S., Yunihastuti, E., Vanar, S., Imran, D., Smyth, K., Kamarulzaman, A., **Wadley, A.L.,** Kamerman, P., Price, P. Predicting Neuropathy Risk Before Stavudine Prescription: an Algorithm for Minimizing Neurotoxicity in Resource-Limited Settings. 16th Conference on Retroviruses and Opportunistic Infections, Montreal, Canada 2009

Wadley, A.L., Cherry, C.L., Price, P., Kamerman, P,R. HIV-associated sensory neuropathy: a comparison of rates and risk factors in Australia and South Africa 5th International Aids Society Conference, Cape Town July 2009.

Wadley, A.L., Cherry, C.L., Price, P., Kamerman, P,R. A comparison of rates and risk factors for HIV-SN in Australia and South Africa University of the Witwatersrand Cross-Faculty Postgraduate Research Symposium 20-21 October 2009.

ABSTRACT

HIV-associated sensory neuropathy (HIV-SN) is one of the most common neurological problems of HIV. It is frequently painful and reduces quality of life. HIV-SN can be caused both by HIV itself and by exposure to neurotoxic antiretrovirals such as stavudine. The South African Department of Health now recommends use of tenofovir in place of stavudine as first line treatment. However many people remain on stavudine and or live with the side effects. Stavudine is still prescribed in many other resource-poor countries. This thesis presents the first systematic study of clinical and genetic risk factors for the development of symptomatic HIV-SN in Black Southern Africans.

I recruited 404 Black HIV-positive Africans from the Virology Clinic of the Charlotte Maxeke Academic Hospital, Johannesburg and assessed HIV-SN using the AIDS Clinical Trials Group (ACTG) Brief Peripheral Neuropathy Screen. HIV-SN was defined as present if the patient had both symptoms and signs of peripheral neuropathy. If present, the distribution and intensity of symptoms were recorded. Of those exposed to stavudine, 57% (226/395) had HIV-SN. Pain was the most common symptom and was experienced by 74% (172/226). Of these, 76% (128/172) reported their pain as moderate to severe. As in previous studies, increasing age and height were independently associated with risk of HIV-SN. However nadir and current CD4 T-cell counts and sex were not associated with SN.

Patients donated blood for DNA extraction and single nucleotide polymorphisms (SNPs) were selected from the literature and genotyped using Illumina Golden Gate™ technology. 342 individuals were assessed for genetic associations with HIV-SN and a subset of 159 positive for HIV-SN were assessed for associations with painful HIV-SN. I completed four genetic analyses:

SNPs and haplotypes from *TNF* and adjacent genes from the major histocompatability complex on chromosome six were assessed for association with

HIV-SN. I found no association with TNF-1031, even though this had associated with risk of HIV-SN in Caucasian, Chinese and Malay cohorts. Novel associations were identified between HIV-SN protection and 5 other SNPs (*BAT1* rs3130059, rs2523504; *ATP6V1G2* rs2071594; *NFKBIL1* rs2071592, rs2071591). Associations were also found with haplotypes: FV15-23 weakly associated with risk and FV30-31 associated with protection against HIV-SN in this cohort. Analysis of 8 SNPs not previously assessed produced two novel associations with *LTA* SNPs (rs1041981, rs909253), where the minor alleles conferred protection against HIV-SN. Analysis of linkage disequilibrium (LD) suggests that there is linkage disequilibrium within the TNF block, that it differs between ethnicities and that TNF-1031 is unlikely to be a causative SNP for risk of HIV-SN.

SNPs from other cytokines and chemokines implicated in the pathogenesis of HIV-SN and the associated pain were assessed in Chapter 5. The major allele of the anti-inflammatory gene *IL4* (rs2243250) associated with risk of HIV-SN. This allele has been associated with higher CD4 T-cell counts, so I have proposed a role for high IL-4 in early stage HIV-SN. A 3-SNP haplotype of *IL10* associated with protection against HIV-SN whilst another *IL10* haplotype showed a trend for risk of painful HIV-SN. These data and the involvement of TNF haplotype (Chapter 4) suggest an inflammatory etiology for HIV-SN.

Polymorphisms of *UCP2* (rs659366) and *UCP3* (rs1800849) have previously associated with risk of diabetic neuropathy. These SNPs encode uncoupling proteins 2 and 3 which regulate reactive oxygen species and may affect development of neuropathy via the effects of oxidative stress and mitochondrial dysfunction. Alleles of these SNPs did not associate with HIV-SN in this cohort. Patterns of linkage disequilibrium may differ between the two ethnicities or UCP2 and UCP3 may associate with a mechanism particular to diabetic neuropathy.

I also assessed a ‘pain protective haplotype’ and SNPs of *GCH1* which have been associated with decreased pain intensity in radicular pain following lumbar discectomy. Associations of the 3-SNP ‘pain protective’ haplotype (rs10483639*C, rs3783641*A and rs8007267*T) and a 6-SNP haplotype containing this motif with protection against pain were significant but dependent on age, sex and CD4 T-cell count. Association of another 3-SNP haplotype (rs10483639*G, rs3783641*T and rs8007267*C) with increased risk of pain in HIV-SN was also not independent of age, sex and CD4 T-cell count. The weaker associations here compared to Caucasian cohorts may be a result of differing LD between ethnicities or demonstrate different pain mechanisms between HIV-SN and radicular pain following lumbar discectomy.

My results highlight the prevalence of HIV-SN and frequency of pain in this Southern African cohort. The genetic studies identify a likely inflammatory component and identify genes worthy of further investigation both in HIV-SN and the associated pain.

ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisors Peter Kamerman and Patricia Price. I am so grateful for the time, energy and patience you have given me in the last four years. Additionally I would like to thank you for the help with funding my study and the support to attend conferences and give my first talks. I'd also like to thank you for the fastest turn-around times ever known! Thank you for steering me along the way and developing my passion for research.

To my collaborators Kate Cherry and Zané Lombard: Thank you Kate for teaching me the neuropathy screen, for answering all my questions and the help with writing. You have been a true example for how to mentor and to give effective criticism gently! Zané, thanks for the help in the lab, with the genotyping, data cleaning and for being patient whilst I was coming to terms with genetics.

To Constance Chew and Punita Pitamber thank you for your assistance with the SNP selection and genotyping, and for teaching a physio everything she knows about genetics and lab work!

Thank you to the staff and students of the BFRG for the help, advice, tea...and G and Ts: Tanya Swanepoel, Stella Iacovides, Sam Kerr, Robyn Hetem and Tapiwa Murenje. Thanks also to the director of the BFRG, Andrea Fuller, for your support, flexibility and for replying to that first email!

I am extremely grateful to the Medical Faculty Research Endowment Fund and the Brain Function Research Group both of the University of the Witwatersrand for their funding for this project. I would also like to thank Flo Mtsweni for her help with recruitment and interpreting for the patients. Additionally, I am very thankful to the

staff and patients of the Virology Clinic at the Charlotte Maxeke Academic Hospital, Johannesburg.

Thank you to my friends for their support during this time including the Preggibellies girls and also Em, Tanya and Mich for support, pep talks and soup. Thank you to my family for their encouragement and belief in me. To my little boy Alasdair who accompanied me internally for 9 months of the journey and since then has provided fun, laughs, distraction and purpose. Finally, to my darling Mark who inspires me and encourages me to fulfil my potential; thank you.

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LIST OF ABBREVIATIONS

ACTG	AIDS Clinical Trials Group
ADT	Assay Design Tool
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ATN	Antiretroviral toxic neuropathy
ATP6V1G2	V-type proton ATPase subunit G 2
BAT1	Gene encoding Spliceosome RNA helicase BAT1
BH4	Tetrahydrobiopterin
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-C motif) receptor
CI	Confidence interval
CMV	cytomegalovirus
d4T	stavudine
ddC	zalcitabine
ddI	didanosine
DoH	Department of Health
DSP	Distal sensory polyneuropathy
DRG	Dorsal root ganglion
GCH1	Guanosine triphosphate cyclohydrolase 1
Gp120	Glycoprotein 120
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus infection
HFE	Human hemochromatosis protein
HIV	Human immunodeficiency virus

HIV-SN	HIV-associated sensory neuropathy
HLA	Human leukocyte antigen
HWE	Hardy Weinberg equilibrium
IENFD	Intraepidermal nerve fibre density
IL	Interleukin
LD	Linkage disequilibrium
LTA	Lymphotoxin alpha
MAC	Mycobacterium avium complex
MAF	Minor allele frequency
MHC	Major histocompatability complex
MHF	Minimum haplotype frequency
NFKBIL1	NF-kappa-B inhibitor-like protein 1
NRTI	Nucleoside reverse transcriptase inhibitor
OR	Odds ratio
PCR	Polymerase chain reaction
PI	Protease inhibitor
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SN	Sensory neuropathy
SNP	Single nucleotide polymorphism
TB	Tuberculosis
TNF	Tumor necrosis factor
UCP	Uncoupling protein
UTR	Untranslated region
Vpr	HIV-1 viral protein R
WHO	World Health Organisation

AUTHOR CONTRIBUTIONS

The design of the clinical part of the study repeated that previously used by Kate Cherry. I assessed 482 patients with the assistance of Flo Mtsweni as an interpreter. Blood samples were taken by the nursing sister taking blood in the clinic.

Single nucleotide polymorphisms (SNPs) were selected with the assistance of Constance Chew. Zané Lombard submitted the SNPs to Illumina for assessment for genotyping. I extracted DNA from 404 samples and carried out DNA quanitification and normalisation. The pre-PCR, PCR and post-PCR phases of the genotyping process were carried out by Zané Lombard and Punita Pitamber and I observed this process.

Data analysis was carried out by me with guidance from Peter Kamerman and Kate Cherry. Exceptions to this include the multivariate and ROC analysis in Chapter 3, and multivariate analysis of the haplotypes in Chapter 4 which was carried out by Kate Cherry. Designation of FV haplotypes to individuals in Chapter 4 was carried out by Constance Chew.

I wrote the manuscripts for the papers emanating from Chapters 3 and 7 and all authors edited it. All other chapters have been written by me and reviewed by Peter Kamerman and Patricia Price.

CHAPTER 1

INTRODUCTION

1.1 Overview of HIV-associated sensory neuropathy (HIV-SN)

"I've given up on my feet ever getting better, I have no hope. I asked the doctor once if my legs were going to get better, and he said no I should just focus on taking my ARVs and eating healthy, but my legs will always be sore. So, that is when I lost hope, and accepted the pain."

Quote from interviews with Southern Africans with HIV-SN (Shaikh 2011)

Imagine being unable to bear wearing shoes, feeling like you are walking on thorns or being unsure if your shoes are on or not (Shaikh 2011). Imagine there being no treatment. This is what it can feel like to have HIV-associated sensory neuropathy (HIV-SN).

HIV-SN is one of the most, if not *the* most, common neurological complications of HIV (Keswani et al. 2002). The symptoms of HIV-SN typically are pain, pins and needles and numbness. Symptoms are bilateral, symmetrical and have a "glove and stocking" distribution. When present, pain is often described as cramping, hot, burning, tight or itching (Shaikh 2011) and is frequently experienced as moderate to severe in intensity (Smyth et al. 2007; Maritz et al. 2010). These symptoms reduce quality of life and can affect mobility, ability to work, mood and personal relationships (O'Connor 2009; Doth et al. 2010; Ellis et al. 2010; Lucey et al. 2011). Clinically, pain is under diagnosed and under treated in HIV (Mphahlele et al. 2012) and currently there is no proven effective, commercially available treatment for the pain associated with HIV-SN (Phillips et al. 2010; Clifford et al. 2012).

HIV-SN experienced in patients who are not receiving antiretroviral therapy (ART) is assumed to be caused by the virus itself or the immune response to HIV and is sometimes classified as HIV-associated distal sensory polyneuropathy (DSP). Neuropathy that develops following ART initiation is sometimes classified as antiretroviral toxic neuropathy (ATN), to differentiate it from the neuropathy that

develops before ART. DSP and ATN are clinically indistinguishable and differentiation between the two categories is primarily based on the timing of onset in relation to ART initiation. HIV-SN is a global term covering both DSP and ATN. Pathogenesis of the two forms of HIV-SN is discussed in Section 1.3.

In 2009 out of a global figure of 33 million, there were an estimated 22.5 million people living with HIV in Sub-Saharan Africa. South Africa bears the largest burden of all the countries in this region with an estimated 5.6 million HIV-positive people in 2009 (UNAIDS 2010) [Figure 1.1].



Figure 1.1 HIV prevalence. *A cartogram of the world map adjusted to show the proportion of people aged 15-49 with HIV living in each country. Note the inflated size of SubSaharan Africa, and especially South Africa.*

Figure taken from worldmapper.org with permission. © SASI group (University of Sheffield)

Of these 5.6 million people with HIV, almost 1 million have been enrolled in ART programmes (UNAIDS 2010). Conservative estimates of HIV-SN prevalence in ART naïve people (DSP) are 13% (Smyth et al. 2007) and in those initiating ART (ATN) are 24% (Wester et al. 2005). Based on these prevalence rates of HIV-SN and estimates of individuals with HIV in South Africa, there could be almost 600,000 individuals with DSP and 240,000 with ATN in South Africa. Thus HIV-SN is a

potential cause of significant impairment of health related quality of life in South Africans with HIV.

To finish, identification of molecular mechanisms to provide new targets for treatment is required. I discuss the current evidence in Section 1.2 and aim to address this goal in Chapters 4-7. To improve management of HIV-SN awareness of the condition needs to be raised amongst clinicians and patients. Encouraging identification of clinical, demographic and disease risk factors to improve diagnosis will go a long way to helping clinicians identify patients at risk of HIV-SN. Appropriate steps can be taken in their monitoring and management to mitigate the risk of developing HIV-SN or the burden of disease should it develop. The current literature regarding risk factors for HIV-SN are described in Section 1.3 and assessed in a Southern African cohort in Chapter 3.

1.2 Pathogenesis of HIV-SN

HIV-SN is a small fibre, length-dependent, distal sensory polyneuropathy involving both myelinated and unmyelinated fibres (Pardo et al. 2001; Ferrari et al. 2006). Axonal degeneration starts distally and progresses proximally, termed a ‘dying back’ pattern (Mah et al. 1988; Miller et al. 1988). Axonal degeneration and reduced intraepidermal nerve fibre density (IENFD) distally [Figure 1.2] (Polydefkis et al. 2002; Skopelitis et al. 2006) are accompanied by loss of dorsal root ganglion (DRG) neurons more proximally (de la Monte et al. 1988; Rance et al. 1988).

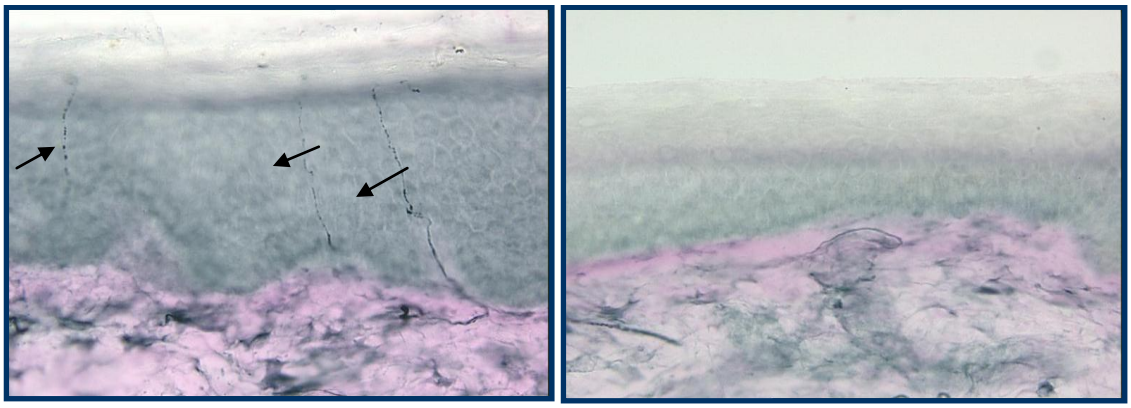


Figure 1.2. Intraepidermal nerve fibre density is reduced in HIV-SN.
On the left is normal skin with numerous nerve fibres innervating the epidermis. The right hand picture demonstrates reduced nerve fibre density in the epidermis of a patient with HIV-SN.
Pictures courtesy of Catherine Cherry.

HIV-SN occurs in the context of someone infected with HIV so here I briefly describe the immune system response to HIV in someone with HIV infection. This information is reviewed in (Appay et al. 2008).

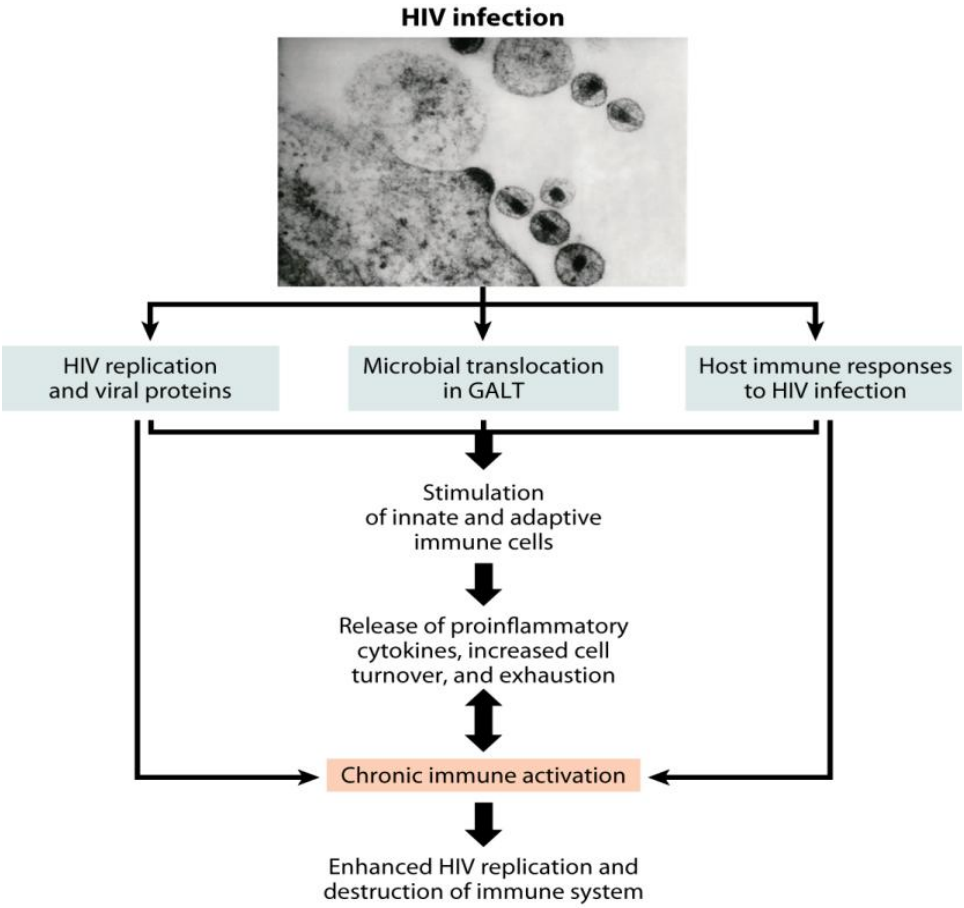


Figure 1.3 Immune response following HIV infection (Moir et al. 2011)
GALT = Gut associated lymphoid tissue

Following exposure to HIV, CD4 T-cells become activated and release interferon gamma and this is one mechanism by which macrophages also become activated. These activated macrophages then secrete proinflammatory cytokines such as TNFα, IL-1β and IL-6. Chronic immune activation develops causing release of proinflammatory cytokines which then further activate CD4 T-cells and a cycle is created (Figure 1.3).

Chronic immune activation results directly from antigenic stimulation by HIV, HIV gene products and indirectly by other viruses such as cytomegalovirus (CMV). Furthermore, systemic inflammation results from gut microbial translocation attributed to depletion of mucosal CD4 T-cells (Brenchley et al. 2006).

Inflammation-related dysfunction and immunosenesence results from chronic immune activation and inflammation. Indeed, chronic inflammation has been linked with neural pathology and the development of chronic pain states (White et al. 2007). The role of chronic inflammation in HIV-SN will be discussed now.

HIV does not directly infect neurons (Gabuzda 1990; Wesselingh et al. 1994; Bachis et al. 2006) but generates an inflammatory response which is neurotoxic leading to neuronal cell death and axonal degeneration by mechanisms involving mitochondrial dysfunction. In models of HIV-SN, pain is frequently used as a measure of neurotoxicity and so the evidence of the pathogenesis of HIV-SN and associated pain is presented together here.

To reiterate HIV-SN is a result of certain toxicities:

- that of HIV itself and the inflammatory response to HIV
- the toxicity of NRTIs alone and in combination with viral proteins

1.2.1 Neurotoxicity of HIV and the inflammatory response

There are two sites for neurotoxic action in HIV-SN: the axon and the soma (Melli et al. 2006). Using models of viral neurotoxicity that have mainly been recreated using the viral coat protein HIV-gp120 two distinct modes of axonal toxicity have been demonstrated (see figure 1.4):

- Directly, gp120 binds to cell surface receptors CXCR4 and CCR5 activating an apoptotic caspase pathway in the axon;
- Indirectly, exposure of the cell body of the DRG to gp120 stimulates apoptosis mediated by Schwann cells leading to axonal degeneration (Melli et al. 2006).

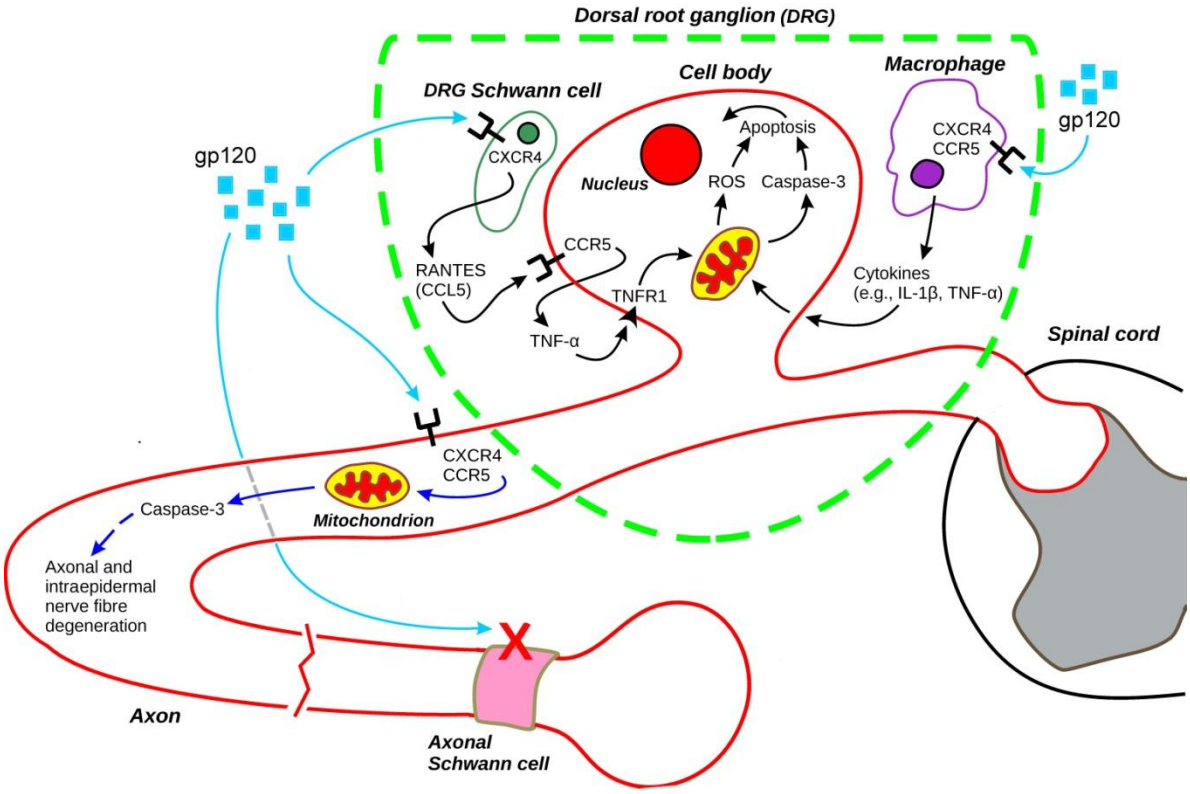


Figure 1.4 Mechanisms of neurotoxicity produced by exposure to HIV-gp120. Taken from (Kamerman et al. 2012a)

In an *in vitro* model where cultured sensory neurons were compartmentalized, separating DRGs from axons, elimination of Schwann cells using the chemotherapeutic agent cytosine arabinoside (which is toxic to Schwann cells) prevented gp120-induced toxicity in the cell bodies but not the axons. Additionally, direct application of gp120 to the compartmentalized axons led to reduced neurite length and neuronal apoptosis, which could be prevented by the addition of zVAD, a caspase inhibitor (Melli et al. 2006). These results demonstrate that direct axonal toxicity is independent of Schwann cells but involves a caspase pathway. Caspase-3 in particular was implicated following inhibition of caspase-3 using a capase-3 antibody which prevented neuritic degeneration and apoptosis (Keswani et al. 2003a; Melli et al. 2006).

The role of TNFα has been implicated in animal models of HIV-SN. Cats infected with feline immunodeficiency virus (FIV) displayed increased TNFα expression in the

peripheral nerves (Kennedy et al. 2004). Additionally, following both epineural and perineural gp120 exposure local TNFα expression increased at the site of application and in the DRG in rats but the cellular origin was not identified (Herzberg et al. 2001; Zheng et al. 2011a). In the indirect mode of neurotoxicity described above and shown in Figure 1.4. Gp120 neurotoxicity is thought to be mediated via a cascade that includes CXCR4, CCR5 and TNFα (Keswani et al. 2003a). Schwann cells are integral to this mechanism of neurotoxicity and release CCL5 which having bound to CCR5 stimulate the release of TNFα (Melli et al. 2006).

In addition to the direct and indirect neurotoxicity, perineural exposure of gp120 has been associated with activated macrophages which surround and infiltrate the nerve and DRG [see Figure 1.4] (Herzberg et al. 2001; Kennedy et al. 2004; Wallace et al. 2007a; Hahn et al. 2008) and reduced IENFD (Wallace et al. 2007a). Additionally in a SIV model macrophage infiltration of the peripheral nerve is associated with reduced nerve conduction velocity (Laast et al. 2011). Activated macrophages release cytokines and following macrophage infiltration increased cytokine expression of IL1β, TNFα and IL-6 are seen in the DRGs of rats and HIV-positive patients at autopsy including those with HIV-SN (Yoshioka et al. 1994; Rizzuto et al. 1995; Nagano et al. 1996; Zheng et al. 2011a). Similar macrophage activation was seen using an *in vitro* model of HIV-1 viral protein [Vpr] (Acharjee et al. 2010).

Figure 1.4 also highlights the role of the chemokine receptor, CXCR4. *In vitro* binding of gp120 to CXCR4 receptors led to mitochondrial dysfunction as measured by loss of mitochondrial membrane potential and subsequent apoptosis in an HEK-293 cell line (Roggero et al. 2001). These results have been repeated in neuronal cells. When dissociated human fetal derived DRG neurons were exposed to supernatants of macrophages infected with CXCR4 or CCR5-tropic strains of HIV, mitochondrial transmembrane potential was reduced indicating mitochondrial dysfunction (Hahn et al. 2008). Interestingly different mechanisms of mitochondrial dysfunction appear to occur in the cell body and the axon (see Figure 1.4). In the cell bodies the role of reactive oxygen species (ROS) was demonstrated by inhibition of ROS by the

antioxidant Trolox resulting in maintenance of mitochondrial membrane potential (Hahn et al. 2008). By comparison, in the axons of macaques infected with a simian model of HIV (SIV) dysfunction of mitochondrial respiratory chain complexes was associated with damaged mitochondrial DNA with subsequent apoptosis of the mitochondria (Lehmann et al. 2011). Common to mitochondrial apoptosis both in cell bodies and the axon is the release of caspase-3 which can be prevented by a caspase inhibitor (Keswani et al. 2003a). Further discussion of oxidative stress in neuropathy is described in Section 1.3.2.2.

Nerve exposure to gp120 causes allodynia in rodents (Wallace et al. 2007a; Bhangoo et al. 2009; Zheng et al. 2011a) and gp120 has two main modes of inducing hypernociception as shown in Figure 1.5:

- Gp120 binds to CXCR4 on axon terminals generating neuronal excitation;
- macrophage infiltration around the nerve causes release of CCL2 and TNFα leading to hypernociception in the DRG and the axons (Oh et al. 2001; Wallace et al. 2007a; Bhangoo et al. 2009; Zheng et al. 2011a)

Mechanical allodynia was evident for 20-30 days after epineural gp120 exposure which corresponded with the length of time TNFα was expressed in the nerve trunk (Herzberg et al. 2001). The role of TNFα in gp120-induced hypernociception was more convincingly demonstrated when following perineural gp120 exposure TNFα expression increased in the spinal dorsal horn and DRG neurons and the resultant allodynia was ameliorated by the administration of soluble TNF receptor or TNFα siRNA (Zheng et al. 2011a).

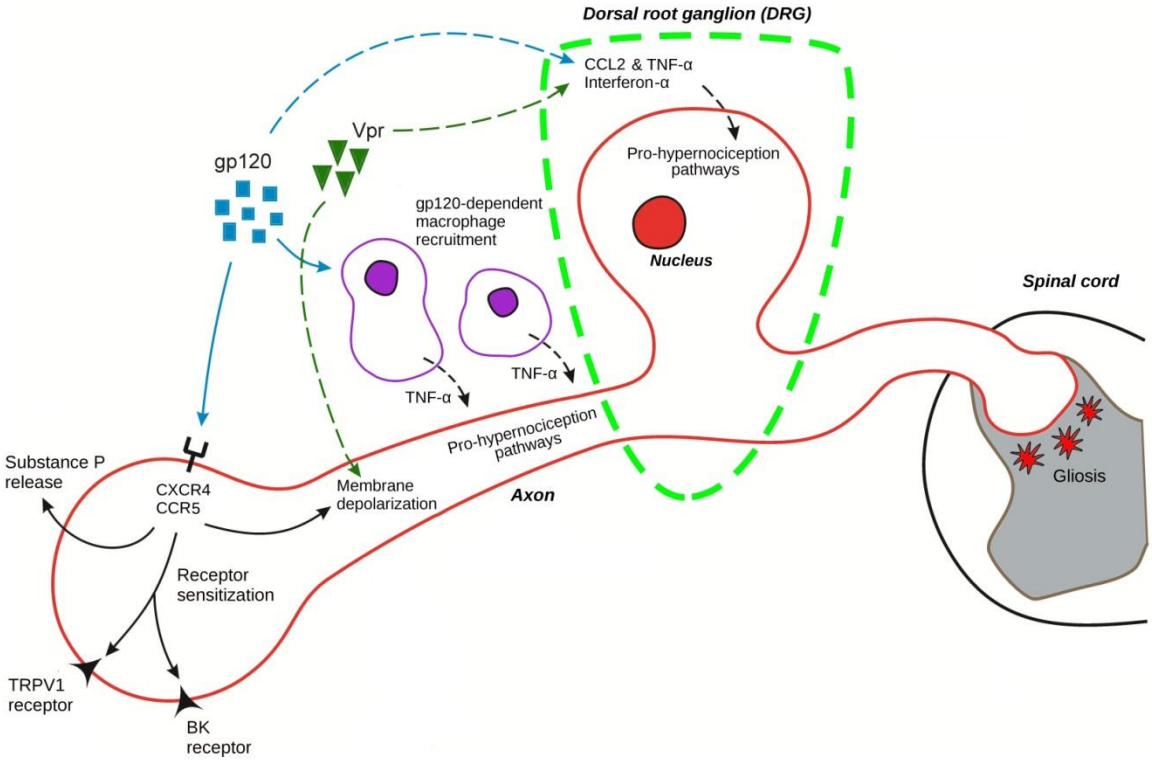


Figure 1.5 Viral proteins of HIV with cytokines and chemokines generate hypernociception in peripheral nerves and spinal dorsal horn. Taken from (Kamerman et al. 2012a)

Oh and colleagues (2001) found that CXCR4 and CCR5 were expressed by nociceptive neurons and intradermal injection separately of gp120, CCL5, CXCL12 and CCL22 that bind to CXCR4 and CCR5 led to allodynia in rats. Generation of peripheral sensitization was demonstrated by both chemokines and gp120 stimulating release of substance P from neurons that also had activated TRPV1 and bradykinin receptors (Oh et al. 2001).

Further evidence of chemokine involvement in gp120-induced hypernociception has come from increased CCL2 and CCR2 expression in DRG neurons following perineural gp120 exposure and additionally, administration of a CCR2 receptor antagonist reversed the accompanying mechanical allodynia (Bhangoo et al. 2009). Expression of CCR2 in gp120 exposed rats was no more increased following the

additional administration of ddC (Bhangoo et al. 2009) suggesting that this response may be particular to gp120.

Centrally, gliosis occurs for about 30 days following peripheral gp120 exposure (Herzberg et al. 2001) and glia have been implicated in the hyperalgesia and allodynia following central gp120 exposure as glial inhibition by fluorocitrate and CNI-1493 prevented gp120-induced thermal hyperalgesia and mechanical allodynia (Milligan et al. 2000). These inhibitors also block proinflammatory cytokines so raising the possibility of other mechanisms of action. However, the role of glia was more convincingly demonstrated by use of the selective microglia inhibitor, minocycline, which delayed the onset of acute gp120-induced allodynia (Ledeboer et al. 2005). The mechanism suggested was TNFα, IL-1β and IL-10 release by the microglia as measured by reduced mRNA expression of the cytokines in the lumbar dorsal spinal cord. In another model of persistent neuropathic pain, administration of minocycline also delayed the onset of allodynia associated with chronic sciatic inflammatory neuropathy suggesting a role for microglia in chronic neuropathic pain states. However, the role of these cytokines in a model of gp120-mediated peripheral neuropathy has not been shown, however, microglial activation does occur (Ledeboer et al. 2005; Wallace et al. 2007a). Although not demonstrated this insinuates that TNFα, IL-1β and IL-10 may play a role in the chronic neuropathic pain associated with gp120 exposure.

Another cytokine implicated in HIV-SN pain is interferon-α which has been implicated in a model using the alternative viral protein, HIV-1 viral protein R (Vpr) (Acharjee et al. 2010). Cultures of human DRGs exposed to Vpr displayed neurite retraction and increased transcripts of interferon-α mRNA. Immunosupressed mice expressing Vpr demonstrated mechanical allodynia and also expressed greater numbers of interferon-α mRNA transcripts in the DRGs and sciatic nerves (Acharjee et al. 2010). However, a definite role for interferon-α in HIV-SN was not shown.

1.2.2 Neurotoxcity from NRTIs alone and combination of viral proteins and NRTIs

Experimental exposure to NRTIs, and in particular ddC, produces signs and behaviours suggestive of neural dysfunction. These signs and behaviours are more completely recreated and more closely resemble the clinical condition of HIV-SN when models incorporate both viral proteins and NRTIs (Wallace et al. 2007b; Bhangoo et al. 2009). It has been hypothesised that the increase in inflammatory cytokines secondary to HIV infection sensitise the neurons making them more susceptible to the toxicity of NRTIs (Keswani et al. 2002; Cossarizza et al. 2004). Clinically, this may explain the progression of a pre-existing subclinical neuropathy to a symptomatic neuropathy following ART initiation (Keswani et al. 2002; Kallianpur et al. 2009). Here I discuss the experimental evidence for neural dysfunction created by both NRTIs alone and in combination with viral proteins.

Animal models of NRTI-induced neuropathy have been challenging and where successful, models most commonly employed ddC. This drug is no longer produced for human consumption due to its toxicity. Robust rodent models of HIV-SN using other NRTIs have been more difficult to establish (Warner et al. 1995; Weber et al. 2009) and/or have not produced (Keswani et al. 2006; Zhu et al. 2007)(Keswani et al. 2006; Zhu et al. 2007)(Keswani et al. 2006; Zhu et al. 2007)all the signs of SN, for example reduced IENFD and neuronal loss in a ddI model (Keswani et al. 2006; Zhu et al. 2007; Wallace et al. 2007b). This is consistent with the lesser neurotoxicity of other NRTIs compared to ddC in patients with HIV (Yarchoan et al. 1988; Lambert et al. 1990; Browne et al. 1993).

Animal derived DRG cultures have been also been used to model NRTI-induced neurotoxcity. Neuronal injury, measured by soma atrophy and neurite retraction in non-HIV exposed DRG cultures from mice and cats has been described following exposure to ddC, d4T and ddI (Keswani et al. 2006; Zhu et al. 2007). A single dose of intra-peritoneal ddC in rats caused alterations in myelin structure distal to the DRG and upregulation of CXCR4 receptors in the DRG (Bhangoo et al. 2007).

Additionally, following exposure to ddC, ddI and d4T, loss of mitochondrial membrane potential was observed (Keswani et al. 2006) which, as previously mentioned, is a sign of mitochondrial dysfunction.

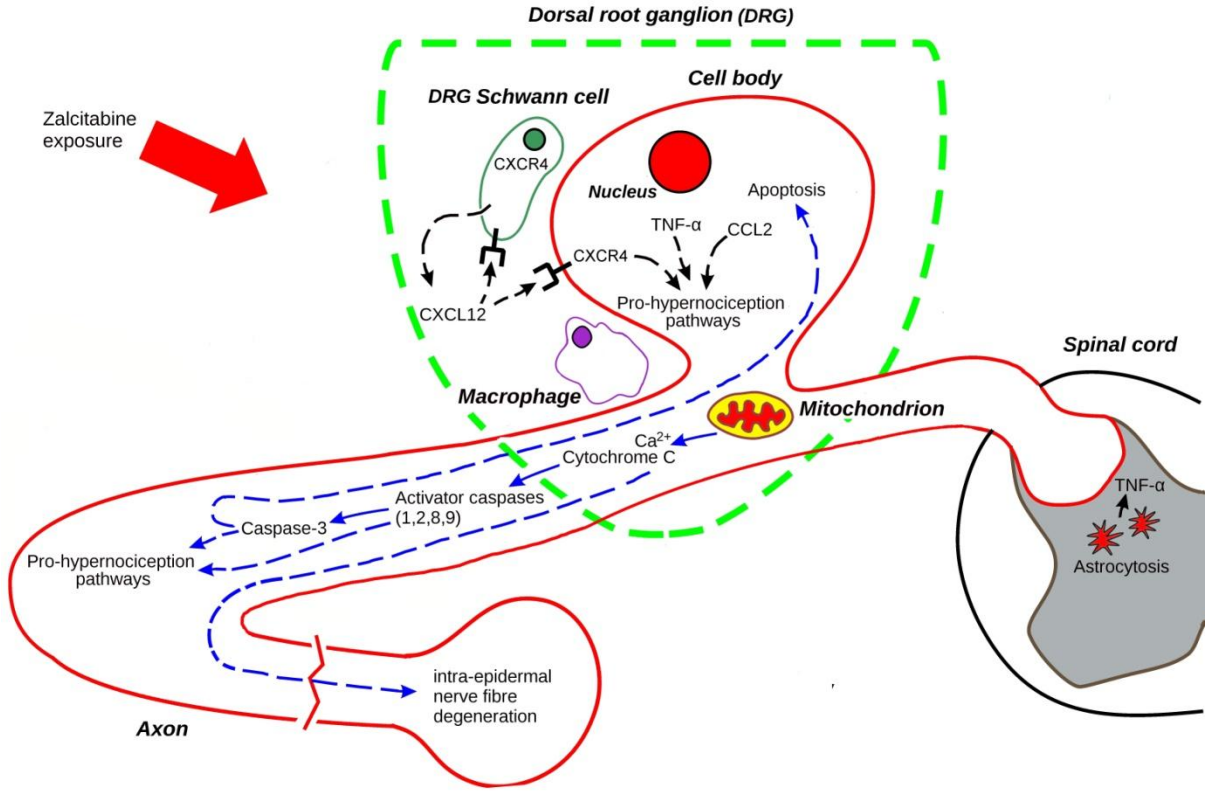


Figure 1.6 Generation of hypernociception following zalcitabine exposure (Kamerman et al. 2012a)

Further evidence of ddC-induced neural dysfunction has been demonstrated following administration of ddC that led to subsequent dysfunction in myelination of peripheral nerve fibres (Bhangoo et al. 2007) and reduced IENFD (Wallace et al. 2007b) in rats which has been associated with increased pain intensity in humans (Polydefkis et al. 2002; Zhou et al. 2007). Indeed, administration of ddC in rats, whether orally, intravenously or intraperitoneally, single or repeated doses has led to behaviours suggestive of mechanical hyperalgesia and allodynia (Joseph et al. 2004; Joseph et al. 2006; Bhangoo et al. 2007; Wallace et al. 2007b; Zheng et al. 2011b) and suggested mechanisms are shown in Figure 1.6.

Mechanical allodynia followed gp120 exposure and worsened after ddC administration (Wallace et al. 2007b; Bhangoo et al. 2009), supporting the hypothesis that HIV and NRTIs have an additive or synergistic effect. However, whilst there was no difference between control rats and those exposed only to gp120, thermal thresholds reduced in those exposed to gp120/ddC (Bhangoo et al. 2009). These data suggest that there may be some mechanisms of hypernocieption particular to NRTIs. Indeed whilst there was upregulation of CXCR4 receptors on DRG neurons in gp120/ddC exposed rats there was no appreciable upregulation in those only exposed to gp120. Additionally, following administration of AMD3100, a CXCR4 antagonist, allodynia was reversed in gp120/ddC exposed rats but not in gp120-only rats (Bhangoo et al. 2009). Indeed, reversal of allodynia in the gp120/ddC rats was similar to that seen in ddC-only treated rats given AMD3100 (Bhangoo et al. 2007) suggesting that CXCR4-mediated hypernociception is particular to ddC-induced hypernocieption.

Another algogenic mechanism implicated in HIV-SN is CXCL12/CXCR4 signalling. CXCL12 (SDF-1) is the chemokine which binds to CXCR4. CXCL12 mRNA expression in the DRG Schwann cells increased following ddC-only exposure with associated increased calcium signaling suggesting a role for CXCL12/CXCR4 signalling in the generation of ddC-induced hypernociception (Bhangoo et al. 2007).

In some of the most convincing evidence to date of TNFα's role in NRTI-associated pain a single high dose of ddC intraperitoneally in non-HIV exposed rats produced mechanical hypersensitivity within 3 days which lasted 7 weeks (Zheng et al. 2011b). Following ddC administration astrocytosis occurred which led to increased TNFα mRNA and protein in the spinal dorsal horn. A concurrent increase in TNFα protein was also observed in the DRG. Blocking of TNFα by administration of TNF soluble receptor and separately, TNF siRNA attenuated the allodynia.

CCL2 has been implicated in pain generation in models of neuropathic pain following DRG compression and sciatic nerve ligation (Tanaka et al. 2004; Sun et al. 2006). CCL2 expression was significantly increased in DRGs of rats following exposure to gp120 and ddC individually and even more so following co-administration (Wallace et al. 2007b). However, expression of CCL2's one receptor, CCR2 did not show increased expression following ddC exposure (Bhangoo et al. 2007) and Wallace and colleagues did not inhibit CCL2 so its involvement in HIV-SN pain requires confirmation.

Intracellular calcium too has been linked to NRTI-induced pain: Repeated oral administration of ddC and single intravenous doses of ddC, d4T and ddI caused mechanical hyperalgesia and allodynia in rats (Joseph et al. 2004). Administration of two intracellular calcium buffers (Quin-2 and TMB-8) ameliorated the hyperalgesia and allodynia suggesting a calcium-dependent aspect to NRTI-induced hypernociception. Furthermore, inhibition of mitochondrial electron transport chain complexes ameliorated the TNFα-generated hyperalgesia caused by a single intravenous dose of ddC and implicated cytochrome c and caspase pathways in ddC-induced mitochondrial dysfunction (Joseph et al. 2006). As intracellular calcium is regulated by mitochondria (Carafoli 2012) it is feasible that mitochondrial dysfunction may lead to disruption of intracellular calcium regulation and subsequent pain.

Reduced mitochondrial DNA and axonal degeneration are seen in HIV-positive individuals both with and without exposure to NRTIs but the extent of the dysfunction is greater in those exposed to NRTIs (Dalakas et al. 2001; Cote et al. 2002; Cossarizza et al. 2004). HIV uses viral reverse transcriptase to replicate and NRTIs block this enzyme so halting viral replication. However, NRTIs also inhibit mitochondrial DNA polymerase gamma, the enzyme responsible for mitochondrial DNA maintenance and synthesis (Kakuda 2000). This action results in tissue damage, particularly in metabolically active cells like the nerves (Cossarizza et al. 2004), and more specifically in impedance of neurite regeneration leading to the

signs and symptoms of neuropathy (Cui et al. 1997). Neural damage may also result from a reduction in energy production and an increase in reactive oxygen radicals which would typically be neutralised by the mitochondria (Brinkman et al. 2000).

In summary, gp120 and certain NRTIs activate neurotoxic pathways in both the DRG and axon which result in neural degeneration and pain. These pathways frequently involve macrophage infiltration, proinflammatory cytokine release and subsequent mitochondrial dysfunction. These pathways have only been demonstrated to date in experimental studies and in autopsy patients. Indeed retrieving live human tissue affected by HIV-SN is difficult but genetic studies can provide circumstantial evidence for the role of proteins in disease *in vivo*. So next I describe genetic evidence available for mitochondria, TNFα, and other proteins implicated in HIV-SN.

1.3 Genetic risk factors

Genetic variation within regions encoding inflammatory responses and mitochondrial function have been implicated in the development of HIV-SN (Kamerman et al. 2012b). Single nucleotide polymorphisms (SNPs) and haplotypes in genes encoding cytokines, including TNFα have associated with SN. Additionally, SNPs in genes encoding mitochondrial DNA, uncoupling proteins and HFE have been implicated in the development of HIV-SN. The evidence will be discussed now followed by evidence of GCH1, a gene associated with reduced pain intensity in neuropathic pain but not previously assessed in HIV-SN.

1.3.1 Genetic evidence of a role of inflammation in HIV-SN

As discussed in Section 1.2 an inflammatory etiology to HIV-SN has been suggested. In support of this, cytokine genotype has been associated with presence of HIV-SN (Affandi et al. 2008; Cherry et al. 2008; Chew et al. 2010) (Table 1.1). In the paper by Cherry and colleagues (2008) a variety of single nucleotide polymorphisms (SNPs) from genes encoding cytokines was assessed. Of the SNPs

demonstrating an association with HIV-SN, rs3212227 in the region of IL12B 3'UTR (which encodes *IL12 p40*) was associated with protection against HIV-SN in Caucasians. Other cytokine SNPs from *IL1A*, *IL1B, IL6, IL10* and *IL18* showed no association. Interestingly despite the implication of chemokines in HIV-SN and the associated pain no genetic studies have assessed chemokine SNPs.

The *BAT1* and *TNFA* genes reside on chromosome six in the major histocompatability complex (MHC). SNPs TNF-1031 [rs1799964] and BAT1(intron10) [rs9281523] have been associated with risk of HIV-SN in Indonesians, Caucasians and Chinese populations (Affandi et al. 2008; Cherry et al. 2008; Chew et al. 2010). The association of a *TNF* SNP supports the evidence described in Section 1.2 associating TNF with the pathogenesis of HIV-SN and the associated pain. This region, which contains many immune-related genes, has been implicated in rheumatoid arthritis, type 1 diabetes and venous leg ulcers (Martinez et al. 2000; Tan et al. 2010; Kumar et al. 2012). The 8.1 ancestral haplotype (HLA-A1, B8, DR3) is present in approximately 15% of Caucasians and is uniquely marked by the SNP described here from BAT1(intron10). This haplotype is associated with several immunopathological disorders and has been shown to be a better disease marker than individual polymorphisms (Price et al. 1999; Allcock et al. 2004; Valente et al. 2009a).

There is high linkage disequilibrium (LD) in the MHC and so detection of causative SNPs for disease has been difficult. Due to the association between TNFA-1031 and HIV-SN in the Caucasian, Chinese and Indonesian studies (Affandi et al. 2008; Cherry et al. 2008; Chew et al. 2010) the region in the MHC surrounding *TNFA* was further investigated. Previously a TNF block of 38 SNPs in a 45kb range of this SNP had been identified from 999 individuals in healthy populations of multiple ethnicities (Figure 1.7) and 31 haplotypes (FV1-FV31) generated (Valente et al. 2009a). Now, a smaller selection of 11 haplotypes, which all contained the minor allele of TNF-1031 were analysed for association with HIV-SN in HIV-positive cohorts with and without HIV-SN (Chew et al. 2010). The 11 haplotypes were identifiable by six SNPs from

the promoter regions of *NFKBIL1* and *TNF*, and promoter and intron regions of *BAT1*. Association was found with a haplotype, FVa6,7,8, in Malay and Chinese cohorts with HIV-SN following correction for age and height (Chew et al. 2010).

There is significant ethnic variability in the haplotypes found in this region (Valente et al. 2009b). Whilst similarities were found between a haplotype containing the BAT1(intron 10) SNP in Caucasian and Gambian cohorts (Price et al. 2003), these similarities cannot be generalized to other African cohorts and haplotypes due to genetic variability within Africa (Tishkoff et al. 2009) and further African studies are required.

Table 1.1 Genetic studies supporting an inflammatory role in HIV-SN

Author	N	Ethnicity	Independent associations	
			Increased risk of SN	Reduced risk of SN
Affandi et al., 2008	96 (33 SN+, 66 SN-)	Indonesian (ethnically Malay)	TNFA-1031*2	-
Cherry et al., 2008	36 (16 SN+, 20 SN-)	98% Caucasian	TNFA -1031*2 BAT1 (intron10)	IL12B(3'UTR)
Chew et al., 2010	175 (57 SN+, 118 SN-)	Malay (n=64) Chinese (n=74) Caucasian (n=37)	TNFA-1031*2[a] FVa6,7,8[a,b,c]	-

[a] *Association in Chinese individuals;* [b] *Association in Malay individuals*

[c] *Haplotype named FVa6,7,8 comprises six SNPs in promoter regions of BAT1, NFKBIL1 and TNFA-1031*

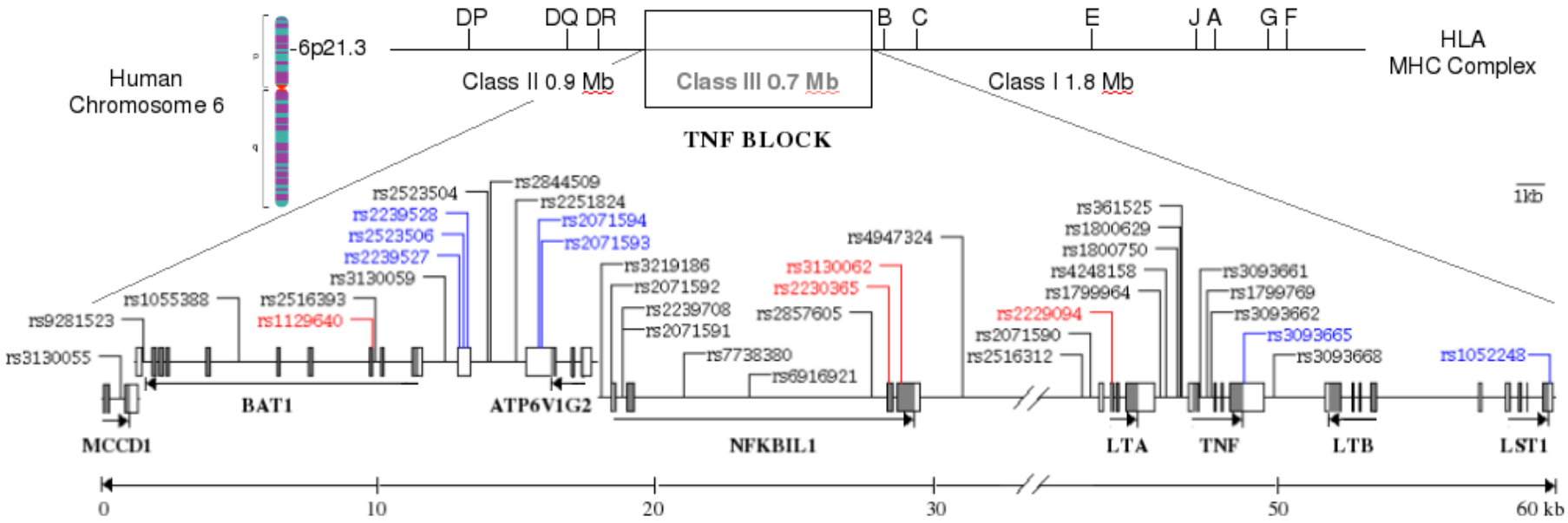


Figure 1.7 Genes and SNPs of the TNF block within the MHC
SNPs were identified in seven of the eight genes of this genomic segment. The direction of transcription is indicated by arrows above the name of each gene.

Exons are shown by boxes. Translated mRNA is indicated by black boxes. Color key for SNPs: Blue: in untranslated region; Red: in translated region; Black: in intronic region. The diagram is to scale.

Figure and legend taken from (Valente 2008).

1.3.2 Genetic evidence of mitochondrial dysfunction in HIV-SN

1.3.2.1 Mitochondrial DNA

Combinations of mitochondrial DNA SNPs form haplogroups that have been associated with neurodegenerative disorders (Howell et al. 2005). Studies using the AIDS Clinical Trials Group Study 384 subjects investigated mitochondrial markers for association with HIV-SN. Mitochondrial haplogroup T associated with an increased prevalence of HIV-SN in Caucasians compared to controls (Hulgan et al. 2005). This association was independent of age, sex, choice of ART and baseline CD4 T-cell and viral load counts. Next, the group assessed polymorphisms within mitochondrial haplogroup T, MTND1*LHON4216C [4216C] and MTND2*LHON4917G [4917G], which have been associated with Leber's Hereditary Optic Neuropathy (LHON) (Brown et al. 1992). The 4917G polymorphism associated with increased presence of HIV-SN, independently of the aforementioned risk factors (Canter et al. 2008). Mitochondrial haplogroup T is only common in Caucasians of North European descent, but the importance of mitochondrial risk for HIV-SN have been realised with data from the Hulgan group showing that mitochondrial subhaplogroups in Africans, specifically subhaplogroup L1c, independently associated with presence of HIV-SN in African Americans (also from the ACTG 384 trial group) (Canter et al. 2010).

1.3.2.2 Uncoupling proteins 2 and 3

Uncoupling proteins (UCP) are mitochondrial inner membrane proteins which dissipate the proton gradient during respiration. Protons then re-enter the cell without creating ATP in the process. In this way ATP synthesis and respiration are 'uncoupled' (Harper 1997). Despite their name, the primary role of UCP2 and UCP3 is more likely regulation of reactive oxygen species (ROS) created during respiration (Negre-Salvayre et al. 1997; Arsenijevic et al. 2000; Vidal-Puig et al. 2000).

Polymorphisms of *UCP2* and *UCP3* associated with diabetic neuropathy in Caucasians with type 1 diabetes (Rudofsky et al. 2006) and there are three aspects

that link uncoupling proteins potentially with the pathogenesis of HIV-SN: oxidative stress, mitochondrial dysfunction and calcium regulation.

Although oxidative stress, measured by plasma F2-isoprostanes, was not found to be associated with developing HIV-SN (Hulgan et al. 2006), oxidative stress is integral to the initiation of neuronal apoptosis and the pathophysiology of neuropathy (Low et al. 1997; Luo et al. 1998; Park et al. 1998; Stevens et al. 2000) and mitochondrial dysfunction and oxidative stress are suggested to contribute to HIV-SN (Hahn et al. 2008; Lehmann et al. 2011). Thus the association warrants investigation in a model of HIV-SN.

Gp120 exposure causes large increases in intracellular calcium thought to lead to the development of HIV-SN (Keswani et al. 2003a; Verkhratsky et al. 2008; Hoke et al. 2009) and UCP3 is involved in regulating intracellular calcium via the sarcoplasmic reticulum (De Marchi et al. 2011).

UCP2 is expressed in many tissues including neurons (Fleury et al. 1997; Rousset et al. 2004) whereas UCP3 is expressed mainly in skeletal tissue but has also been shown to be present in DRGs (Vidal-Puig et al. 1997; Krook et al. 1998; Vincent et al. 2004). The minor alleles of the SNPs associated with diabetic neuropathy (-866G/A *UCP2*; C-55T *UCP3*) have also associated with increased mRNA expression in other cell lines in Caucasians and Indians (Schrauwen et al. 1999; Krempler et al. 2002). Current data do not establish whether the association between these two SNPs and diabetic neuropathy in Caucasians is causative, or may relate to LD with another gene. These SNPs have not been investigated in an African cohort with or without HIV-SN.

1.3.2.3 HFE mutations

Altered iron regulation leads to increased oxidative stress and subsequent neuronal dysfunction and neurodegenerative disorders (Zecca et al. 2004; Connor et al. 2006; Salvador 2010). HIV-1 interferes with iron homeostasis via downregulation of the human hemochromatosis protein HFE (Drakesmith et al. 2005). Kallianpur et al (2006) hypothesised that alterations in iron homeostasis caused during HIV infection may cause neuronal damage and potentially HIV-SN and they found a mutation in the *HFE* gene (C282Y) associated with protection against HIV-SN (Kallianpur et al. 2006) in individuals on stavudine and didanosine. This was not replicated in another study using a small heterogenous patient population (Costarelli et al. 2007). Another *HFE* mutation (187 C>G) has been associated with another ARV side effect involving mitochondrial dysfunction: lipoatrophy (Hulgan et al. 2008). Neuropathy studies of the HFE mutations to date have mostly involved Caucasians as the mutations only occur frequently in this ethnicity (Hulgan et al. 2008), and thus the broader implications for HIV-SN in other populations are unclear.

1.3.3 Genetic factors affecting pain in HIV-SN

Increased viral load, which associates with lower IENFD (Simpson et al. 2002), is a measure of more severe HIV disease and so it is not surprising that both increased viral load (Simpson et al. 2002) and reduced IENFD have been associated with worse pain severity on several scales (Polydefkis et al. 2002; Herrmann et al. 2006; Zhou et al. 2007). Pain intensity therefore, could be considered a measure of disease severity in HIV-SN and additionally, increasing pain intensity in HIV-SN associates with worsening quality of life (Keltner et al. 2012). Here I discuss genetic factors which may affect pain in HIV-SN.

Polymorphisms in *GCH1* have been associated with intensity of neuropathic pain. This gene encodes the rate limiting enzyme GTP cyclohydrolase (GCH1) essential to the production of tetrahydrobiopterin (BH4) which in turn is involved in the processing of the algesic molecules catecholamines, nitric oxide and serotonin

(Thony et al. 2000). Increased pain results from increased BH4 concentrations. *GCH1* transcription and translation influence BH4 concentrations and can be reduced in association with a 15-SNP haplotype in *GCH1 (*Tegeder et al. 2006*)*.

BH4 is induced in primary sensory neurons and dorsal root ganglions following axonal injury. Also increased sensitivity to pain in nociceptive, neuropathic and inflammatory pain models follow intrathecal injection of BH4. This is prevented by administration of a GCH1 antagonist (Tegeder et al. 2006). GCH1 was upregulated in the dorsal root ganglion following peripheral nerve injury (Costigan et al. 2002).

1.3.3.1 GCH1 genotype and pain

A 15-SNP haplotype and five SNPs independently associated with reduced neuropathic pain in a model using leg pain after lumbar discectomy surgery (Tegeder et al. 2006). The group subsequently showed that a three-SNP haplotype could identify the 15-SNP 'pain protective' haplotype with 100% sensitivity and 100% specificity in Caucasian populations (Lotsch et al. 2007).

Three further studies found positive associations with *GCH1* and pain intensity in Caucasians and mixed race cohorts (Tegeder et al. 2008; Campbell et al. 2009; Kim et al. 2010) [Table 1.2]. However an equal number of negative studies have been published based on models of dental pain (Kim et al. 2007), visceral pain (Lazarev et al. 2008) and chronic widespread pain (Holliday et al. 2009) in Caucasians [Table 1.3]. The initial study was criticised for potential population stratification and errors in data analysis (Kim et al. 2007). The conflicting results may also arise because pain sensitivity can be specific to the cause of the pain (Tegeder et al. 2008; Kim et al. 2009). Costigan et al (2012) have reported that the *GCH1* haplotype has the greatest effect in neuropathic pain rather than pain associated with nociceptive or inflammatory pain.

Table 1.2 Studies reporting an association between GCH1 haplotype and SNPs and pain intensity

Author/year	n	Ethnicity	Pain phenotype measurement	Pain model	Findings
Tegeder et al, 2006	168 Unclear	Caucasian Unclear	Z score created from aggregated subjective measures (non-validated) of pain during function Z scores from heat, ischemic and pressure pain thresholds and tolerances	Clinical: neuropathic Experimental: nociceptive	15 SNPs assessed. 5 independently associated with persistent leg pain post discectomy for lumbar root pain (rs nos. 8007267, 3783641, 8007201, 4411417, 752688). 15-SNP haplotype associated with pain protection and 2 SNPs (rs nos. 8007267 & 3783641) unique to this haplotype. Homozygotes for this haplotype had significantly reduced pain. Healthy volunteers carrying the pain protective haplotype had reduced pain sensitivity to nociceptive inputs.
Tegeder et al, 2008	32	Caucasian	Von Frey hairs, Pressure algometer,	Experimental: nociceptive, with capsaicin-induced peripheral sensitisation	Carriers of 'pain protective' 15-SNP haplotype had higher thresholds to mechanical and heat pain in presence of sensitisation. No difference between thresholds in those without pre-existing sensitisation.
Campbell et al, 2009	39	19 Caucasian 12 African American 8 Asian	VAS	Experimental: nociceptive	3 SNPs (rs nos. 3783641, 4411417, 752688) independently associated with reduced intensity of capsaicin pain. Together the 3 SNPs accounted for 35% of inter-individual variance in pain.
Kim et al, 2010	69	Caucasian	NRS ODI	Clinical: primarily nociceptive	1 SNP (rs998259, minor allele T) associated with reduced pain and disability scores following surgery for lumbar degenerative disc disease. A particular haplotype was associated with improvements in disability post surgery.

Table 1.3 Studies reporting no association between GCH1 and pain intensity

Author/year	n	Ethnicity	Pain phenotype measurement	Pain model	Findings
Kim and Dionne, 2007	735 experimental, 221 clinical	Caucasian	VAS	Experimental and clinical: nociceptive	38 SNPs analysed. No association found between GCH1 SNPs or haplotypes (from the 38 SNPs) and pain intensity from cold or heat pain or following 3rd molar extraction.
Laserev et al, 2008	396	Caucasian	Non-validated 5-point questionnaire combining severity with chronicity of pain	Clinical: visceral, nociceptive	No association with 2-SNP haplotype and pain patterns in chronic or recurrent acute pancreatitis
Holliday et al, 2009	Unclear	Not recorded – "predominantly from Caucasian geographic area"	Pain questionnaire and body charts (using American College of Rheumatology Criteria)	Clinical: central sensitisation	3-SNP haplotype (rs nos. rs10483639, rs3783641 and rs8007267) not associated with susceptibility to chronic widespread pain

1.4 Non-genetic risk factors for HIV-SN

Risk factors for neuropathy can be divided into three broad categories, namely demographic and anthropomorphic, clinical, and disease-related (Table 1.4). In this section I will describe key factors within these broad categories. Several factors described are addressed in my study and are investigated in Chapter 3.

Table 1.4 Risk factors for HIV-SN

Demographic & anthropomorphic	**Clinical**	**Disease related**
Age	Drug exposure	CD4 T-cell count
Sex	Other illnesses	Viral load
Height		Length of infection
Race		AIDS defining illnesses
		Other measures of disease severity

Rates of and risk factors for HIV-SN are summarised in Tables 1.5 – 1.8:

- Table 1.5 shows these factors before HIV treatment
- Table 1.6 describes after the commencement of ART but before HAART.
- Table 1.7 shows rates and risk factors in the post-HAART era
- Table 1.8 describes post-HAART factors in African studies.

Diagnostic criteria for HIV-SN were inconsistent between studies and so the criteria used in each study have been described in the tables. Objective assessments of large fibre neuropathy (e.g. quantitative sensory testing [QST] and nerve conduction velocities [NCV]) and small fibre neuropathy (QST and intraepidermal nerve fibre density [IENFD]) are not practical in large-scale epidemiological research and neither NCV nor IENFD have good diagnostic efficiency (Polydefkis et al. 2002; Zhou et al. 2007). Neuropathy-free individuals and those with symptoms of neuropathy, but no

signs, have similar findings on QST and investigation of IENFD (Cherry et al. 2005) suggesting that symptoms should not be relied on alone. However, symptoms are associated with the presence of signs (Cherry et al. 2005; Robinson-Papp et al. 2010) and the combination is the preferred method for research studies (Cherry et al. 2005; England et al. 2005). This approach is used in the studies I have undertaken.

Rates of DSP measured before the introduction of ART were 1.5-42% (So et al. 1988; Barohn et al. 1993; Norton et al. 1996; Smyth et al. 2007) and incidence rates of ATN measured in SN-free individuals as they initiated ART were 24-50% (Maschke et al. 2000; Wester et al. 2005; Robinson-Papp et al. 2009). The variability of SN prevalence pre-ART (Table 1.5) demonstrates the disease stage of the cohorts used. Higher rates were recorded in patients with AIDS admitted due to disease complications (So et al. 1988; Norton et al. 1996) with lower rates being recorded in ambulatory, otherwise healthy, patients (Barohn et al. 1993; Smyth et al. 2007). As ART was introduced prevalence rates of SN increased (Tables 1.6 and 1.7) with several studies demonstrating increasing rates with dual and triple therapy regimens (Bacellar et al. 1994; Maschke et al. 2000; Smyth et al. 2007). Although ART coverage varies in African studies, incidence rates of 36-38% and HIV-SN prevalence of 49% have been reported in cohorts assessed using signs and symptoms (Forna et al. 2007; Sacktor et al. 2009; Maritz et al. 2010). This demonstrates the burden of disease is also high in Africa.

Table 1.5 Before ART HIV-SN is more common in people that progress to AIDS

Authors	**Associations with HIV-SN**	**Prevalence/ incidence**	**N**	**Ethnicity**	**Diagnostic criteria/tool**
Barohn et al, 1993	Advanced HIV disease – CD4 count <300 cells/mm^3	1.5%*	798	ND	Clinical ax using symptoms ± signs
So et al, 1988	Advanced HIV disease: >5 months duration of systemic illness, greater weight loss	35%*	37	ND	Clinical ax using symptoms ± signs
Norton et al, 1996	Mycobacterium avium complex infection	42%*	134	ND	Clinical ax using symptoms ± signs
Smyth et al, 2007	Mycobacterium avium complex infection	13%*	100	ND	Screening tool: ACTG BPNS

ND = not described; ax = assessment; ACTG BPNS = AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen

Table 1.6 Disease severity remained a risk factor for HIV-SN in the era of ARVs, but pre-HAART

Authors	Associations with HIV-SN	Prevalence/ incidence	N	Ethnicity	Diagnostic tool used	Neurotoxic NRTI exposure
Bacellar et al, 1994	Advanced HIV disease – CD4 <200 cells/mm^3	34%†	2641	79% Caucasian, 14% African American 6% Hispanic 1% Other	Clinical ax using symptoms ± signs	ND
Childs et al, 1999	Viral load >10,000 copies/mL, CD4 count <750 cells/mm^3	ND	1604	88% White 12% ND	Clinical ax using symptoms ± signs	62% on ART, mainly AZT but also d4T, ddI, ddC; %ND
Tagliati et al, 1999	↑age, ↓CD4 count (<100 cells/mm$^{3)}$	38%*	251	40% Hispanic 29% Caucasian 19% Black	Clinical ax using symptoms ± signs	ND
Schifitto et al, 2002	History of AIDS diagnosis, ↓CD4 count	35%†	272	51% Caucasian 49% ND	Clinical ax using signs only (but described as symptomatic or asymptomatic)	23%

*ND = not described; ax = assessment; † = incidence; * = prevalence*

Table 1.7 Demographic factors and comorbidities became risk factors following the introduction of HAART

Authors	Associations with HIV-SN	Prevalence/incidence	N	Ethnicity	Diagnostic tool used	Neurotoxic NRTI exposure
Affandi et al, 2008	↑ age, ↑ height, carriage of TNF-1031*2	34%*	96	Indonesian	Symptoms + signs BPNS	100% d4T
Ances et al, 2009	Diabetes mellitus type 2, ↑ triglycerides	55%*	130	ND	Signs only	59%
Anziska et al, 2011	↑ age, diabetes, Hepatitis C, African American race	36%*	1369	64% African American 20% Hispanic 9% Caucasian	Signs only	100% HAART, ~6% including dNRTIs
Cherry et al, 2006	Exposure to d4T and ddI	USA 49%* Aus 55%*	147	#13%/97% Caucasian 86%/0% African American 1%/0% Hispanic 0%/1% Asian	Symptoms + signs BPNS	d4T ever: USA 72% Aus 79%
Cherry et al, 2009	↑age and height	Melbourne 42%* KL 19%* JK 34%*	294	34% Caucasian 33% Malaysian 32% Indonesian	Symptoms + signs BPNS	d4T ever: Melbourne 4% KL 47% JK 100%
Evans et al, 2011	↑ age, ↑ height, diabetes, exposure to d4T, ddI, ddC, or a PI	23%*	2140	44% Caucasian 32% Black 21% Hispanic 3% Other	Symptoms + signs BPNS	~24%

*d4T = stavudine; ddI = didanosine; ddC = zalcitabine; PI = protease inhibitor; Aus = Australia; KL = Kuala Lumpur; JK = Jakarta; * = prevalence* # = 1st percentage from USA, 2nd from Australia

Lichtenstein et al, 2005	Age >40, diabetes, white race, nadir <50 cells/mm^3, CD4 T-cell count 50-199 cells/mm^3, VL >10,000 copies/mL, exposure to ddI, d4T, NVP, lamivudine, ritonavir, nelfinavir, saquinavir	13%†	2515	55% Caucasian	Symptoms only	53%
(Nakamoto et al. 2010)	↑age, ↓nadir CD4 T-cell count	31%†	61	51% Caucasian 49% ND	Signs only	57%
Robinson-Papp et al, 2009	↑ age, ↑ CD4 T-cell count, Hispanic race, intravenous drug users	50%†	104	41% Hispanic 38% Black 21% Caucasian	Signs only	50%
Skopelitis et al, 2006	AIDS diagnosis, ↑age, ↓nadir CD4 T-cell count, exposure to 2 neurotoxic NRTIs	36%*	100	98% Caucasian 2% African	Signs only - abnormal nerve conduction velocity and amplitude studies. Clinical SN – symptoms +/- signs	45%
Skopelitis et al, 2007	↑ nadir CD4 T-cell count, more advanced HIV stage, prior exposure to neurotoxic NRTI combinations	37%*	94	95% Caucasian	Signs only – abnormal IENFD	45% single agent, 12% 2 neurotoxic agents
Smyth et al, 2007	↑ age, exposure to d4T, ddI, indinavir	44% 2001* 42% 2006	100	ND. Australian study – likely mainly Caucasian	ACTG BPNS	82% exposure 2001, 58% in 2006
Watters et al, 2004	Age ≥ 50	43%*	174	ND. Hawaiian study	Symptoms + signs	70% of those with HIV-SN

*ND = not described; d4T = stavudine; ddI = didanosine; ddC = zalcitabine; PI = protease inhibitor; VL = viral load; † = incidence; * = prevalence*

Table 1.8 Risk factors reported in African studies are variable demonstrating the variety of ART coverage and content

Authors	**Associations with HIV-SN**	**Prevalence/ incidence**	**N**	**Ethnicity**	**Diagnostic tool used**	**Neurotoxic NRTI exposure**
Forna et al, 2007	Age ≥35 years, TB treatment at baseline	36%†	1029	Black Africans	Symptoms + signs	100% HAART containing d4T
Isezuo et al, 2009	Stage IV HIV disease	2.2%*	322	Black Africans	ND	18% on HAART including d4T
Maritz et al, 2010	ART use, ↑ age, prior TB, ↑ systolic BP, ↑ waist-hip ratios, ↑ serum triglyceride levels	49%*	598	ND but South African	Symptoms + signs BPNS Modified Total Neuropathy Score.	58% on HAART, 34% on d4T currently, 45% d4T ever
Sacktor et al, 2009	Not assessed	38%†	102	Black Africans	Symptoms + signs	100% HAART containing d4T
Van Griensven et al, 2010	↑age, advanced clinical HIV disease, use of 40mg d4T	8%†	2190	Black Africans	Assessed “clinically”	100% HAART containing d4T
Wester et al, 2005	Pre-existing dx of SN, those on d4T+ddI regimens	37%†	153	Black Africans	ND	100% HAART: 64% on regimen containing d4T and ddI

*ND = not described; TB = tuberculosis; † = incidence; * = prevalence*

1.4.1 Demographic and anthropomorphic risk factors

1.4.1.1 Age

Like diabetic neuropathy (Young et al. 1993; Tesfaye et al. 1996) increasing age has a well documented association with HIV-SN (Morgello et al. 2004; Watters et al. 2004; Lichtenstein et al. 2005; Cherry et al. 2006; Wright et al. 2008; Ances et al. 2009; Robinson-Papp et al. 2009; Maritz et al. 2010; Vance et al. 2010; Anziska et al. 2011). This is consistent with declining neurological function with age (Watters et al. 2004; Cherry et al. 2009). Whilst no studies have assessed risk of HIV-SN in a geriatric population the positive association with age suggests that little HIV-SN should be expected in HIV-positive children and indeed, SN-positive children were reported as having a mean age of 14 compared to a mean age of 8 in non-SN children (Floeter et al. 1997). With the aging HIV population expected with widespread access to effective ART, we should expect an increased prevalence of HIV-SN, independent of changes to modifiable risk factors such as exposure to neurotoxic ART, due to the positive association of HIV-SN with age.

1.4.1.2 Sex

It has been reported that women are more predisposed to alcoholic neuropathy (Ammendola et al. 2000) but men more predisposed to diabetic neuropathy (Booya et al. 2005; Aaberg et al. 2008). Numerous studies have found no association between sex and HIV-SN (Schifitto et al. 2002; Cherry et al. 2006; Skopelitis et al. 2006; Skopelitis et al. 2007; Affandi et al. 2008; Sacktor et al. 2009). However, two studies have found an association, one finding males more at risk (Morgello et al. 2004) and one females (Mehta et al. 2011). Male sex remained a risk factor after logistic regression in the cohort described by Morgello and colleagues (2004), but the factors corrected for were not described. Of note, height was not measured and therefore could not have been corrected for. As males are likely to be taller this may have made them more at risk of SN (Cherry et al. 2009). Mehta et al (2011) found women 9.6 times more likely to develop neuropathy than men on univariate analysis and this association remained after correction for age, height, ART regimen, isoniazid exposure, WHO stage and CD4 T-cell count. This is surprising considering

the neuroprotective effects of female sex hormones oestrogen and progesterone which have been documented to promote axonal sprouting, synaptic transmission and myelination following neuronal insult (De Nicola et al. 2006; Crawford et al. 2010). However, the women in Mehta et al's (2011) cohort had a trend for greater body mass indices (BMI) than the men, a risk factor for SN (Ances et al. 2009; Maritz et al. 2010) [see section 1.4.2.2] and had lower haemoglobin levels than the men. Anaemia is associated with lower immune status (Lichtenstein et al. 2008), which in turn has been associated with HIV-SN in non-ART treated cohorts (Barohn et al. 1993; Bacellar et al. 1994; Childs et al. 1999; Tagliati et al. 1999) [see section 1.4.3] and these participants were yet to initiate HAART. Although SN did not associate with WHO clinical staging, rates of SN were lower at higher haemoglobin levels although this was not significant (Mehta et al. 2011).

1.4.1.3 Height

Recent studies have found increasing height to be associated with symptomatic HIV-SN (Affandi et al. 2008; Cherry et al. 2009; Evans et al. 2011). Height has also been associated with other forms of neuropathy (Sosenko et al. 1986; Gadia et al. 1987; Robinson et al. 1992; Cheng et al. 2006). Two mechanisms have been suggested: Cheng et al (2006) hypothesise that taller people have an increased axon surface area which would lead to a greater toxic exposure from HIV resulting in nerve dysfunction. These data are supported by *in vitro* work of Keswani et al, who showed that viral coat proteins are capable of causing damage to neurites in cultured DRG neurons (Keswani et al. 2003a; Keswani et al. 2004). Cherry et al (2009) suggest that a disruption in neuronal energy generation may lead to neuropathy. Such a disruption might occur following mitochondrial dysfunction and the effect may be more pronounced in the longer nerves of a tall person. Indeed both animal and human studies show HIV infection and treatment with neurotoxic NRTIs (e.g. stavudine and zalcitabine) disrupt mitochondrial function and lead to axonopathy (Keswani et al. 2003b; Keswani et al. 2006; Lehmann et al. 2011), so it is possible that these two hypotheses are linked. Furthermore, Lehmann et al. (2011) demonstrate that the common mt DNA^{4977} deletion mutation was increased in distal

nerve segments of humans infected with HIV-SN compared to proximal nerve segments so supporting the hypothesis of a length dependent neuropathy.

1.4.1.4 Race

As different genotypes associate with HIV-SN (Affandi et al. 2008; Cherry et al. 2008; Chew et al. 2010) it is plausible that ethnicity may play a role in HIV-SN (see section 1.3.2). Four studies have found an association between HIV-SN and race; albeit different races. Lichtenstein et al (2005) found Caucasians[1] independently associated with increased risk of HIV-SN but did not describe the other race categories. Similarly, Simpson et al (2006) described that compared to Black Africans and Hispanics, Caucasians had worsening neuropathy over the study period, but the effect was not evident in multivariate analysis (Simpson et al. 2006). In comparison, Robinson-Papp et al (2009) found (on univariate analysis) that Hispanics were at greater risk of developing ATN on ART than African Americans and Caucasians and recently Anziska et al (2011) found African American women more at risk of HIV-SN than women of European or Hispanic descent. This association was independent of other potential risk factors including age, diabetes, alcohol use, use of neurotoxic NRTIs (stavudine or didanosine), CD4 T-cell count and viral load. Additionally Black race was independently associated with increased risk of HIV-SN compared to Caucasians, Chinese and Malays (Cherry et al. 2011). However, 'black race' in this study (Cherry et al. 2011) included both Black Africans and African Americans. Due to significant admixture in the latter group it is likely that Black Africans and African Americans form genetically distinct populations which should not be grouped (Tishkoff et al. 2009). Thus, if ethnicity does influence susceptibility to SN the data currently are equivocal, which may reflect that any ethnic effect is small.

[1] 'Caucasian' refers to individuals of European ancestry and is used here for brevity

1.4.2 Clinical risk factors

1.4.2.1 Drug exposure

Antiretroviral therapy started with monotherapy or dual therapy in the early 1990's. Sensory neuropathy soon became the dose-limiting factor for several nucleoside reverse transcriptase inhibitors (NRTIs), including zalcitabine (ddC), stavudine (d4T) and didanosine (ddI) (Lambert et al. 1990; Browne et al. 1993; Blum et al. 1996). In fact, zalcitabine was withdrawn in 2006 due to its neurotoxic effects (Roche 2006). The combination of stavudine and didanosine increased the rate of HIV-SN by 3.5 times compared to didanosine alone, and by 7.8 times when these two drugs were administered with hydroxyurea, a DNA synthesis inhibitor (Moore et al. 2000). The combination of three or more drugs administered simultaneously is known as highly active antiretroviral therapy (HAART) and was introduced in resource-rich countries from 1996 and guidelines for their delivery in resource-limited countries released in 2002 (WHO 2002). Viral control was greatly improved through the implementation of HAART and drug resistance became rare. However the prevalence of HIV-SN increased in one clinic where patients were surveyed in 1993 (pre-HAART) and again in 2001 (post-HAART) (Smyth et al. 2007).

Of the ARV drugs currently in use the NRTIs stavudine and didanosine have both been associated with neuropathy clinically (Lichtenstein et al. 2005; Wester et al. 2005; Cherry et al. 2006). Didanosine is used less frequently in second line regimens but stavudine remains in first line regimens in many resource-limited countries (WHO 2010). Initially 40mg bd doses of stavudine were used for patients over 60kg, but stavudine has repeatedly been found to cause neuropathy particularly at doses greater than 30mg bd (Lichtenstein et al. 2005; Laurent et al. 2008; van Griensven 2010; Menezes et al. 2011; Maskew et al. 2012; Pahuja et al. 2012). As 30mg bd is as effective for virological control as 40mg bd and has fewer side effects this dosage has been adopted as standard policy (WHO 2006).

Following introduction of non-stavudine based regimens, rates of HIV-SN have not dropped as may be expected (Smyth et al. 2007; Ellis et al. 2010). In individuals where a first line regimen has failed protease inhibitors are often prescribed in second-line therapy (WHO 2010b) and the protease inhibitor, indinavir, has been associated with HIV-SN (Lichtenstein et al. 2005; Pettersen et al. 2006; Smyth et al. 2007; Cherry et al. 2009). One study here is worthy of note: Pettersen and colleagues (2006) studied the effect of indinavir both clinically and *in vitro*. The clinical study showed both use of and exposure to indinavir associated with HIV-SN. In the *in vitro* study DRGs from Sprague Dawley rats were infected with a recombinant infectious HIV-1 clone and then exposed to indinavir. Mean neurite length, number of neurons with processes and neuronal soma size were assessed. There were no significant reductions in these measures in the DRGs post-exposure to HIV or indinavir alone but following exposure to both, the reductions in neurite length were significant suggesting an additive or synergistic effect of HIV and indinavir. The requirement of concurrent HIV infection for indinavir toxicity contrasts with that of stavudine where six percent of individuals prescribed stavudine for post-exposure prophylaxis developed SN following d4T-exposure, despite none of them going on to develop HIV infection (Winston et al. 2005). The mechanisms for the neurotoxicity of PIs are unclear but regimens incorporating PIs are now ritonavir boosted (WHO 2010b). Ritonavir, also a PI, enhances the action of the main PI in a regimen (hence the term ‘boosting’) allowing a lower dose to be used, which may have reduced the incidence of side effects including neuropathy (Lichtenstein et al. 2005). Indeed in a cohort which included both boosted and unboosted PIs no independent association was detected with HIV-SN (Cherry et al. 2009).

Two additional studies have found HIV-SN associated with protease inhibitor use but have not specified the drugs in use at the time (Ances et al. 2009; Evans et al. 2011). Other protease inhibitors such as saquinavir, ritonavir and nelfinavir have also been associated with HIV-SN (Lichtenstein et al. 2005; Pettersen et al. 2006).

1.4.2.2 Comorbid medical conditions

Comorbid conditions may increase the risk of developing HIV-SN potentially due to added neural insults. An example is diabetes mellitus, which is itself a cause of peripheral polyneuropathy and has been associated with increased risk of HIV-SN (Lichtenstein et al. 2005; Ances et al. 2009; Evans et al. 2011). Components of metabolic syndrome other than diabetes have also been associated with HIV-SN including hypertension, obesity and lipid dysregulation (Ances et al. 2009; Maritz et al. 2010). Whilst Maritz and colleagues corrected for diabetes in multivariate analysis Ances et al did not. As it is unclear how hypertension, obesity and lipid dysregulation might cause SN their association may rather be with the glucose dysregulation that occurs concurrently in metabolic syndrome.

Tuberculosis (TB) has only been documented as a risk factor for HIV-SN since studies in Africa have been published (Forna et al. 2007; Maritz et al. 2010; Shurie et al. 2010) where the load of TB infection is high. This suggests that TB may also be a risk factor in other regions, such as India, where a high prevalence of TB also exists. Starting treatment for tuberculosis (TB) concurrently with ART or within a two week period increased chances of stavudine substitution within the first two months of ART seven-fold, mostly due to development of neuropathy (Westreich et al. 2009). Both TB and the TB treatment isoniazid have been associated with increased risk of HIV-SN (Forna et al. 2007; Maritz et al. 2010; Shurie et al. 2010). However, it is not clear from these studies which exactly is the primary cause (Grant et al. 2010; Maritz et al. 2010) because even in the case of pyridoxine administration, used to counteract the neurotoxicity of isoniazid, this treatment has still been associated with HIV-SN (Forna et al. 2007).

In addition to TB, mycobacterium avium complex (MAC) infection is also an AIDS defining illness and has been associated with HIV-SN (Norton et al. 1996; Smyth et al. 2007). However, the association is contentious (Woolley et al. 1997) and the

association may rather be with disease severity as indicated by MAC infection than by the MAC itself.

Peripheral neuropathy occurs in hepatitis C virus infection (HCV), particularly in those with cryoglobulinemia (Yoon et al. 2011; Takada et al. 2012). HCV may have an additive or synergistic effect on risk for HIV neuropathies (Brew 2003; Estanislao et al. 2005) and indeed one recent study did find HCV associated with HIV-SN in a large cohort of American women (Anziska et al. 2011). This study used both presence of HCV antibodies and measurable HCV viral load to denote infection. However, the HIV-SN/HCV association has been disputed elsewhere including in large multi-centre studies (Ances et al. 2009; Cherry et al. 2010) [see Appendix 2]. These earlier studies used less sensitive measures to confirm presence of HCV infection and so it is possible that an association, reported by Anziska et al (2011) was missed. Consequently the association between HIV-SN and HCV remains controversial.

1.4.3 Disease-related risk factors

Peripheral neuropathy in AIDS patients was described within two years of AIDS being identified (Snider et al. 1983; Levy et al. 1985), and a few years later it was also described in those with severe HIV infection (Cornblath 1988; So et al. 1988). In the pre-HAART era disease severity was associated with HIV-SN (see Table 1.5 and 1.6), where increasing disease severity was measured by low CD4 T-cell count (Barohn et al. 1993; Bacellar et al. 1994; Childs et al. 1999; Tagliati et al. 1999), high viral load (Childs et al. 1999) or an AIDS diagnosis (Schifitto et al. 2002; Isezuo et al. 2009).

Probably because most patients on HAART have effective virological control, measures of disease severity generally no longer associate with HIV-SN since the introduction of HAART [see Table 1.7] (Morgello et al. 2004; Wester et al. 2005; Cherry et al. 2006; Evans et al. 2011). However, some studies describe an

association with nadir CD4 T-cell count (Watters et al. 2004; Skopelitis et al. 2006; Robinson-Papp et al. 2009; Ellis et al. 2010). The majority of these studies find a positive association between low nadir CD4 T-cell count and HIV-SN (Watters et al. 2004; Skopelitis et al. 2006; Ellis et al. 2010; Maritz et al. 2010) and corresponds with previously published associations between HIV-SN and increasing disease severity. However, Robinson-Papp et al (2009) found that patients with higher CD4 T-cell counts had a higher risk of neuropathy [70 (SD 164) *vs* 169 (SD 219) cells/ul]. It is likely this can be explained by greater exposure to potentially neurotoxic NRTIs. However, an alternative explanation is neuropathy may occur as a result of immune reconstitution syndrome (IRIS) [reviewed in (Price et al. 2009a)]. IRIS causes pre-existing (usually infectious) diseases to become worse on ART. As the immune system recovers from its previous suppression, an inflammatory response may be mounted potentially causing an asymptomatic condition to become symptomatic. Neuropathy from Guillan-Barré syndrome has been described in this context (Piliero et al. 2003; Capers et al. 2011) but not HIV-SN.

Length of time since HIV diagnosis has been associated with HIV-SN in some cohorts (Maschke et al. 2000; Ances et al. 2009) but not in others (Skopelitis et al. 2006; Affandi et al. 2008). Record of HIV duration is likely confounded by variability in time elapsed between contraction of HIV and when a patient presents for testing and therefore subject to significant error.

1.5 Thesis aims

HIV-SN is painful and significantly affects quality of life, yet is under recognised and under treated. Identification of clinical, demographic and disease risk factors will help clinicians identify patients at risk of HIV-SN so they can monitor and manage these patients to mitigate risk and/or reduce the burden of disease. This thesis identifies clinical and genetic risk factors for the development of symptomatic HIV-SN in Southern Africans.

Risk factors for HIV-SN in patients receiving ART include increasing age and exposure to neurotoxic NRTIs. Some studies also implicate increasing height, race, diabetes, TB, hepatitis C and measures of HIV disease severity. However, HIV-SN studies have largely been restricted to American, European or Asian cohorts. There have been a few recent studies in African cohorts but both diagnostic criteria for neuropathy and exposure to neurotoxic NRTIs have been variable. It may be important that African patients present later for treatment, are more often treated with stavudine and include more women compared to populations in resource-rich countries. I set the scene for the thesis by addressing the following questions in Chapter 3:

- What is the prevalence of HIV-SN in an HIV-positive Southern African cohort exposed to stavudine-based ART?
- Which clinical, disease-related, demographic and anthropomorphic factors associate with presence of HIV-SN?

Hypothesis: age, height, sex and nadir CD4 T-cell count associate with the presence of HIV-SN in a stavudine-treated cohort of HIV-positive individuals of African ancestry.

Genetic studies have contributed to the evidence of involvement of mitochondria and TNFα to the pathogenesis of HIV-SN. However, no genetic studies have been completed in an African population. The minor allele of a *TNFA* SNP, TNF-1031, has been associated with HIV-SN and haplotypes containing the *TNFA* SNP and other immune-related genes have also associated with HIV-SN in Caucasians and Asians. This requires confirmation in Africans. In Chapter 4 I address the following questions:

- Do alleles of TNF-1031 and BAT1(intron10) SNPs associate with HIV-SN in Southern Africans?
- Do alleles of other SNPs within the TNF block associate with HIV-SN?
- Do previously identified TNF block haplotypes associate with HIV-SN?
- Do alleles of other putative haplotype tag-SNPs from the TNF block region associate with HIV-SN?

- Does LD within the region differ in Africans and what does this infer about potential causative SNPs?

Hypothesis: minor alleles of TNF-1031, BAT1(intron10) and previously-identified TNF block haplotypes will associate with HIV-SN in a cohort of HIV-positive individuals of African ancestry on ART.

Alleles of SNPs in cytokine genes other than *TNFA* have been assessed in Caucasian, Chinese and Malay cohorts with only the minor allele of an *IL12* SNP generating a negative association. Chemokines are also involved in the pathogenesis of experimental HIV-SN but have never been associated genetically. In Chapter 5 I address the following questions:

- Do SNPs of cytokines and chemokines: *IL1A, IL1B, IL4, IL10, IL12, IL18, CCL5* associate with presence of HIV-SN?
- Do SNPs of cytokines and chemokines: *IL1A, IL1B, IL4, IL10, IL12, IL18, CCL5, CCL2, CCR2* associate with presence or intensity of pain in HIV-SN?

Hypothesis 1: alleles of cytokine and chemokine SNPs will associate with the presence of HIV-SN in a cohort of HIV-positive individuals of African ancestry on ART.

Hypothesis 2: alleles of cytokine and chemokine SNPs will associate with both presence and intensity of pain in HIV-SN in a cohort of HIV-positive individuals of African ancestry on ART.

Uncoupling proteins 2 and 3 regulate ROS and so are implicated in mitochondrial function. Alleles of *UCP2* and *UCP3* SNPs have been associated with diabetic neuropathy in Caucasians. Uncoupling proteins have not been investigated in HIV-SN either experimentally or genetically. In Chapter 6 I answer the following question:

- Do alleles of *UCP2* or *UCP3* SNPs associate with risk of HIV-SN in Southern Africans?

Hypothesis: alleles of SNPs in *UCP2* (rs659366) and *UCP3* (rs1800849) will associate with HIV-SN in a cohort of HIV-positive individuals of African ancestry on ART.

Finally, *GCH1*, which has associated with reduced intensity of neuropathic pain in another model of neuropathic pain has not previously been assessed in HIV-SN related pain. In Chapter 7 I address the following questions

- Do SNPs of *GCH1* associate with pain protection in HIV-SN?
- Do haplotypes of *GCH1*, including the 'pain protective' haplotype associate with reduced presence or intensity of pain in HIV-SN?Hypothesis

Hypothesis: individual SNPs and a haplotype in *GCH1* will associate with reduced intensity of pain in HIV-SN in a cohort of HIV-positive individuals of African ancestry on ART.

In Chapter 8, I bring together the clinical and genetic risk factors I find in my studies of Southern Africans and discuss the implications and directions for future research.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical aspect

Black African adults who had a confirmed diagnosis of HIV infection and had been on antiretrovirals for at least six months were screened for neuropathy at the Virology Clinic of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, between July 2008 and April 2009. The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, South Africa (protocol number M080220; see appendix 2) and written, informed consent was obtained from all participants. An interpreter fluent in English as well as commonly spoken local African languages facilitated consent and study procedures. The sample was a convenience sample.

Demographic (age, sex, ethnicity) and clinical information (current and nadir CD4 T-cell counts, duration of HIV infection, AIDS-defining illnesses), antiretroviral treatment history and other potential causes of neuropathy (diabetes mellitus, alcoholism, vitamin B12 deficiency, exposure to isoniazid and chemotherapy) were obtained through participant self-recall and their medical files. I personally reviewed all medical records in their entirety. Participants' heights and weights were recorded and a venous blood sample was taken for hepatitis C serology (Abbott AxSYM HCV version 3.0 microparticle enzyme immunoassay, Abbott Laboratories, Abbott Park, IL) and DNA extraction. The hepatitis C blood samples were taken to the National Health Laboratory Service on the same day as collection and the DNA blood samples were stored at 4 ° C until I began DNA extractions. The first 39 participants had saliva samples taken for DNA extraction but following concerns over DNA quantity with ethical clearance I switched to blood samples.

Inclusion criteria

- Age 18 or over
- Confirmed HIV infection
- On antiretrovirals for at least 6 months prior to assessment
- Black African

2.1.1 Neurological assessment

Participants were screened for symptomatic SN using the Aids Clinical Trials Group (ACTG) Brief Peripheral Neuropathy Screen (Cherry et al. 2005) [Appendix 3]. This is a validated tool which was effective in identifying those diagnosed with HIV-SN by IENFD and QST (Cherry et al. 2005) and has been used in a South African population previously (Maritz et al. 2010).

Participants were considered to have a HIV-SN if they had at least one current or prior symptom suggestive of neuropathy (pain, aching, burning, numbness or pins-and-needles) and presented with at least one neuropathic clinical sign of neuropathy bilaterally (reduced vibration sense or absent ankle reflexes). This tool has been validated against objective measures and therefore, has been used as the “gold standard” epidemiological definition of HIV-SN throughout this thesis. Vibration sense was assessed using a 128Hz tuning fork, which was placed on the interphalangeal joint of each great toe; vibration sense of ten seconds or less was considered abnormal (Figure 2.1). If participants acknowledged the presence of symptoms, the anatomical distribution and intensity of the symptoms were recorded. Symptom intensity was rated on an 11-point rating scale anchored at “0” (no symptom experienced) to “10” (worst imaginable). Pain was classified as moderate to severe with a score of four or greater on this scale. All assessments were performed by me, a clinically-trained investigator who had received training on the use of the tool by the validator, Dr Catherine Cherry.

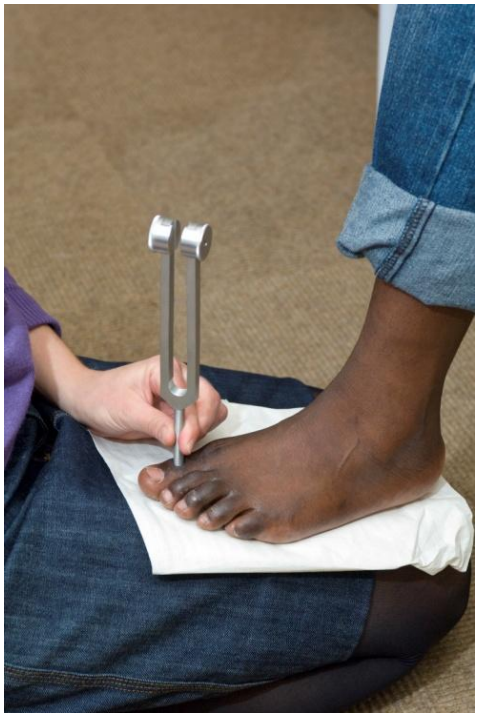


Figure 2.1 Measuring vibration sense

2.1.2 Cohort characteristics

Four hundred and four black Southern African HIV-positive patients were recruited. Nine of these patients had not used stavudine and so were excluded from the clinical analysis of HIV-SN presented in Chapter 3. A further 53 patients were excluded from genetic analyses for a variety of reasons (see Figure 2.2).

Results of the genetic analyses are presented in Chapters 4-7. To avoid repetition the demographics of the cohort are presented here. The whole cohort of 342 was used in the analysis of association between the SNPs and the presence of HIV-SN, whilst the subset of 159 patients with HIV-SN was used to analyse associations between the SNPs and presence and intensity of pain (Figure 2.2). In the 342-patient cohort, 75% (257/342) were female with a mean age of 39 (SD 8) and a current median CD4 T-cell count of 388 cells/µl (range 27-1091). The HIV-SN cohort (n=159) had a similar demographic make-up with 78% (124/159) female, mean age = 41 (SD 8) and median current CD4 T-cell count = 399 cells/µl (range 81-1091).

The HIV-SN cohort had a median pain score of 5 (range 0-10) on the 11-point pain rating scale.

Most were South African (93%, 318/342) with the remaining 7% from other Southern African countries in the Niger-Kordofanian ethno-linguistic grouping (Zimbabwe n=12, Mozambique n=9, Malawi n=2, Zambia n=1) (Tishkoff et al. 2009).

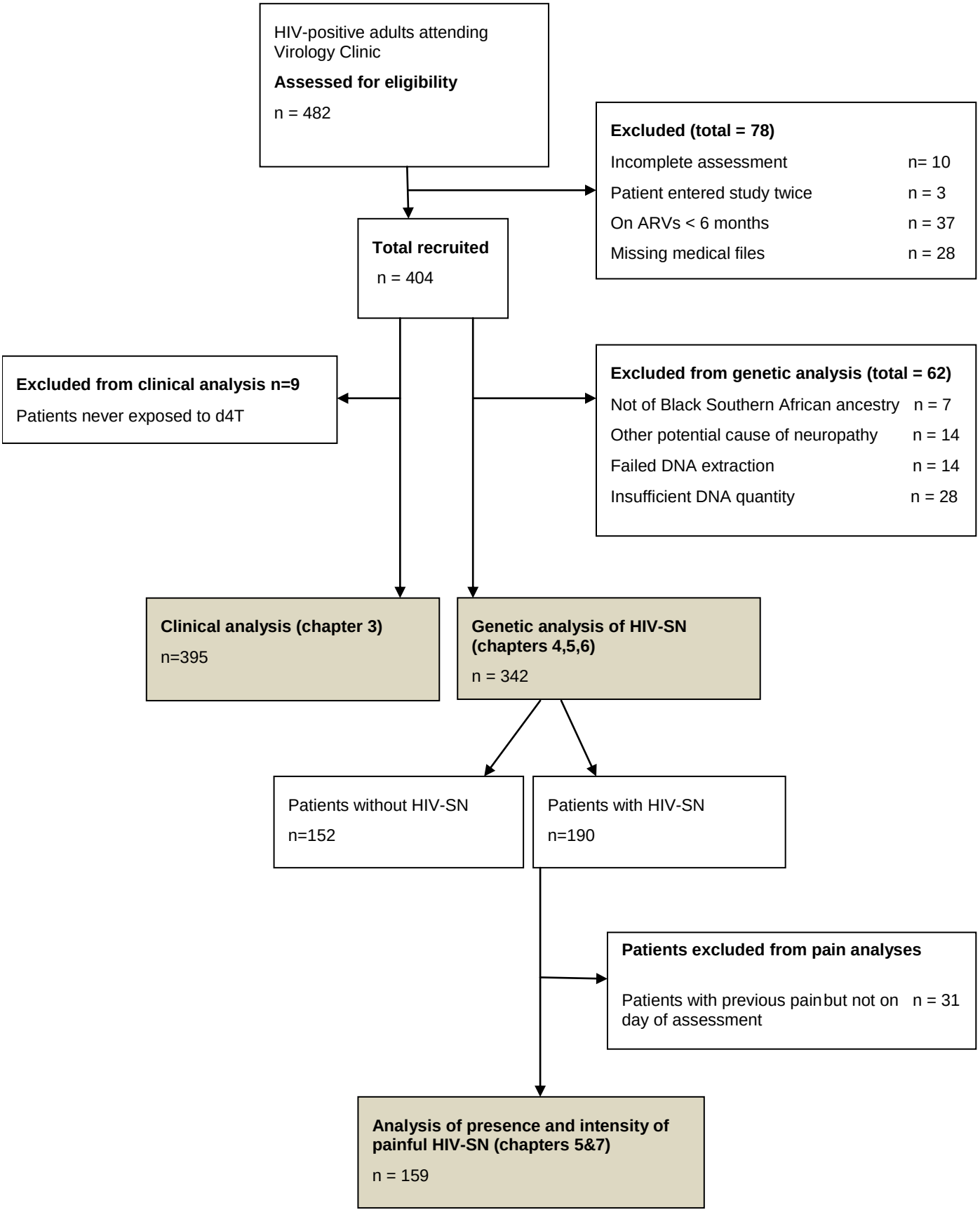


Figure 2.2 Recruitment and exclusion of patients

2.2 Genetic aspect

The DNA extraction and genotyping were carried out in the Division of Human Genetics, National Health Laboratory Service & University of the Witwatersrand, Johannesburg, South Africa under the supervision of Dr Zané Lombard.

SNPs were chosen based on a survey of the literature and reflected an association with inflammatory or neuropathic disease. The SNPs were from genes in four categories:

i) from the TNF block in the major histocompatability complex (MHC) where SNPs and haplotypes have previously associated with HIV-SN (See Chapter 1, Section 1.3.1) [Table 2.1] and 8 additional SNPs from the region not previously assessed [Table 2.2]

ii) other cytokines and chemokines implicated in animal and autopsy studies (see Chapter 1, Section 1.3.1) [Table 2.3],

iii) *UCP* (associated with diabetic polyneuropathy and discussed in Chapter 1, Section 1.3.2.2) [Table 2.4]

iv) *GCH1 (*associated with reduced neuropathic pain and discussed in Chapter 1, Section 1.3.3.1) [Table 2.5].

SNPs are identified via rs numbers and are generally described on the forward strand, except for SNPs from the TNF block, which are described using the Ensembl database [Ensembl release 67, May 2012] (Flicek et al. 2012) and were retrieved using the web-based data-mining tool Biomart (Kinsella et al. 2011) for consistency with previously published papers.

Table 2.1 SNPs forming TNF block haplotypes from the MHC on chromosome 6

Gene	SNP ID	Common name	Chromosomal position
MCCD1	rs3130055		31605378
BAT1	rs9281523*	BAT1int10	31606224
	rs1055388		31609716
	rs2516393		31614723
	rs3130059		31617263
	rs2239528	BAT1-348	31618084
	rs2523504	BAT1-1101	31618837
	rs2844509	BAT1-1167	31618903
Within 2kb of *BAT1*	rs2251824		31619836
ATP6V1G2	rs2071594		31620699
	rs2071593		31620778
NFKBIL1	rs2071592	NFKBIL1-62	31623319
	rs2071591		31623778
	rs7738380		31625994
	rs6916921		31628405
	rs2857605		31632830
	rs2230365	NFKBIL1+446	31633427
	rs3130062	NFKBIL1+738	31633891
Within 2kb of *NFKBIL1*	rs4947324		31636109
LTA	rs2516312	LTA-1085	31647414
	rs2071590	LTA-752	31647747
TNF	rs1799964	TNF-1031	31650287
	rs4248158	TNF-806	31650512
	rs1800750		31650942
	rs1800629	TNF-308	31651010
	rs1799769*	TNFD509	31652006
	rs3093662	TNF+671	31652168
	rs3093665	TNF+1873	31653370
	rs3093668		31654474
LST1	rs1052248		31664560

**Insertion deletion not a SNP*

Table 2.2 Further SNPs from the MHC on chromosome 6 not previously assessed for association with HIV-SN

Gene	SNP ID	Common name	Chromosomal position
BAT1	rs1129640	BAT1+348	31614602
NFKBIL1	rs45457097	-	31634524
LTA	rs1041981	LTA+265	31648763
	rs909253	LTA+252	31648291
	rs2229094	T+496C	31540556
	rs2239704	LTA-379	31648120
	rs3093544	LTA+927	31649758
TNF	rs3093661	TNF-240	31651736

Table 2.3 Cytokine and chemokine SNPs assessed

Gene	SNP	Common name	Chromosome	Chromosomal position
IL4	rs2243250	IL4-590	5	132037052
IL10	rs1800872	IL10-592	1	205013029
	rs1800896	IL10-1082	1	205013519
	rs1800890	IL10-3575	1	205015987
IL12	rs3212227	IL12B (3'UTR)	5	158675527
IL1A	rs17561	IL1A+4845	2	113253693
	rs1800587	IL14-889	2	113259430
IL1B	rs1143634	IL1B +3953	2	113306860
	rs16944	IL1B -511	2	113311337
IL18	rs187238	IL18-137	11	111540197
CCL2	rs3760396	CCL2-927C	17	29605553
	rs4586	CCL2 Cys35Cys	17	29607381
CCR2	rs1034382	CCR2 -4385	3	46343405
CCL5	rs2107538	RANTES-403	17	31231892
	rs2280789	RANTES-In1.1	17	31231115

Table 2.4 UCP SNPs on chromosome 11 have previously been associated with diabetic neuropathy

Gene	SNP ID	Common name	Chromosomal position
UCP2	rs659366	G-866A	73372402
UCP3	rs1800849	C-55T	73397813

Table 2.5 SNPs assessed from the GCH1 gene on chromosome 14 previously associated with intensity of neuropathic pain

Gene	SNP ID	Common name	Chromosomal position
GCH1	rs10483639	c.4279	54376206
	rs3783641	c.343+8900	54429888
	rs4411417	c.509+5836	54390312
	rs752688	c.627-708	54381318
	rs8007201	c.509+1551	54394597
	rs8007267	c.-9610	54448740

2.2.1 DNA extraction and quantification

The initial 39 DNA samples were extracted from saliva, but thereafter the protocol was changed to DNA extraction from blood, for all following samples, after concerns over DNA quantity and quality. DNA was extracted from the saliva samples using a QIAamp DNA mini kit (QIAGEN; Valencia, CA). The kit is a silica-based spin-column extraction method and includes all necessary reagents. Using a high salt concentration the DNA is attracted to the silicon membrane of the spin column. Proteins are removed using the buffers and centrifugation. Finally DNA is eluted from the membrane of the spin column into TE buffer (Figure 2.3). DNA was extracted from the blood samples using the salting out method (Miller et al. 1988). This is a

two-day procedure where following cell lysis concentrated sodium chloride salt solution is used to precipitate protein from the solution. Thereafter, ethanol is used to precipitate the DNA, which is then dissolved in TE buffer. The concentration of the DNA samples was determined using spectrometry on a Tecan Infinite 200 NanoQuant. Finally, the samples were normalized to 50ng/µl for genotyping using the Tecan Freedom Evo®, an automated system which reads concentrations from a spreadsheet and dilutes the sample appropriately. All DNA samples were stored at 4°C following extraction.

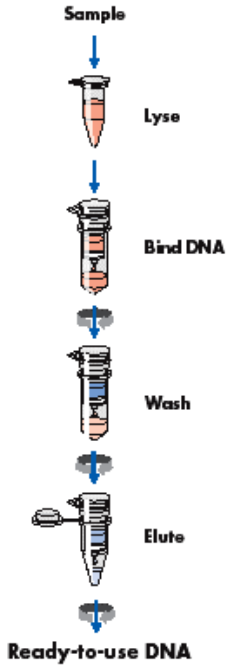


Figure 2.3 Process of DNA extraction using the QIAamp DNA mini kit
Picture from qiagen.com

2.2.2 Genotyping

All SNPs selected for genotyping were submitted to Illumina Technical Support for evaluation by the Illumina® Assay Design Tool (ADT). The tool uses an algorithm which assesses minor allele frequency, sequence and position of SNPs for likelihood of successful GoldenGate genotyping. All SNPs were scored from 0-1 where a score

of 0 was deemed incompatible with the GoldenGate assay and 1 compatible with the genotyping assay. I used a cutoff of 0.8 and excluded any SNPs achieving an ADT score of less than this in the assay design (Table 2.6).

Table 2.6 SNPs deemed incompatible with successful genotyping by the Illumina Assay Design Tool

SNP	**Gene**	**ADT SNP score**
rs3219186	*NFKBIL1*	-99*
rs844509	*BAT1*	0.24
rs1024611	*CCL2*	0.59
rs1801157	*CXCL12*	0.54
rs2239527	*BAT1*	0.59
rs2523506	*BAT1*	0.56
rs361525	*TNF*	0.60
rs3131637	Within 10kb of *NFKBIL1*	0.55

**Insertion/deletion failure code*

All SNPs deemed successful by the ADT were genotyped using the Goldengate® assay on the Illumina BeadXpress™ genotyping platform. This platform is geared for low to mid-throughput genotyping. An Illumina genotyping protocol was followed (Lin et al. 2009). There are three phases that occur as part of the genotyping: pre-PCR, PCR, post-PCR.

In the pre-PCR phase the DNA is made single stranded by incubating at 95°C. Single strands of DNA are necessary to allow the primers to bind thereby allowing identification of SNP sites and replication in the PCR phase. Biotin, which is attached to beads with magnetic properties, is added to the samples and binds with the DNA.

The biotin is attached to beads with magnetic properties and thus the DNA can be held magnetically whilst cell debris and process solutions are washed away. 2-propanol precipitates the DNA, which is then resuspended. Allele-specific extension primers are added to the samples. These primers contain an ‘address sequence’ which allows each SNP to be identified. The primers attach to one or other of the SNPs resent in the sample. The two different primers for each SNP have different coloured fluorophore-labelled probes, which identify the alleles of each SNP at the end stage (Figure 2.4). The allele-specific primer extensions have a universal tail that does not bind to the strand. This universal tail is the section amplified by the PCR and allows multiple SNPs to be amplified in one assay. Attached to the primers are Veracode beads; these are minute glass beads imprinted with a barcode linked to the SNP information. PCR is then carried out, amplifying the DNA and primers so that there is sufficient quantity to be detected in the final stage.

Following PCR the final part of the process is executed: the plate scanning. The BeadXpress reader excites the fluors attached to the VeraCode beads and records data via a dual laser capture process: each VeraCode bead is scanned both for its ‘address’ barcode, which identifies the SNP and also for the fluorescence emanating from the allele associated fluorophores, so identifying the alleles present.

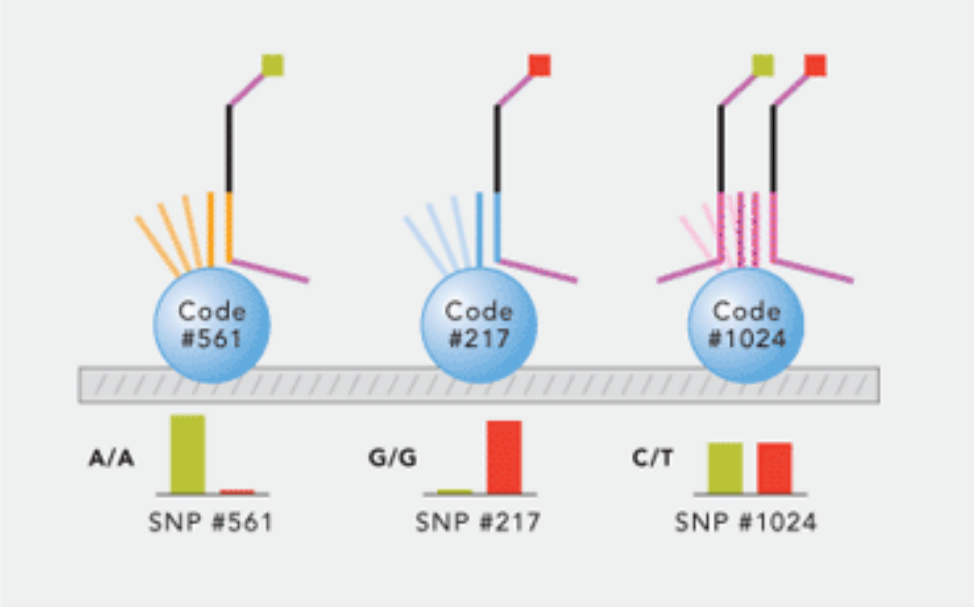


Figure 2.4 VeraCode beads are inscribed with a barcode identifying the SNP. Fluorophores attached to the DNA are attached to the beads. There are different colour fluoroprobes for each allele. Homozygotes emit one strong colour signal whereas heterozygotes have a lower intensity, mixed colour signal.

Picture from Illumina.com

Cluster plots generated for each SNP were inspected individually to check the software genotyping calls. Outliers were either assigned to the most appropriate group or eliminated if the call was ambiguous (see Figure 2.5). This checking process was essential to ensure that the analysis was not confounded by incorrect genotyping calls (Tindall et al. 2010) and four SNPs were removed (Table 2.7).

Table 2.7 SNPs which failed genotyping

Gene	SNP	Chromosome	Position	Reason for exclusion
LTA	rs1800683	6	31648049	Het/homo close
IL10RA	rs2229113	11	117374879	CC genotype failure
CCL2	rs1024610	17	29604343	Het/homo close
CCL5	rs2280788	17	31231517	Complete failure to genotype

Het/homo close = heterozygote and homozygote clusters too close to call accurately

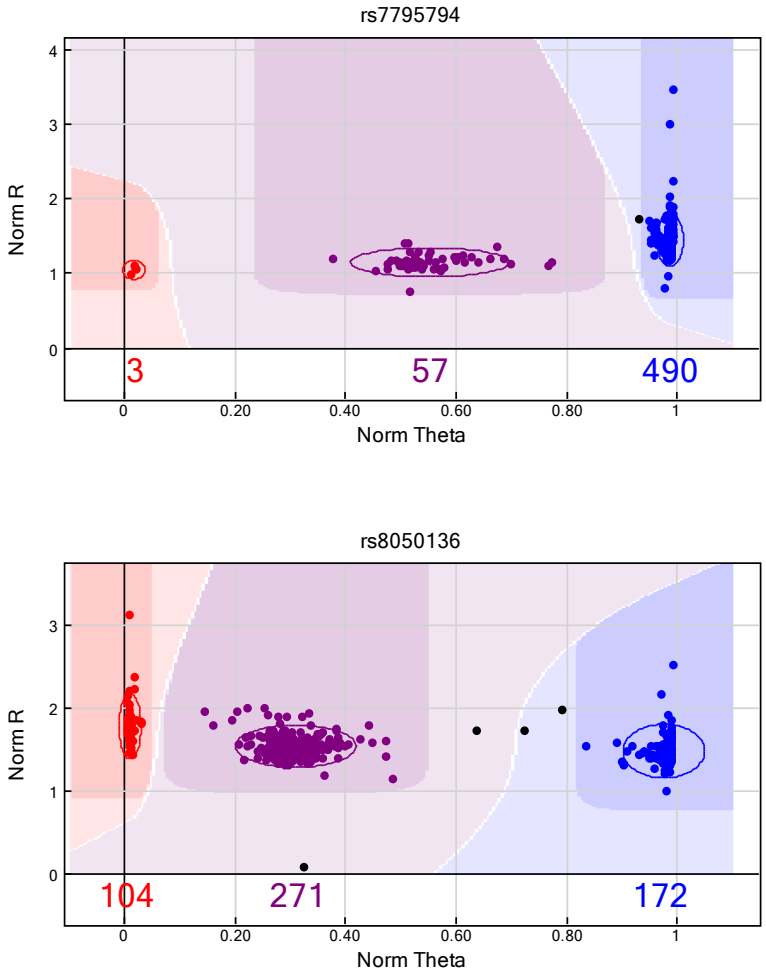


Figure 2.5 Examples of cluster plots generated by the Illumina system, which show signal intensity for each allele. The top picture shows clear groupings in the three groups. The bottom picture shows outliers in black which would be removed.

2.3 Data handling and statistical analysis

Analysis of clinical data in Chapter 3

Normally distributed, continuous data are presented as mean (SD), and nonparametric data as median (range). Univariate analyses of risk factors associated with neuropathy were undertaken using Chi^2 tests (dichotomous variables), unpaired *t*-tests (parametric, continuous variables), and Mann-Whitney tests (non-parametric, continuous variables). Multivariate analysis was performed by my collaborator Dr Catherine Cherry using multiple logistic regression modelling with a reverse selection procedure. Variables were included in the model if they have previously been associated with HIV-SN or if they were associated with SN ($p < 0.1$) in the current study. The variable least strongly associated with SN was then removed in a step wise fashion until the removal of any more variables substantially impaired the resulting model. Receiver operating characteristic (ROC) analyses were used to determine optimal cutoff levels for continuous variables that were associated with neuropathy in this cohort. Cutoffs were chosen based on correctly classifying the maximum number of cohort participants according to their neuropathy status as defined by the ACTG tool.

Analysis of genetic data in Chapters 4-7

Statistical analysis of the genetic data in Chapters 4-7 was performed using PLINK software (Purcell et al. 2007; Purcell 2009). Standard quality control filters (Clarke et al. 2011) comprised: minor allele frequency (MAF) >0.01, SNP missingness rate < 0.05, individual missingness rate < 0.2 and Hardy-Weinberg equilibrium (HWE) $<1 \times 10^{-4}$. Allelic (chi-square), genotypic, dominant and recessive models (Table 2.8) were used to assess for associations between alleles of individual SNPs and randomly generated haplotypes and the presence of HIV-SN. TNF haplotypes presented in Chapter 4 only were generated using the PHASE algorithm (Stephens et al. 2001) by Dr C Chew. All other haplotypes presented in this thesis I generated using the phasing algorithm in PLINK. Associations with pain were completed only in those with HIV-SN. Due to the smaller sample size of this group only an allelic model was

run for assessing associations with pain. Logistic regression was completed for analysis of association of SNPs or haplotypes and presence of HIV-SN or pain. Linear regression was used for assessment of pain intensity. Multivariate analysis corrected for age, height and CD4 T-cell count in HIV-SN analyses, and age, sex and CD4 T-cell count in pain analyses. Point-wise empirical p values are given and where relevant family-wise empirical p values, which adjust for multiple comparisons. The point wise process used an iterative process and the family-wise process was based on 1000 permutations. P values <0.05 were considered significant. Generation of haplotypes using the PHASE algorithm is described in Chapter 4.

Table 2.8 Detail of genetic models used

Test	
Allelic	A versus a
Genotypic	AA versus Aa versus aa
Dominant	(AA, Aa) versus aa
Recessive	AA versus (Aa, aa)

'A' represents the major allele and 'a' the minor allele

CHAPTER 3

RISK FACTORS AND SYMPTOM CHARACTERISATION OF HIV-SN

Data presented in this chapter have been published:

WADLEY AL, CHERRY CL, PRICE P, KAMERMAN P

HIV neuropathy risk factors and symptom characterization in stavudine-exposed South Africans

Journal of Pain and Symptom Management, 41: 700-706, 2011

And presented at a conference:

Third International Conference on Neuropathic Pain, Athens, Greece. 25-30 May 2010

3.1 Introduction

Following the introduction of antiretroviral therapy (ART), in most published studies the incidence (Robinson-Papp et al. 2009; Sacktor et al. 2009) and prevalence (Cherry et al. 2006; Smyth et al. 2007; Affandi et al. 2008; Cherry et al. 2009) of HIV-SN have increased. This increase has been linked to the use of ART regimens containing particular nucleoside reverse transcriptase inhibitors (NRTIs), notably stavudine (Wester et al. 2005; Cherry et al. 2006; Skopelitis et al. 2007; Smyth et al. 2007; Cherry et al. 2009).

The situation of HIV treatment in South Africa is improving. Access to ART in South Africa has increased significantly since 2004, over 80% of pregnant women now receive ART and policy has changed to replace stavudine with tenofovir in first line regimens (Nyirenda et al. 2007; Department of Health 2010; UNAIDS 2011). However, tenofovir supply is inconsistent (Business_Day 2012), many patients remain on stavudine and live with persistent complications such as SN and additionally trials for lower dose stavudine continue (Maskew et al. 2012; Venter et al. 2012). As such, despite the WHO recommending the phasing out of stavudine it will still be some time before this occurs.

One recent study assessing HIV-SN in a South African cohort found stavudine use, increasing age, prior TB and components of metabolic syndrome associated with HIV-SN (Maritz et al. 2010). Other than this study there are few reports of HIV-SN in African populations using well defined diagnostic criteria (Chapter 1, Table 1.8), therefore I determined the risk factors for HIV-SN in a Black South African cohort with universal exposure to stavudine and identified associations with risk of HIV-SN in such patients. There is a dearth of symptom descriptors in the literature and so I also characterised the intensity and distribution of three common symptoms of peripheral neuropathy: pain, numbness and pins-and-needles, to establish the symptom experience of HIV-SN.

3.2 Methods

The methodology for patient recruitment and neurological assessment was as described in Chapter 2. Only stavudine-exposed individuals were included in this analysis and recruitment of the sample of 395 individuals is shown in Figure 3.1.

Patients were interviewed and their medical notes reviewed. It is possible that medical records were incomplete if patients sought treatment at another institution. However, patient interview was used to minimise the chance that any relevant major events were overlooked.

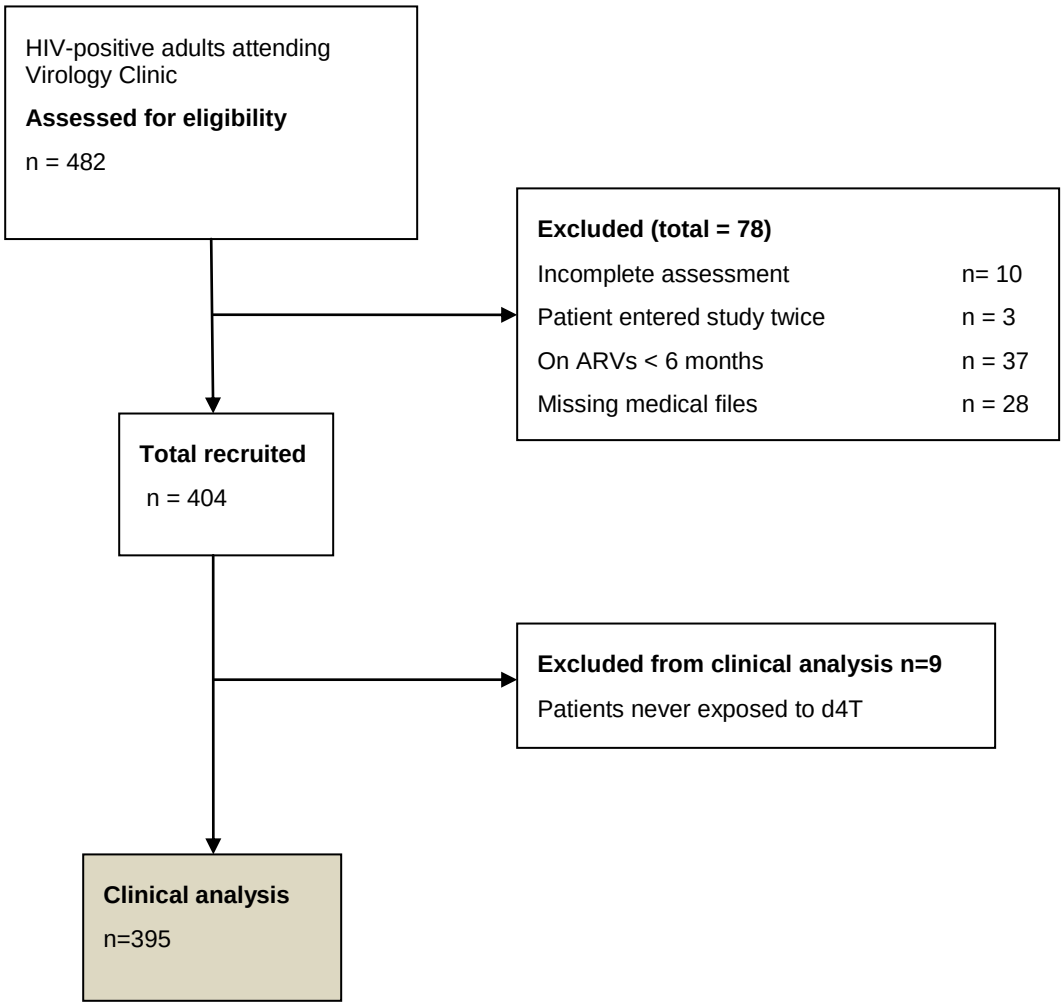


Figure 3.1 Recruitment and exclusion of participants used in this analysis

3.3 Results

Three hundred and ninety-five patients participated in the study, of whom 226 (57%) had a clinical diagnosis of symptomatic HIV-SN. All participants identified themselves as Black Africans, were 18-years or older, had a confirmed HIV infection and had been on stavudine-based ART for at least six months.

Demographic and clinical data

Demographic and clinical data are shown in Table 3.1. Non-HIV-related neuropathy risk factors such as diabetes (England et al. 2004) were uncommon. Hepatitis C testing of the first 300 participants showed no association with HIV-SN (Cherry et al. 2010) [see appendix 2]. Nadir CD4 T-cell counts were not available for 18 participants who had commenced antiretroviral treatment at other clinics. Although 24% (n = 96) of participants had been exposed to stavudine at a dose of 40mg twice daily, no participant was receiving more than 30 mg stavudine orally twice daily at the time of screening. No patients had been prescribed a protease inhibitor or zalcitabine.

Table 3.1 Participant demographic, disease and treatment characteristics

Variable	
Number of participants	395
Female gender	295 (75%)
Age in years	39 (8)
Height in cm	59 (8)
Weight in kg	69 (14)
Current CD4 (cells/µl) (n=386)	383 (27-1091)
Nadir CD4 (cells/µl)	94 (1-403)
Months since HIV diagnosis	45 (6-240)
History of an AIDS defining illness	176 (45%)
Seropositive for Hepatitis C	9 (2%)
History of tuberculosis*	160 (41%)
Diabetes	12 (3%)
Stavudine use ever	395 (100%)
Current stavudine use	115 (29%)
Stavudine use (months; med and IQR)	15 (9-27)
Didanosine use ever	37 (9%)
Current didanosine use	25 (6%)

Dichotomous variables are presented as number (%), normally distributed continuous variables are presented as mean (standard deviation) and non-normally distributed continuous variables are presented as median (range).

**All patients with a history of tuberculosis had been treated with isoniazid and most (93%) had documented pyridoxine supplementation*

Prevalence and characteristics of HIV-SN

In addition to the 226 (57%) with symptomatic HIV-SN, 14% (55/395) had symptoms of neuropathy but not signs, while 11% (42/395) had objective signs of peripheral nerve damage but not symptoms. These latter patients may be classified as having asymptomatic HIV-SN. Pain was the symptom most often reported by participants diagnosed with symptomatic HIV-SN (76%, 172/226) [Table 3.2]. Seventy-four percent (74%, 128/172) of participants with pain, 76% (82/108) of participants with pins-and-needles, and 77% (81/105) of participants with numbness, reported experiencing the symptom at moderate to severe intensity based on a numerical rating scale score of ≥4. Thirty-nine percent (39%, 89/226) reported two or more symptoms. All participants with HIV-SN experienced symptoms in their feet. Pain proximal to the feet was reported by less than half of those with pain (Figure 3.2).

Table 3.2 Prevalence and features of HIV-SN observed in this study

Feature	**Number (%)**
Symptomatic HIV-SN (symptoms and signs)	226 (57)
Symptoms, but no signs	55 (14)
Signs, but no symptoms	42 (11)
Symptom prevalence in HIV-SN	
Pain, aching or burning	172 (76)
Pins and needles	105 (46)
Numbness	108 (48)
Sign prevalence in HIV-SN	
Reduced vibration sense	99 (44)
Absent ankle jerks	191 (85)

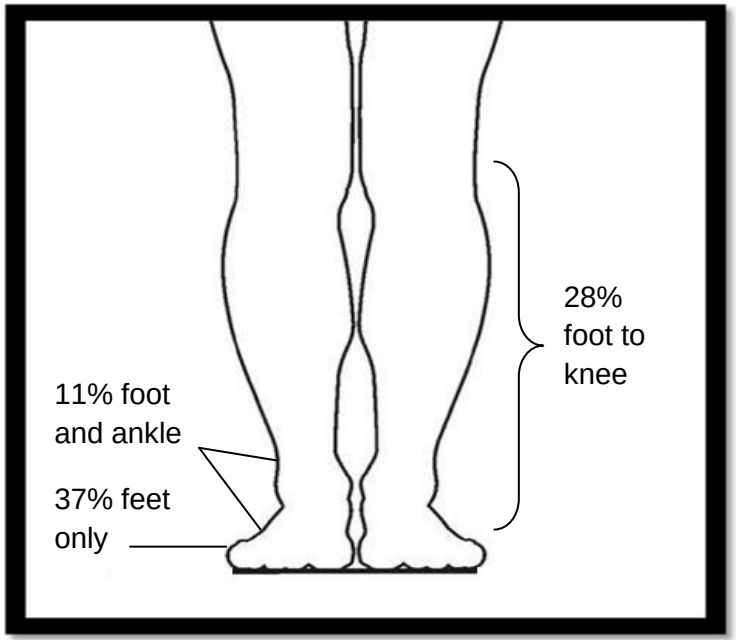


Figure 3.2 Location of pain in those with painful HIV-SN
Seventy six percent of those with symptomatic HIV-SN experienced pain of which, the majority was experienced in the feet

Risk factors associated with HIV-SN

Increasing age ($p < 0.001$) and increasing height ($p = 0.005$) were the only factors significantly associated with HIV-SN on univariate analyses (Table 3.3). Factors included in the multiple regression model were age, height, didanosine exposure and a history of an AIDS defining illness (based on $p<0.1$ on univariate analysis) as well as a nadir CD4, diabetes mellitis or isoniazid use [based on associations with HIV SN in other cohorts] (England et al. 2004; Wright et al. 2008; Cherry et al. 2009). The only factors independently associated with neuropathy risk among stavudine exposed African HIV patients were age and height (Table 3.4).

Table 3.3 Univariate associations between HIV-SN status and demographic/clinical variables.

Variable	**SN free (n=169)**	**SN (n=226)**	**p value**
Female gender	128 (76%)	167 (74%)	0.7
Age in years	36.4 (7.6)	40.8 (8.0)	<0.0001
Height in cm	157.6 (8)	160.1 (8)	0.003
Weight in kg	68.2 (3)	69.6 (4)	0.3
Months since HIV diagnosis	45 (6-204)	46 (7-240)	0.4
Current CD4 T-cell count	386 (45-1079)	380 (27-1091)	1.0
Nadir CD4 T-cell count	96 (2-403)	91 (1-253)	0.7
AIDS defining illness	68 (40%)	108 (48%)	0.1
Tuberculosis (isoniazid use)	65 (38%)	95 (42%)	0.5
Hepatitis C seropositivity	4 (2%)	5 (2%)	0.9
Didanosine ever	11 (7%)	26 (12%)	0.09
Documented history of diabetes mellitus	4 (2%)	8 (4%)	0.5

Dichotomous variables are shown as number (%) and compared with SN status using a Chi^2 test. Normally distributed variables are shown as mean (standard deviation) and compared with SN status using an unpaired t test. Non-normally distributed continuous variables are shown as median (range) and compared with SN status using a Mann-Whitney U test.

Table 3.4 Multiple logistic regression model of factors associated with prevalent HIV-SN in this cohort.

Variable	Odds Ratio	95% CI	p
Age in years	1.08	1.05 – 1.11	<0.001
Height in cm	1.04	1.01 – 1.07	0.005

Factors included in the analysis were those associated with HIV-SN on univariate analysis (age, height, having had an AIDS defining illness and exposure to didanosine) and factors that have previously been found to be risk factors for HIV-SN in other cohorts (nadir CD4 T-cell count, isoniazid exposure and diabetes mellitus). The final model was obtained using a reverse selection procedure, and has model r^2= 0.07 and model p<0.0001.

ROC analysis comparing age and neuropathy status in all 395 participants yielded an area under the curve (AUC) of 0.66 (95% confidence interval 0.61-0.72). An age cutoff of ≥38 years had a sensitivity of 68% and a specificity of 63% for predicting neuropathy. When comparing height and the presence of neuropathy with ROC analysis, the AUC was 0.59 (95% confidence interval 0.53-0.65), and a height cutoff of ≥158cm had a sensitivity of 64% and a specificity of 51% for predicting neuropathy. When age and height were combined as predictors, the prevalence of neuropathy was 35% in younger, shorter participants and 76% in older and taller participants (Table 3.5).

Table 3.5 Prevalence of HIV-SN by age and height groups among African patients who had been treated with stavudine.

	Height < 158cm	Height ≥ 158cm
Age < 38 years	35%	45%
Age ≥ 38 years	64%	76%

3.4 Discussion

We identified the prevalence, associations with risk and described the symptom experience of symptomatic HIV-SN in a stavudine-exposed cohort of South African patients. Patients most likely to develop a symptomatic HIV-SN after stavudine exposure may now be identified and the expected severity of their symptoms better understood. The prevalence of symptomatic HIV-SN was 57% showing this is a common problem in African patients exposed to stavudine-based ART. Within our cohort duration of exposure to stavudine was not associated with prevalent SN risk, possibly because those that developed SN were likely to be switched to non-neurotoxic agents. This prevalence is higher than that of a similar South African cohort recently assessed (Maritz et al. 2010) but our cohort was entirely exposed to ART and importantly to stavudine-based ART. Of note, three-quarters of individuals with HIV-SN in our cohort reported moderate to severe pain demonstrating potential for substantial suffering. Age and height were the only independent risk factors associated with HIV-SN in our cohort. Although these factors do not explain all the variation in SN status following stavudine exposure (model r^2=0.07), simple stratification of patients by age (≥ 38 years) and height (≥ 158 cm) can predict those at highest risk for HIV-SN (Table 5). HIV-SN is therefore a clinically important condition and efforts to reduce new cases of HIV-SN are critical, particularly in clinics where stavudine use continues.

Routinely recording age and height are quick and easy tasks that can be part of a normal patient assessment. Age and height cutoffs identified through ROC analysis illustrate well how HIV-SN risk increases with increasing age and height, and this has important clinical implications. In any setting where the use of stavudine is an economic necessity, we suggest that priority access to less toxic antiretroviral agents should be given to older and taller (and particularly both older *and* taller) patients. In this way, rates of SN are likely to be reduced. Improved patient and doctor awareness of HIV-SN and increased frequency of follow up of patients at risk of HIV-SN may also facilitate prompt regimen revision if neuropathy symptoms develop. Early switching off stavudine when neuropathy develops may improve the likelihood of symptom resolution (Moore et al. 2000; Pettersen et al. 2006).

It is worth noting that 57% of patients presented with symptomatic HIV-SN despite most patients only ever having been exposed to twice daily 30mg doses of stavudine. Treatment guidelines worldwide have changed to reflect the reduced toxicity of 30mg rather than 40mg stavudine doses (WHO 2006). However, our data show an unacceptably high prevalence of HIV-SN, even among patients exposed to this “less toxic” dose. Makinson et al (2008) recently reported the effectiveness of 20mg doses of stavudine (Makinson et al. 2008). Although stavudine use is being phased out (WHO 2010b), this will take time as the alternatives are more expensive (Long et al. 2010). Should the effectiveness of 20mg doses be confirmed and if the safety profile of this dosage is more acceptable, use of a 20mg twice daily stavudine dose should be considered to reduce toxicities during the phasing out of stavudine.

Our findings that increasing age and height are associated with increased HIV-SN risk are consistent with several other studies (Watters et al. 2004; Zhou et al. 2007; Affandi et al. 2008; Cherry et al. 2009; Robinson-Papp et al. 2009). Indeed, where height has been assessed and found not to be associated with HIV-SN, the diagnosis of “neuropathy” was not identical to that used in this thesis (Maritz et al. 2010). We found an association between height and HIV-SN risk despite our cohort being 5cm shorter on average than other ethnic groups studied (Cherry et al. 2009), and the height cut-off values identified in our cohort through ROC analysis were 12cm lower. This finding that height was a risk, despite the shorter height of our cohort may be explained by longer leg length relative to the trunk length in black compared to white individuals (Nyati et al. 2006). Our findings corroborate that factors associated with HIV-SN in untreated HIV patients such as nadir CD4 T-cell count (Bacellar et al. 1994) are less important in the context of antiretroviral therapy (Cherry et al. 2006; Skopelitis et al. 2007; Zhou et al. 2007). Lastly we did not find an association between isoniazid use and HIV-SN in our cohort. Isoniazid is neurotoxic (Argov et al. 1979). Although co-administration of pyridoxine (as provided to most patients with tuberculosis in our cohort) minimizes the risk of SN (Carlson et al. 1956) isoniazid/pyridoxine use was associated with moderate to severe neuropathy

in another African HIV cohort (Forna et al. 2007). However in that study, the cohort had not been universally exposed to stavudine. It may be that the greater neurotoxicity of stavudine and the greater exposure to it in our cohort disguised the neurotoxicity of isoniazid/pyridoxine.

Symptoms of HIV-SN are described as following a 'glove and stocking' distribution, with the feet being the primary site affected (Cornblath et al. 1988; So et al. 1988; Ferrari et al. 2006). However, the distribution of HIV-SN symptoms is rarely described in the literature. Consistent with earlier reports on HIV-SN symptoms in the pre-HAART era (Cornblath et al. 1988), all patients with HIV-SN in our study experienced symptoms in their feet, and over one third of these patients felt symptoms only in their feet, usually the soles. However, the proximal extension of symptoms above the ankle was greater in our cohort than in untreated HIV-positive patients (Cornblath et al. 1988), which may be related to our cohort being exposed to neurotoxic antiretroviral drugs.

The most common symptom experienced in our cohort was pain, with the majority (74%) experiencing moderate to severe pain. This contrasts with early reports of HIV-SN in untreated patients, where symptoms "were usually mild", "painful dysethesias uncommon" and non-painful paresthesias most frequently experienced (So et al. 1988; (Tagliati et al. 1999). This extent and severity of pain observed in our cohort will likely have a major impact on quality of life (Ellis et al. 2010) and may reduce adherence to antiretroviral drugs (van Oosterhout et al. 2005; Ferradini et al. 2006; Hitchcock et al. 2008).

The limitations of this study include that it was cross-sectional. Although this provides an accurate picture of patients attending the clinic currently, it is not possible to establish a temporal link between the condition and exposure to the risk factors. The other temporal relationship difficult to assess was whether patients had developed SN before or after stavudine therapy was started. Thus I used the

umbrella term HIV-SN to cover both existing and incident cases of SN on ART. I relied on patient recall and data recorded in the medical file to assess risk factors for HIV-SN rather than collecting data prospectively and monitoring for incident cases of HIV-SN. Furthermore, age and height did not fully explain the variation in neuropathy status in our cohort, and therefore other factors not measured here must also be important. For example, host genetics may influence HIV-SN risk in patients on ART (Hulgan et al. 2005; Affandi et al. 2008; Cherry et al. 2008) and the following chapters assess associations between cytokine polymorphisms and SN in this cohort. In addition, we used a relatively simple clinical tool to diagnose patients with symptomatic HIV-SN. This tool has been validated against objective measures in the context of HIV infection (Cherry et al. 2005), but it is possible that milder cases of HIV-SN were missed with our chosen diagnostic criteria. Furthermore, although this tool assesses the major symptoms that have been associated with HIV-SN (pain, pins-and-needles and numbness) (So et al. 1988; Robinson-Papp et al. 2009), this also means that our characterisation of neuropathy symptoms among African patients exposed to stavudine is limited to this short symptom list derived from patients in other settings. Finally, it is possible that patients without neuropathy symptoms would have been less inclined to participate in the study leading to selection bias. However, attempts were made to screen patients consecutively in the queue.

In conclusion, HIV-SN is a common problem that frequently presents with moderate to severe pain and I found increasing age and height to be associated with HIV-SN. In resource-limited settings where stavudine frequently still forms the backbone of antiretroviral therapy, prioritising older and taller patients for access to alternative agents could be an effective, inexpensive way to reduce patient suffering.

CHAPTER 4

INDIVIDUAL SNPs AND HAPLOTYPES FROM THE TNF BLOCK REGION ASSOCIATE WITH HIV-SN

4.1 Introduction

As discussed in Chapter 1, Section 1.3.1, carriage of the minor allele of TNFA-1031 associates with increased risk of HIV-SN in Caucasians, Chinese and Malays (Affandi et al. 2008; Cherry et al. 2008; Chew et al. 2010). The minor allele of a SNP in an adjacent gene [BAT1(intron10)] also associates with greater risk of HIV-SN in Caucasians (Cherry et al. 2008). These two SNPs lie in the central major histocompatability complex (MHC) on chromosome 6 in a region of high linkage disequilibrium, known as the TNF block (Ackerman et al. 2003).

TNF block haplotypes have been characterised in a series of papers by my collaborators. Based on 38 SNPs typed in 999 donors of multiple ethnicities, 31 haplotypes were identified but no ethnic population had more than 19 haplotypes. Haplotypes were named FVx (Valente et al. 2009a). When Chew et al (2010) analysed associations between HIV-SN and FV haplotypes containing the minor allele of TNFA-1031 (rs1799964) a family of haplotypes (FVa6,7,8) was predictive of SN risk in Chinese and Malay cohorts (Chew et al. 2010). Thus, both individual SNPs and haplotypes in the central MHC are predictive of HIV-SN in cohorts of different ethnicities indicating the potential importance of this region of chromosome 6 for HIV-SN.

In this chapter I assess whether alleles of TNFA-1031, BAT1(intron10) or other TNF block SNPs associate with HIV-SN in my cohort. Using the samples that I generated for the present project, 30 SNPs within the TNF block were genotyped using Illumina GoldenGate technology (directed by Dr Zané Lombard at Division of Human Genetics and the National Health Laboratory Service, Johannesburg) and FV haplotypes were reconstructed in accordance with the published FV nomenclature (Valente et al. 2009a) using the PHASE algorithm by Constance Chew (PhD thesis 2012 University of Western Australia, see Figure 4.1). I also genotyped eight SNPs not included in the FV series of TNF block haplotypes (rs1129640, rs45457097, rs1041981, rs909253, rs2229094, rs2239704, rs3093554, rs3093661). I then

examined LD between these SNPs and aligned them with known haplotypes previously associated with SN (Chew et al. 2010).

Haplotypes	rs3130055	rs9281523	rs10553880	rs2516393	rs3130059	rs2523506	rs2239528	rs2523504	rs2844509	rs2251824	rs2071594	rs2071593	rs2071592	rs2071591	rs7738380	rs6916921	rs2857605	rs2230365	rs3130062	rs4947324	rs2516312	rs2071590	rs1799964	rs4248158	rs1800750	rs1800629	rs1799769	rs3093662	rs3093665	rs3093668	rs1052248	South Africa HIV+ (n=339)	Busselton (n=194)	Perth (n=208)	Chinese (n=50)	Malay (n=50)	Caucasian HIV+ (n=52)	Chinese HIV+ (n=49)	Malay HIV+ (n=51)
FV1	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-	-	-	-	2	-	2	2	1.73%	3.87%	4.33%	NF	NF	2.88%	NF	0.98%
FV1.1	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-	2	-	-	2	-	2	2	3.90%	0.26%	NF	NF	NF	0.96%	NF	NF
FV2	-	-	-	-	-	2	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-	-	-	-	2	-	2	2	NF	NF	NF	NF	1.00%	NF	NF	NF
FV3	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-	-	-	-	2	-	2	-	NF	NF	NF	2.00%	3.00%	NF	NF	NF
FV4	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	2	-	2	-	-	2	-	-	-	0.43%	1.29%	0.48%	NF	NF	0.96%	NF	NF
FV5	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	2	2	-	2	2.60%	1.80%	2.16%	NF	NF	1.92%	NF	0.98%
FV6,7	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	2	NF	1.55%	1.44%	9.00%	7.00%	1.92%	7.14%	1.96%
FV7.1	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	2	0.72%	NF	NF	NF	NF	1.92%	NF	1.96%
FV8	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	2	9.39%	3.87%	4.57%	NF	5.00%	4.81%	1.02%	0.98%
FV9	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	-	NF	NF	NF	4.00%	1.00%	NF	1.02%	0.98%
FV10	-	-	-	-	-	2	-	-	-	2	-	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	2	1.45%	9.79%	12.98%	2.00%	8.00%	2.88%	10.20%	0.98%
FV11	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	2	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	NF	6.19%	7.45%	14.00%	3.00%	6.73%	11.22%	5.88%
FV12	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	2	-	-	-	-	-	2	2	2	-	-	-	-	-	-	-	NF	NF	NF	NF	1.00%	NF	NF	NF
FV13	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	2	-	-	-	-	-	2	-	-	-	-	-	-	-	-	2	NF	0.52%	0.24%	NF	NF	NF	NF	NF
FV14	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	2.17%	7.99%	9.13%	3.00%	1.00%	7.69%	NF	NF
FV15	-	-	-	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	NF	4.12%	4.57%	NF	1.00%	0.96%	NF	NF
FV16	-	2	-	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	12.72%	12.89%	12.50%	15.00%	8.00%	7.69%	9.18%	0.98%
FV16.1	-	2	-	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.87%	0.26%	NF	NF	NF	NF	NF	NF
FV17	-	-	-	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	2.31%	NF	NF	1.00%	NF	1.92%	1.02%	NF
FV18	-	-	-	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35.40%	11.08%	13.46%	28.00%	27.00%	8.65%	16.33%	14.71%
FV18.1	-	-	-	-	2	-	-	2	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.89%	NF	NF	NF	NF	0.96%	NF	1.96%
FV19	2	-	2	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NF	NF	NF	7.00%	2.00%	NF	6.12%	0.98%
FV20,21	-	-	-	-	2	-	-	-	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	5.06%	1.03%	NF	NF	NF	0.96%	NF	NF
FV22	-	-	-	-	2	-	-	-	-	-	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.58%	2.32%	1.68%	NF	NF	0.96%	NF	2.94%
FV23	-	-	-	-	2	-	-	-	-	-	2	-	-	2	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NF	NF	NF	NF	5.00%	NF	1.02%	3.92%
FV24	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	2	NF	NF	NF	NF	NF	NF	NF	NF
FV25,26	2	-	2	-	-	-	2	2	-	-	-	2	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	2	0.87%	5.67%	2.40%	3.00%	11.00%	3.85%	4.08%	10.78%
FV27	2	-	2	-	-	-	2	2	-	-	-	2	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	2	NF	NF	NF	NF	NF	NF	NF	NF
FV28	2	-	2	2	-	-	2	2	-	-	-	-	-	-	-	-	2	-	2	-	-	2	-	-	-	-	-	-	-	-	-	NF	5.93%	7.69%	NF	NF	2.88%	NF	0.79%
FV29	2	-	2	2	-	-	2	2	-	-	-	-	-	-	-	-	2	-	-	-	-	2	-	-	-	-	-	-	-	-	-	0.58%	10.05%	8.17%	8.00%	13.00%	6.73%	9.18%	10.78%
FV29.1	2	-	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	0.87%	0.77%	0.72%	NF	NF	1.92%	NF	NF
FV30	-	-	2	2	-	-	2	2	-	-	-	-	-	-	2	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	NF	2.32%	1.20%	NF	NF	NF	NF	NF
FV30.1	-	-	2	2	-	-	2	2	-	-	-	-	-	-	-	-	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-	2.89%	NF	NF	NF	NF	NF	NF	NF
FV31	-	-	2	-	-	-	2	2	-	-	-	2	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	4.34%	2.84%	2.64%	NF	NF	3.85%	NF	NF
Cumulative Frequency																																91.76%	96.39%	97.84%	96.00%	97.00%	74.04%	77.55%	62.56%
2n																																692	388	416	100	100	104	98	102

Figure 4.1 FV haplotypes and frequencies for Southern Africans and other previously assessed cohorts in the TNF block region

Southern African specific haplotypes not previously described by the FV series are denoted as FV X.1. '-' denotes major alleles, '2' denotes minor alleles. Red shading denotes haplotype tagSNPs. Green shading denotes new FVX.1 haplotypes identified in Southern Africans. Taken from (Chew 2012)

4.2 Methods

Methodology is as described in Chapter 2. Thirty SNPs from the TNF block plus eight newly-selected SNPs were assessed for association with HIV-SN. The recruitment of individuals used in the analysis are shown in Figure 4.2. Alleles were designated using the Ensembl database [Ensembl release 67, May 2012] (Flicek et al. 2012) and retrieved via the web-based data-mining tool Biomart (Kinsella et al. 2011) in accordance with published literature. LD between SNPs was visualised using a plot built in Haploview using the confidence interval method (Barrett et al. 2005). Analyses performed by Constance Chew (PhD UWA 2012) and my collaborator Dr Catherine Cherry are included (and acknowledged as 'CC analysis') as a framework to aid the interpretation of the associations with individual SNPs.

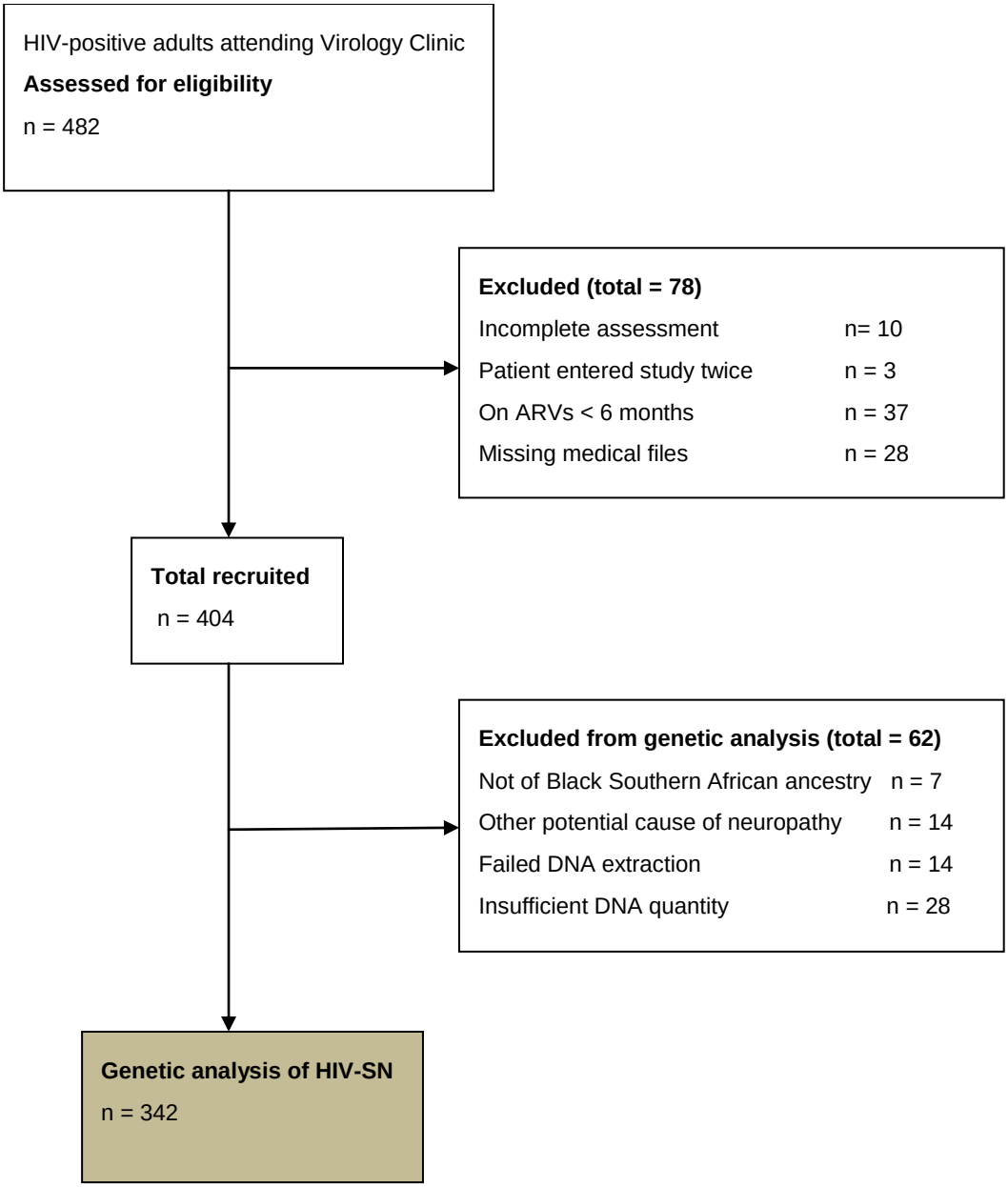


Figure 4.2 Recruitment of individuals used in the analysis of association between SNPs from the TNF block and HIV-SN

4.3 Results and Discussion

Due to the several analyses presented here I have combined the Results and Discussion with a Conclusions section at the end.

DNA from 342 individuals assessed for SN (as described in Chapter 2, Section 2.1.1) was successfully genotyped and included in the analysis. MAFs for the SNPs used to define the FV series are shown in Table 4.2. SNPs with MAFs < 0.01 were excluded from the SNP analysis due to lack of power. The cases and controls of all SNPs assessed were in Hardy Weinberg equilibrium except rs9281523. Three of the eight SNPs not previously genotyped were not in HWE (rs1129640, rs3093554 and rs3093661). Controls without HIV-SN were included in this calculation, so the breach of HWE may reflect an effect of TNF genotype on susceptibility to HIV. The median genotyping success rate was 98%. Three individuals had missing genotype data that exceeded 20% (Chapter 2, Section 2.3) and were excluded, thus the sample size for this study was 339.

A LD plot was generated depicting LD between SNPs from the TNF block and the eight additional SNPs from the same region. The cohort of 339 individuals was used, containing patients with and without HIV-SN. The plot was generated in Haploview and used the confidence interval method. The entire diagram is shown here (Figure 4.3) and demonstrates blocks of high LD (red blocks) throughout the region. Selected sub-regions are magnified later to highlight particular points.

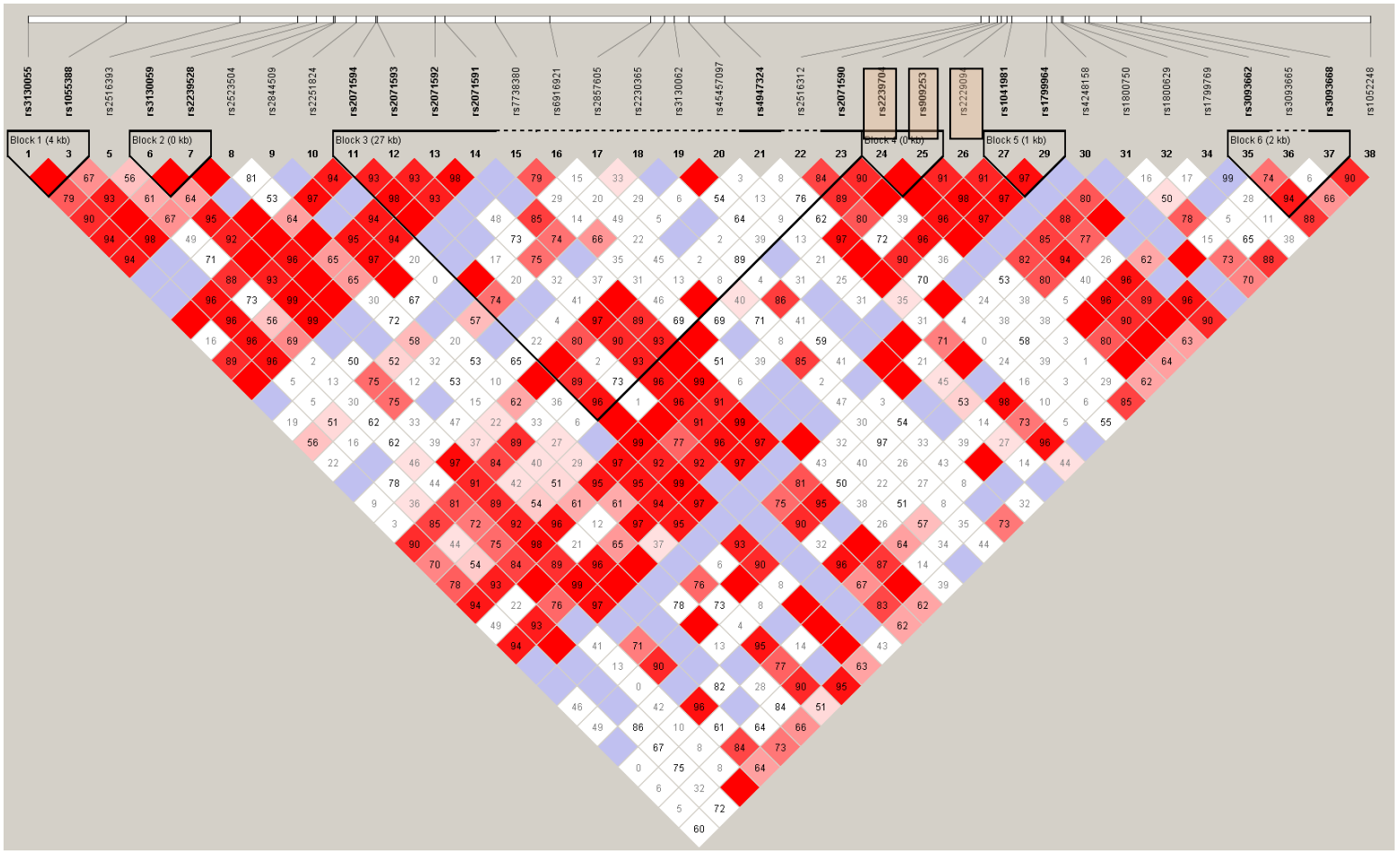


Figure 4.3 LD plot depicting linkage between SNPs from the TNF block region in this Southern African cohort.

Strong LD (D'= 1, LOD ≥2) is shown in red with lower LD shown in descending shades of pink. D'= 1 but LOD < 2 shown in blue, weak LD (D' <1, LOD <2) in white. [LOD = logarithm(base 10) of odds). D' is shown as a percentage within the block. The newly-assessed SNPs incorporated into the diagram by Haploview are shown in orange boxes.

This study was the first assessment of association between alleles of SNPs in the TNF block and HIV-SN in an African population. I found no association with alleles from SNPs previously associated with HIV-SN [TNF-1031 or BAT1(intron10)] (Table 4.1) so it was essential to analyse other SNPs and their contribution to haplotypes. The lack of association between these 2 previously associated SNPs suggests that LD may differ between the Southern Africans and the ethnicities assessed before.

Table 4.1 SNPs associated with SN in Asians and Caucasians are not associated with HIV-SN in Southern Africans, following univariate analysis

Common name	SNP	n	HIV-SN+	HIV-SN-	Empirical p values			
					Allelic	Genotypic	Recessive	Dominant
TNFA-1031	rs1799964	335	19%	21%	0.56	0.43	0.43	0.43
BAT1(intron10)	rs9281523	336	5%	3%	0.63	1.00	1.00	1.00

Results of various models of univariate analyses and showing the percentage of subjects who were carriers of the minor allele with and without HIV-SN. No associations were found.

From the original TNF block panel, associations with several alleles within *BAT1, ATP6V1G2* and *NFKBIL1* (Table 4.2) were significant after correction for age and height (Table 4.3). All associations showed carriage of the minor allele was associated with reduced risk of SN. These associations are consistent for a role of inflammation in the etiology of HIV–SN as *NFKBIL1* genotypes have been associated with rheumatoid arthritis (Chiba et al. 2011) and haplotypes including *ATP6V1G2* have associated with cardiac myopathy and myocardial infarction (Iida et al. 2003; Shichi et al. 2005). However, no studies have associated abnormal production of either protein with neurological disorders or HIV progression. This suggests that the associations found with individual SNPs may reflect linkage disequilibrium within the TNF block. Indeed, haplotype family analysis revealed three patterns of association which are discussed now.

Table 4.2. Minor alleles of BAT1, ATP6G1V2 and NFKBIL1 from the TNF block associated with HIV-SN in an allelic model of univariate analysis

Gene	SNP	n	Alleles		Genotype frequency			MAF	HIV-SN+	HIV-SN-	P value
			1	2	1,1	1,2	2,2				
MCCD1	**rs3130055**	331	T	C	292	35	4	**0.06**	-	-	-
BAT1	**rs9281523**	336	A	C	322	1	13	**0.04**	-	-	-
	rs1055388	335	T	C	270	62	3	0.10	8%	13%	0.06†
	rs2516393	330	C	A	282	47	1	**0.07**	-	-	-
	rs3130059	337	C	G	138	159	40	0.35	31%	41%	0.009*†
	rs2239528	335	G	A	272	60	3	0.10	8%	12%	0.07†
	rs2523504	335	C	T	248	79	8	0.14	12%	17%	0.04†
	rs2844509	330	T	C	242	81	7	0.14	13%	17%	0.16
ATP6V1G2	rs2251824	335	G	A	258	72	5	0.12	11%	14%	0.22
	rs2071594	329	G	C	129	161	39	0.36	32%	42%	0.004*†
	rs2071593	332	G	A	298	34	0	**0.05**	-	-	-
NFKBIL1	rs2071592	333	T	A	133	160	40	0.36	31%	42%	0.004*†
	rs2071591	334	G	A	134	163	37	0.35	32%	41%	0.01*†
	rs7738380	336	A	G	330	6	0	**0.01**	-	-	-
	rs6916921	333	C	T	328	5	0	**0.01**	-	-	-
	rs2857605	339	T	C	320	18	1	**0.03**	-	-	-
	rs2230365	334	C	T	313	21	0	**0.03**	-	-	-
	rs3130062	334	T	C	331	3	0	**0.004**	-	-	-
	rs4947324	336	C	T	246	87	3	0.14	12%	17%	0.06†

SNPs (marked in bold) with MAFs <0.1 were excluded due to lack of power

** also significant on a dominant test; † associated with reduced risk of HIV-SN*

SNPs obtaining p<0.1 were taken forward to multivariate analysis

Table 4.2 continued

Gene	SNP	n	Alleles		Genotype frequency			MAF	HIV-SN+	HIV-SN-	P value
			1	2	1,1	1,2	2,2				
LTA	**rs2516312**	339	A	G	314	25	0	**0.04**	-	-	-
	rs2071590	342	G	A	276	48	6	**0.09**	-	-	-
	rs4248158	336	C	T	334	2	0	**0.002**	-	-	-
TNF	rs1799964	335	T	C	214	107	14	0.20	19%	21%	0.54
	rs1800750	336	G	A	296	39	1	**0.06**	-	-	-
	rs1800629	330	G	A	232	86	12	0.17	18%	15%	0.46
	rs1799769	332	G	A	222	10	0	**0.02**	-	-	-
	rs3093662	335	A	G	276	58	1	**0.09**	-	-	-
	rs3093665	331	A	C	307	23	1	**0.04**	-	-	-
	rs3093668	335	G	C	295	38	2	**0.06**	-	-	-
LST1	rs1052248	336	T	A	174	135	27	0.28	28%	28%	0.89

SNPs (marked in bold) with MAFs <0.1 were excluded due to lack of power
** also significant on a dominant test; † associated with reduced risk of HIV-SN*

Table 4.3. Alleles of 3 SNPs from the TNF block were independently associated with HIV-SN. Other factors considered in the regression model were age and height

Gene	SNP	Multivariate analysis			
		OR	CI	Empirical p value	Empirical p value adjusted for multiple comparisons*
BAT1	rs1055388	0.61	0.35-1.09	0.11	0.77
	rs3130059	0.50	0.24-0.31	**0.005**	**0.03**
	rs2239528	0.61	0.34-1.09	0.10	0.78
	rs2523504	0.59	0.35-0.98	**0.04**	0.47
ATP6V1G2	rs2071594	0.48	0.29-0.77	**0.003**	**0.03**
NFKBIL1	rs2071592	0.46	0.29-0.75	**0.002**	**0.02**
	rs2071591	0.51	0.32-0.82	**0.007**	0.06
	rs4947324	0.63	0.38-1.04	0.07	0.66

*P values <0.05 in bold *as provided by PLINK*

Haplotype family FV15-23

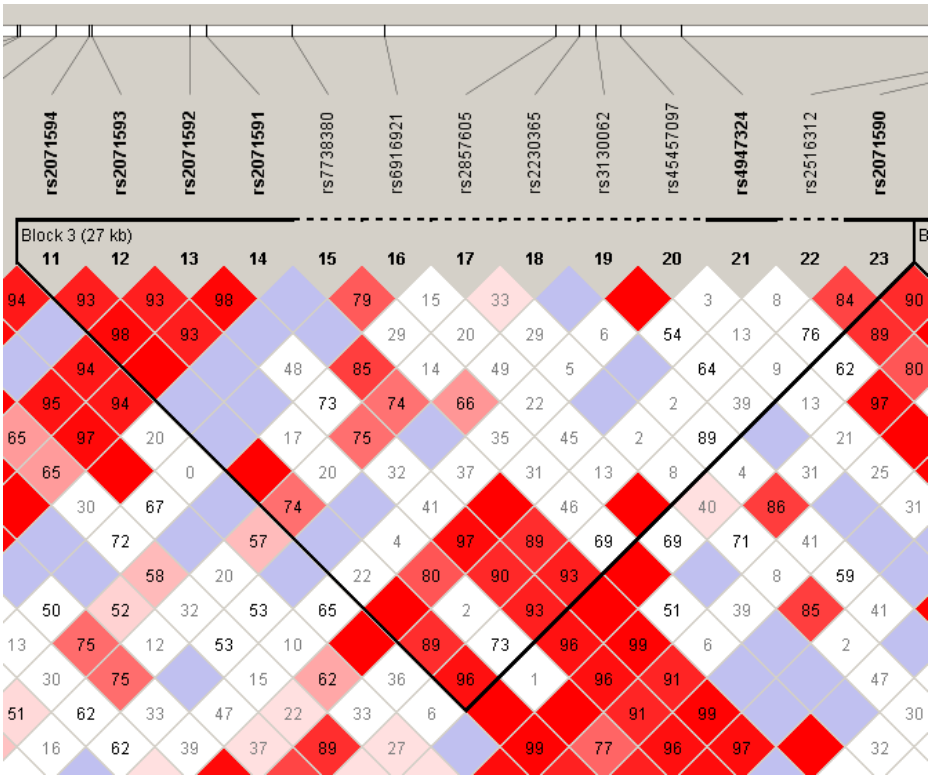
The minor allele of a *BAT1* SNP (rs3130059) associated with protection against HIV-SN. This SNP and the other SN-associated SNPs described in Table 4.3 (rs2071594, rs2071592, rs2071591) occur together in the FV15-23 haplotypes (Figure 4.1). I confirmed that the SN-associated SNPs occurring in FV15-23 (rs2071594, rs2071592, rs2071591) were in LD in this cohort (Figure 4.4).

Within this family of haplotypes, only FV16 and FV18 occur at a frequency of >10% in this Southern African cohort. Biologically this is a plausible association as FV18 has been associated with increased TNF production (Tan et al. 2011).The other allele associated with SN was rs2523504*2 in *BAT1* which marks the FV18.1 haplotype (a haplotype unique to our Southern African cohort, differing from FV18 by

only this SNP) but this haplotype occurred at a frequency of only 3% and so could not be analysed in relation to HIV-SN.

Neither FV16 nor FV18 alone were associated with SN, but the FV15-23 family associated with HIV-SN on univariate analysis (CC analysis - Chi^2; Odds ratio 1.4, 95% CI 1.03 - 1.94, p=0.02). However following logistic regression with a stepwise removal procedure, FV15-23 did not contribute to the final model so the effect is not strong.

Figure 4.4 Magnification of the critical region from the TNF block LD plot shown in Figure 4.3



This section of a haplotype within the TNF block illustrates the tight linkage disequilibrium between the SN-associated SNPs occurring in FV15-23 (rs2071594, rs2071592, rs2071591).

Haplotype family FV30-31

The FV30-31 family comprises 3 rare haplotypes, one of which (FV30.1) is unique to this Southern African cohort (FV30 not found, FV30.1 2.89%, FV31 4.43%). The FV30-31 family associated with resistance to HIV-SN on univariate analysis (CC analysis - Chi^2; OR = 0.45, 95% CI = 0.24 - 0.86, p=0.02). Following logistic regression with a stepwise removal procedure, the family remained associated with a reduced risk of HIV-SN (CC analysis - logistic regression; OR = 0.39, 95% CI 0.2 - 0.78, p = 0.008). The final model containing age, height and FV30-31 generated a p value of 0.0001 and an R^2 of 0.068. This suggests a rare but potent mechanism by which a TNF block genotype may protect against HIV-SN.

All haplotypes in the FV30-31haplotype family contain allele 2 of the *BAT1* SNP (rs2523504) which I found associated with resistance to HIV-SN on multivariate analysis (Table 4.3). This remains a candidate SNP for a role in resistance to SN. Allele 2 of rs2523504 is not in the other family associated with SN, FV15-23, which showed association with risk of SN on univariate analysis.

Although FV30 is not found in this Southern African cohort, in other populations it contains a tag SNP rs7738380 (NFKBIL1) which is lies between the SNPs marking FV15-23 SNPs (Figure 4.2) but is not in linkage disequilibrium with them.

Haplotypes incorporating TNF-1031

The minor allele of the previously associated TNFA-1031 SNP (rs1799964) did not associate with HIV-SN here (Table 4.1). Haplotypes containing this SNP (FVa6,7,8) associated with SN in Malays and Caucasians (Chew et al. 2010) so it is notable that FV6,7 is not found in this South African cohort. A new Southern African haplotype FV7.1 (two SNPs different from FV6,7 and one SNP different from FV8) contained a *LTA* SNP (rs2071590) which was also in LD with the FV15-23 SNPs and FV31 tag

SNP described earlier (Figure 4.3). Of interest, another SNP occurring in FV31 (rs4947324) marked other haplotypes (FV1-5) containing TNFA-1031 (rs1799964).

Assessment of new SNPs in the region

Next, eight novel regional SNPs were assessed. Four SNPs were removed as their MAF made them uninformative (Table 4.4).

Table 4.4 Four novel regional SNPs were removed from the analysis due to an insufficient MAF

Gene	SNP	n	Alleles		Genotype frequency			MAF
			1	**2**	**1,1**	**1,2**	**2,2**	
BAT1	rs1129640	335	C	T	301	5	29	**0.09**
NFKBIL1	rs45457097	301	G	A	281	19	1	**0.03**
LTA	rs1041981	329	A	C	129	158	42	0.37
	rs909253	328	A	G	126	160	42	0.37
	rs2229094	334	T	C	167	144	23	0.28
	rs2239704	329	C	A	272	56	1	**0.09**
	rs3093554	336	A	G	321	3	12	**0.04**
TNF	rs3093661	337	G	A	293	7	37	0.12

Univariate analysis of the remaining four SNPs yielded two SNPs from the *LTA* gene (rs1041981 and rs909253) associated with SN in a dominant model (Figure 4.5). In both cases carriage of the minor allele was associated with reduced risk of SN.

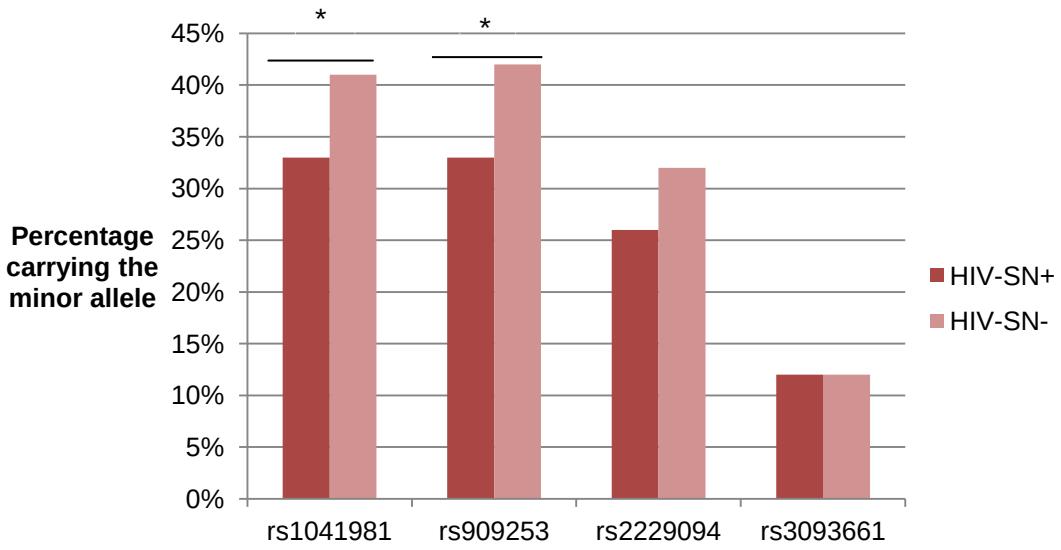


Figure 4.5 The minor alleles of newly assessed LTA SNPs, rs1041981 and rs909253, associated with risk of HIV-SN on univariate analysis

The association between these SNPs and SN risk remained significant after correction for age and height (Table 4.5). As the SNPs put forward to multivariate analysis had not previously been associated with neuropathy (ie: the comparison was not a confirmation of an *a priori* hypothesis), the analysis was corrected to account for multiple comparisons. The association with rs909253 remained significant.

Table 4.5. Multivariate analysis of newly assessed SNPs found two LTA SNPs associated with HIV-SN after correction for age and height

Gene	**SNP**	**Multivariate analysis**			
		OR	**CI**	**Empirical p value**	**Empirical p value adjusted for multiple comparisons**
LTA	rs1041981	0.54	0.34-0.88	**0.01**	0.06
	rs909253	0.48	0.29-0.77	**0.002**	**0.01**
	rs2229094	0.69	0.44-1.08	0.11	0.53

Logistic regression using a dominant model. P values <0.05 in bold

Marked linkage disequilibrium was evident between TNFA-1031 (rs1799964) and the nearby *LTA* SNP (rs1041981). One explanation for the association with *LTA* is that this SNP is in LD with an important previously identified functional area such as *TNF*. Alternatively, this SNP may be part of an as yet unidentified contributing pathway to HIV-SN. Genotyping the previously assessed Caucasian and Asian cohorts for rs1041981 will be an important first step in clarifying this issue.

Due to difficulties genotyping certain SNPs using the SNPlex system, rs909253 and rs1041981 have not been analysed in terms of FV haplotypes. Haplotype coverage will be improved with the addition of these SNPs. Genotyping and incorporating these *LTA* SNPs into the analyses of other ethnicities will determine if these SNPs are markers for populations other than a Southern African and may yield interesting associations with HIV-SN.

4.4 Conclusions

This is the first assessment of the TNF block in an African population and is the largest cohort to have assessed the TNF block and HIV-SN. I found SNPs in immune-related genes in the region associated with reduced risk of HIV-SN (rs3130059*G, rs2071594*C, rs2071592*A, rs909253*G). In addition to the associations with SNPs, I identified associations with haplotype families, some members of which are known to increase TNF production.

Limitations of this study include low MAF of several SNPs in the TNF block. This was expected given the known variability of allele frequency between ethnicities (Chew CSN et al. in press). However, associations between SNPs and HIV-SN in this cohort support the theory that the MHC is a region of interest in this disease. Analysis of the rare FV30-31 family should be repeated with an increased sample size so increasing the power. This was the first identification of an association with SN in this haplotype family in an African cohort and provides a basis for further investigation.

The association of alleles of individual SNPs within the TNF block and HIV-SN support an inflammatory etiology for this condition. The finding that the precise associations differ by ethnic group may be explained by variation in LD in different races. Alternatively, as rs909253 and rs1041981 were not explored in previous HIV-SN genetic studies, I cannot exclude that these SNPs may also be associated with HIV-SN in Caucasians, Chinese and Malays. Incorporating the two new *LTA* SNPs (rs909253 and rs1041981) plus additional population specific SNPs into future haplotype analyses may elicit stronger associations with SN and thus, progress the work towards identifying causative SNPs in HIV-SN still further.

CHAPTER 5

GENETIC ANALYSIS OF CYTOKINE AND CHEMOKINE INVOLVEMENT IN HIV-SN

Data from this chapter have been accepted for publication:

Wadley, A.L., Kamerman, P.R., Chew, C.S.N., Lombard, Z., Cherry, C.L., Price, P.

A polymorphism in *IL4* may associate with sensory neuropathy in African HIV patients.

Molecular Immunology, in press

5.1 Introduction

As described in Section 1.3, it has been hypothesised that proinflammatory cytokines are released following macrophage infiltration around peripheral nerves and are associated with the development of painful HIV-SN (Herzberg et al. 2001; Kennedy et al. 2004; Wallace et al. 2007a). To date, except for autopsy studies, the role of cytokines and chemokines has been demonstrated in animal models only. Here I assess the role of cytokines and chemokines in HIV-SN in humans using a genetic association study.

Several cytokines have been implicated in the pathogenesis of HIV-SN: IL-12p40 has been implicated by virtue of a study of Caucasians finding the minor allele of *IL12B* protective against HIV-SN (Cherry et al. 2008). Reduced levels of IL4 mRNA were observed in the peripheral nerves of HIV-positive patients with neuropathy compared to controls at autopsy (Wesselingh et al. 1994). TNFα, IL-1β and IL-6 protein has also been documented in the DRG of the sciatic nerves of HIV-positive individuals at autopsy (Yoshioka et al. 1994; Nagano et al. 1996). Centrally, intrathecal administration of gp120 into the lumbar dorsal spinal cord of rats caused mechanical allodynia which was attenuated by a TNF functional antagonist and reversed by IL-1ra (receptor antagonist) or an IL-6 neutralizing antibody (Milligan et al. 2000; Milligan et al. 2001; Schoeniger-Skinner et al. 2007).

Other cytokines have not been implicated directly in HIV-SN but in other models of neuropathic pain: In a chronic constriction injury model of neuropathic pain in rats increased mRNA expression of IL10 was observed 45 days post injury (Okamoto et al. 2001). Spinal IL-1α was upregulated in an animal model of radiculopathy of which, the accompanying allodynia was attenuated following administration of IL-1ra (Rothman et al. 2010). IL-18 expression was increased following spinal nerve ligation in rats and the allodynia was reversed following administration of an IL18 neutralizing antibody (Miyoshi et al. 2008).

The chemokine CCL2 and its receptor CCR2 have been directly implicated in the pain associated with HIV-SN as they are upregulated in the DRG in animal models and the accompanying hypernociception may be reversed by administration of a CCR2 receptor antagonist (Wallace et al. 2007b; Bhangoo et al. 2009). CCL5 is involved in Schwann cell-mediated neurotoxicity in the DRG (Chapter One, Section 1.2.1) and in hypernociception induced by a 2.5μl intradermal injection of soluble CCL5 into rats' paws (Oh et al. 2001).

Based on these data I have completed two analyses:

1. associations between pain in HIV-SN and SNPs in *CCL2* and *CCR2*
2. associations between HIV-SN, and pain in HIV-SN, using SNPs in *IL1A*, *IL1B, IL4, IL6, IL10, IL12B, IL18, TNF and CCL5*

5.2 Methods

Methodology is as described in Chapter 2. SNPs were chosen from published associations with HIV or neurological disease (Tables 5.1 and 5.2). Most of the polymorphisms had not previously been tested for association with pain, so these investigations represent an exploratory study. Figure 5.1 details the selection of patients whose data was analysed.

The association of *TNFA* alleles with presence of HIV-SN was assessed in Chapter 4, so only associations with the presence and intensity of pain are presented here.

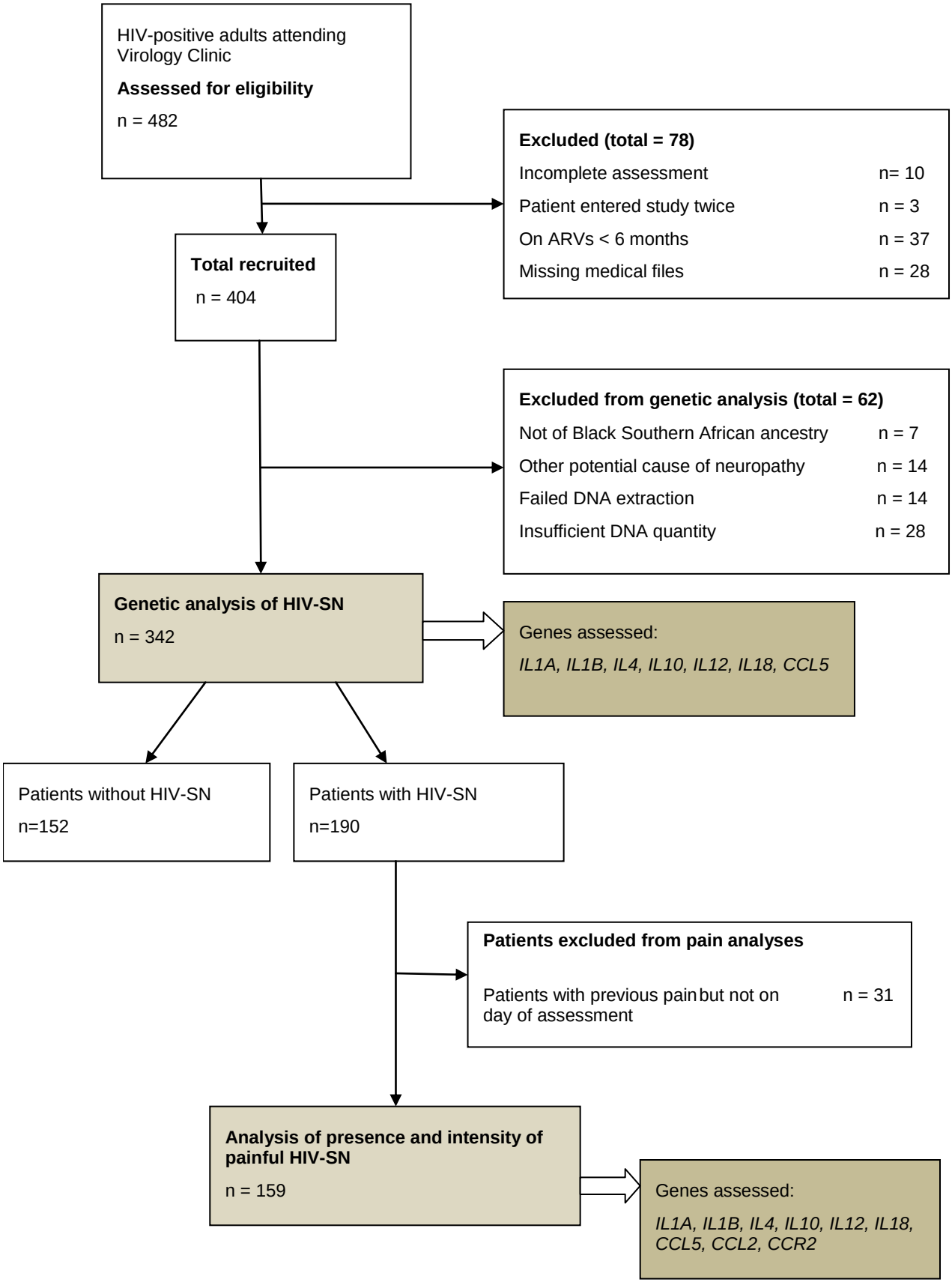


Figure 5.1 Individuals and genes included in the analysis

Table 5.1 Cytokine SNPs assessed and reason for selection

Gene	SNP	Common name	Chromosome	Chromosomal position	Alleles	Reason for SNP selection
IL1A	rs17561	IL1A+4845	2	113253693	G/T	T allele associated with onset and progression of multiple sclerosis in Caucasians (Mirowska-Guzel et al. 2011)
	rs1800587	IL1A-889	2	113259430	C/T	Minor allele associated with development of type 2 diabetes in Caucasians (Luotola et al. 2011)
IL1B	rs1143634	IL1B +3953	2	113306860	C/T	Minor allele also associated with development of type 2 diabetes in Caucasians (Luotola et al. 2011)
	rs16944	IL1B -511	2	113311337	A/G	Minor allele associated with risk of multiple sclerosis in Caucasians (Borzani et al. 2010)
IL4	rs2243250	IL4-590	5	132037052	T/C	T allele associated with risk of rheumatoid arthritis in Caucasians (Pawlik et al. 2005)
IL6	rs1800797	IL6-597	7	22732745	G/A	G alleles associated with presence of neuropathic pain following disc herniation in Caucasians (Noponen-Hietala et al. 2005)
	rs1800795	IL6-174	7	22733169	G/C	
IL10	rs1800872	IL10-592	1	205013029	C/A	A allele associated with type 2 diabetes in North Indians (Saxena et al. 2012)
	rs1800896	IL10-1082	1	205013519	A/G	A allele associated with risk of rheumatoid arthritis in Caucasians (Zhang et al. 2011)
	rs1800890	IL10-3575	1	205015987	T/A	A allele associated with risk of systemic sclerosis in Caucasians (Hudson et al. 2005)
IL12	rs3212227	IL12B(3'UTR)	5	158675527	A/C	Allele 2 associated with protection against HIV-SN in Caucasians (Cherry et al. 2008)
IL18	rs187238	IL18-137	11	111540197	G/C	G allele associated with risk of HIV infection in Indians (Sobti et al. 2011)
TNF	rs1800629	TNF-308	6	31651737	G/A	A allele associated with HIV-associated dementia in cohort with 50% Caucasians, 50% not described (Quasney et al. 2001)
	rs1799964	TNF-1031	6	31650287	T/C	Minor allele associated with HIV-SN in Caucasians, Chinese and Malays (Affandi et al. 2008; Cherry et al. 2008)

Table 5.2 Chemokine SNPs assessed and reason for selection

Gene	SNP	Common name	Chromosome	Chromosomal position	Alleles	Reason for SNP selection
CCL2	rs3760396	CCL2-927C	17	29605553	C/G	Associated with oedema in type 2 diabetes in a cohort with 94% Caucasians, 5% African American, 1% Hispanic (Ruano et al. 2009)
	rs4586	CCL2 Cys35Cys	17	29607381	C/T	Haplotype including rs4586 associated with multiple sclerosis in Caucasians (Ockinger et al. 2010)
CCR2	rs1034382	CCR2 -4385	3	46343405	A/T	T allele associated with sarcoidosis in Caucasians (Valentonyte et al. 2005)
CCL5	rs2107538	RANTES-403	17	31231892	T/C	Minor allele associated with protection against type 1 diabetes in Caucasians (Zhernakova et al. 2006)
	rs2280789	RANTES-In1.1	17	31231115	T/C	Associated with HIV transmission and progression in North Indians (Rathore et al. 2008)

5.3 Results and Discussion

As there are many cytokines and chemokines being dealt with here I have combined the Results and Discussion and placed a Conclusions section at the end of the chapter.

5.3.1 Selection of SNPs

Tables 5.1 and 5.2 detail the cytokine and chemokine SNPs and the reasons for selection. All SNPs were in Hardy Weinberg equilibrium except the *IL6* SNP rs1800795 where cases i.e. those affected with HIV-SN, were not in equilibrium. This may suggest that rs1800795 has an effect on susceptibility to HIV-SN. However, both *IL6* SNPs (rs1800797 and rs1800795) were excluded from the analysis as their MAFs were below 0.01 (Table 5.3). A CCL2 SNP (rs3760396) was also excluded for this reason. The median genotyping success rate was 98%, so the range of sample sizes for this selection of SNPs was 322-338.

Table 5.3 SNPs selected for analysis.

Gene	SNP	Common name	n	Allele		Genotype frequency			MAF
				1	2	1,1	1,2	2,2	
IL1A	rs17561	IL1A+4845	336	G	T	238	91	7	0.16
	rs1800587	IL1A-889	333	C	T	139	144	50	0.37
IL1B	rs1143634	IL1B+3953	333	C	T	253	74	6	0.13
	rs16944	IL1B-511	322	A	G	95	168	59	0.44
IL4	rs2243250	IL4-590	323	T	C	175	118	30	0.28
IL6	**rs1800797**	IL6-597	335	G	A	329	6	0	**0.01**
	rs1800795	IL6-174	333	G	C	326	5	2	**0.01**
IL10	rs1800872	IL10-592	337	C	A	135	154	48	0.37
	rs1800896	IL10-1082	335	A	G	162	140	33	0.23
	rs1800890	IL10-3575	332	T	A	196	116	20	0.31
IL12	rs3212227	IL12B(3'UTR)	333	A	C	154	145	34	0.32
IL18	rs187238	IL18-137	336	G	C	240	87	9	0.16
TNF	rs1800629	TNF-308	330	G	A	232	86	12	0.17
	rs1799964	TNF-1031	335	T	C	214	107	14	0.20
CCL2	**rs3760396**	CCL2-927C	335	C	G	314	19	2	**0.03**
	rs4586	CCL2 Cys35Cys	331	C	T	156	147	28	0.31
CCR2	rs1034382	CCR2-4385	338	A	T	139	155	44	0.36
CCL5	rs2107538	RANTES-403	335	T	C	111	154	70	0.44
	rs2280789	RANTES-In1.1	337	T	C	220	102	15	0.20

Three SNPs excluded due to low minor allele frequencies (MAF) are shown in bold

5.3.2 SNPs tested for associated with pain in HIV-SN

Table 5.4 Alleles of TNFA, CCL2 and CCR2 do not associate with the presence or intensity of pain.

Gene	SNP	Common name	Alleles	Empirical p value	
				Presence of pain	Pain intensity
TNF	rs1800629	TNF-308	G/A	0.49	0.26
	rs1799964	TNF-1031	T/C	0.13	0.47
CCL2	rs4586	CCL2 Cys35Cys	C/T	0.95	0.57
CCR2	rs1034382	CCR2 -4385	A/T	0.66	0.40

Univariate analysis using allelic model

5.3.2.1 TNF

TNF is implicated in the pathogenesis of pain in HIV-SN (Zheng et al. 2011a; Zheng et al. 2011b). The two *TNF* SNPs assessed here formed part of the TNF block analysed in Chapter 4 and had sufficient MAFs for analysis. The A allele of rs1800629 associates with higher TNF production (Wilson et al. 1997) and both this allele and the minor allele of rs1799964 have been associated with severity of cancer pain in Caucasians (Reyes-Gibby et al. 2009; Rausch et al. 2012). Alleles of these two *TNF* SNPs did not associate with the presence or intensity of pain here (Table 5.4) which may indicate different LD from Caucasians (see Chapter 4) or differences in the pathogenesis of pain associated with HIV disease and cancer. There were five other *TNF* SNPs genotyped (see Chapter 4, Table 4.2), but their MAFs were too low for inclusion here.

5.3.2.2 Chemokines

CCR2 and CCL2 have been implicated in HIV-SN related hypernocieption (Wallace et al. 2007b; Bhangoo et al. 2009). Chemokine polymorphisms have not been

assessed in HIV-SN previously, so SNPs were selected for associations with inflammatory and neuropathic conditions (Table 5.2). These data have been published in non-African cohorts and LD differs between ethnicities (Tishkoff et al. 2009). Chemokine SNPs have not previously been associated with pain and no associations were evident here. However one *CCL2* SNP failed genotyping (Chapter 2, Table 2.8) and another had a very low MAF (Table 5.3), so coverage was poor.

5.3.3 SNPs tested for association with both HIV-SN and pain

Table 5.5 The IL4 SNP rs2243250 associated with presence of HIV-SN

Gene	SNP	Common name	Alleles	Empirical p value		
				Presence of HIV-SN	Presence of pain	Pain intensity
IL1A	rs17561	IL1A+4845	G/T	0.15	0.98	**0.09**
	rs1800587	IL1A-889	C/T	0.26	0.27	0.82
IL1B	rs1143634	IL1B +3953	C/T	0.77	0.58	0.84
	rs16944	IL1B -511	A/G	0.77	0.13	0.15
IL4	rs2243250	IL4-590	T/C	**0.02**	0.27	0.30
IL10	rs1800872	IL10-592	C/A	0.14	0.25	0.29
	rs1800896	IL10-1082	A/G	0.25	0.58	1.00
	rs1800890	IL10-3575	T/A	0.22	0.30	0.37
IL12	rs3212227	IL12B(3'UTR)	A/C	0.31	0.56	0.35
IL18	rs187238	IL18-137	G/C	0.72	0.19	0.58
CCL5	rs2107538	RANTES-403	T/C	0.67	0.70	0.27
	rs2280789	RANTES-In1.1	T/C	0.54	0.35	0.67

5.3.3.1. IL1A

There were several reasons to investigate *IL1A*. IL-1α protein increased in the spinal cord of rats following nerve root compression and administration of IL1-ra reduced the accompanying allodynia and astrocytic activity (Rothman et al. 2010). Carriage of the minor allele of rs1800587 associated with risk of type 2 diabetes (Luotola et al. 2011) and diabetes may be a risk factor for HIV-SN (Lichtenstein et al. 2005; Ances et al. 2009; Evans et al. 2011) [see Chapter 1, Section 1.4.2.2]. The minor alleles of rs17561 and rs1800587 are in tight linkage disequilibrium and associated with a poor virological response to ART in Caucasians, but were not in LD and did not associate with this phenotype in African Americans (Price et al. 2009b). This suggests haplotypic differences between Caucasians and Africans. Here neither SNP associated with HIV-SN or presence of pain (Table 5.5). This is in accordance with our earlier study of HIV-SN in a Caucasian cohort (Cherry et al. 2008). However, the T allele of rs17561 achieved a p value <0.1 on an allelic model of univariate analysis assessing association with pain intensity (Table 5.5). However following correction for age, sex and CD4 T-cell count, the association was no longer significant (linear regression; p=0.10).

5.3.3.2 IL1B

IL-1β has been detected in the DRGs of HIV-positive patients at autopsy (Nagano et al. 1996; Jones et al. 2005). IL-1β expression was associated with neurite degeneration and IL-1β transcripts were also detected more frequently in those with HIV-SN than those without (Jones et al. 2005). Additionally IL-1β has been implicated in an animal model of HIV-SN pain (Milligan et al. 2001). The minor allele of rs16944 associated with risk of multiple sclerosis in Italians (Zhang et al. 2011) and the minor allele of rs1143634 associated with development of type 2 diabetes in Caucasians from Finland (Luotola et al. 2011). Neither of the *IL1B* SNPs associated with HIV-SN, as was the case previously in a Caucasian cohort (Cherry et al. 2008).

5.3.3.3 *IL4*

The T allele of rs2243250 associates with higher production of IL-4 and so has been linked with Th2 conditions such as asthma and atopic allergy in Caucasians and Filipinos (Rosenwasser et al. 1997; de Guia et al. 2010). Alleles of rs2243250 showed no association with HIV-SN in a Caucasian population (Cherry et al. 2008), but LD within *IL4* varies between ethnicities (Wang et al. 2004) so this SNP was tested in our cohort.

The T allele associated with risk of HIV-SN here (Figure 5.2, Table 5.5). Following correction for age and height, rs2243250 remained associated but the association did not withstand correction for multiple comparisons (Table 5.6). This is consistent with a report by Uceyler and colleagues (2007) finding high IL-4 serum levels in varying neuropathies in HIV-negative individuals compared to controls.

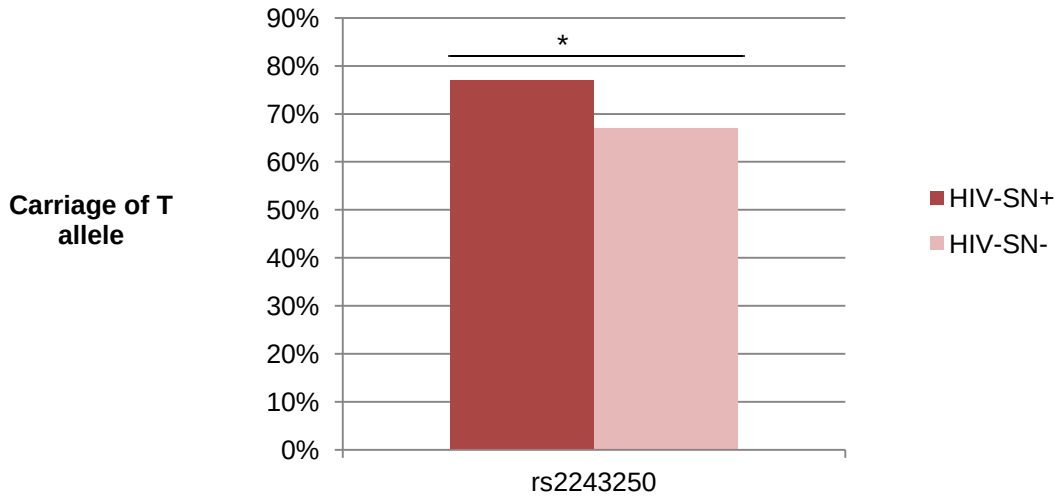


Figure 5.2 The 'T' allele of the IL4 SNP rs2243250 associated with increased prevalence of HIV-SN on an allelic model of univariate analysis

Table 5.6 Multivariate analysis of association of rs2243250 with HIV-SN

	OR	**CI**	**Empirical p value**	**Empirical p value adjusted for multiple comparisons**
Allelic model	0.64	0.45-0.91	**0.01**	0.23
Dominant model	0.62	0.39-0.98	**0.04**	0.58

The C allele of the IL4 SNP rs2243250 remained associated with reduced prevalence of HIV-SN after correction for age and height (in bold) but not after correction for multiple comparisons

In contrast, lower levels of IL-4 mRNA and increased TNFα were found at autopsy in the peripheral nerves of patients with HIV-SN compared to HIV patients without neuropathy (Wesselingh et al. 1994). These patients were at late stage of HIV disease (they had died due to AIDS) – a group which may be biased to include *few* individuals with the high *IL4* producing genotype (homozygotes of the T allele of rs2243250 measured here) since this predominates amongst patients who retain higher CD4 T-cell counts (Wang et al. 2004). Although CD4 T-cell counts did not differ significantly by *IL4* genotype, there was a trend (Kruskal Wallis; p=0.12) and the association may have been minimised by the effects of ART.

As IL-4 and TNFα are mutually antagonistic, it is possible that high TNFα and low IL-4 are seen at end stage HIV disease, whilst high IL-4 (a Th2 environment) is a feature of early stage HIV disease. Patients had been on ART for a median of 29 months (range 6-120) and 52% (179/342) had had a diagnosis of AIDS so the cohort included individuals with both early and late disease.

It is notable that plasma levels of IL-4 were slightly higher in painless rather than painful neuropathy (Uceyler et al. 2007). Hence it is logical that as TNFα levels rise and IL-4 levels drop during HIV disease, neuropathy develops first asymptomatically then symptomatically (Figure 5.3). Indeed, IL-4 may reduce pain in early SN. IL-4

injected in soluble form or stimulated in the lumbar DRG using a vector, reduced neuropathic pain in rats following injection of nociceptive substances in to hind paws and separately in a model of sciatic nerve ligation (Cunha et al. 1999; Hao et al. 2006). One mechanism of this pain reduction may be increased IL-4-induced mu-opioid receptor transcription in the DRG and spinal cord neurons after nerve injury (Kraus et al. 2001; Uceyler et al. 2011).

What is clear is that IL-4 has been insufficiently assessed in HIV-SN and requires further investigation.

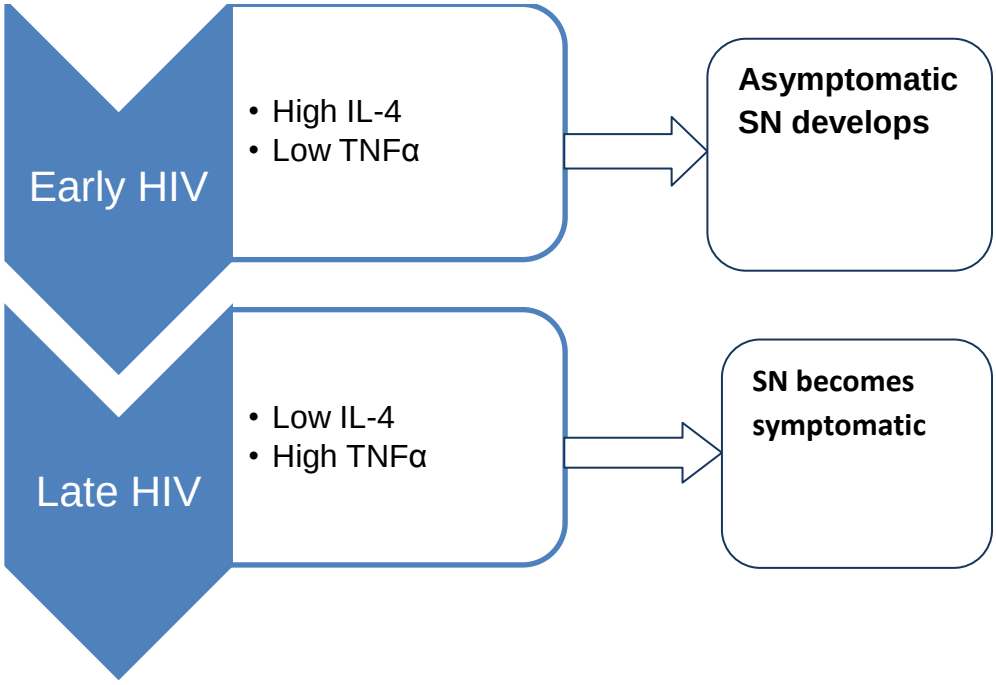


Figure 5.3 Suggested mechanism by which IL-4 and TNFα levels might contribute to development of HIV-SN

5.3.3.4 *IL10*

The G allele of rs1800896 (IL10-1082) associated with higher viral loads in acute HIV infection in Africans (Naicker et al. 2009) and with risk of diabetic neuropathy in Southern Indians with type 2 diabetes (Kolla et al. 2009). The A allele of rs1800872 (IL10-592) also associated with increased susceptibility to HIV in Africans (Naicker et al. 2009). Both these alleles and the T allele of rs1800890 (IL10-3575) associate with higher IL-10 production (Naicker et al. 2009; Banerjee et al. 2011), but did not associate with HIV-SN in this study (Table 5.5) or in Caucasians (Cherry et al. 2008). However *IL10* haplotypes comprising rs1800872, rs1800896 and a third SNP in tight linkage disequilibrium with rs1800872 proved to be better predictors of *ex-vivo* IL-10 secretion than individual alleles (Temple et al. 2003). Here five haplotypes of rs1800872, rs1800896 and rs1800890 accounted for 99% of the cohort but only three haplotypes with frequencies >0.1 were included in the analysis. The CGA haplotype achieved a p value <0.1 on an allelic model univariate analysis (Table 5.7) and following correction for age and height remained associated with protection against HIV-SN (logistic regression; OR = 0.64, p value = 0.02). This haplotype contained the G allele of rs1800896 has associated with risk of diabetic neuropathy (Kolla et al. 2009) and so this result warrants confirmation in other cohorts.

In the analysis of *IL10* haplotypes with presence and intensity of pain, the AAT haplotype achieved p<0.1 on univariate analysis, suggesting an association with presence of pain, but not the intensity of pain. Following correction for age, sex and CD4 T-cell count, the association remained marginal (logistic regression; OR 2.39, p=0.07). In an animal model of neuropathic pain, administration of soluble IL-10 ameliorated the thermal hyperalgesia associated with the chronic constriction injury (Wagner et al. 1998), which would support an association between pain and a high IL-10 producing haplotype. Hence this weak association warrants further study in a larger cohort.

Table 5.7 Univariate analysis of IL10 haplotypes

Haplotype	Frequency	Empirical p value		
		HIV-SN	Risk of pain	Pain intensity
CGA	0.20	**0.09**	0.58	0.98
AAT	0.36	0.22	**0.08**	0.42
CAT	0.31	0.97	0.21	0.17

Univariate analysis showed the CGA and AAT haplotypes achieve p<0.1 on risk of HIV-SN and pain respectively (in bold).

Haplotypes created from alleles at rs1800872, rs1800896, rs1800890

5.3.3.5 *IL12B*

Carriage of the minor allele of a polymorphism in the IL12B(3'UTR) has associated with immunopathological conditions including type 1 diabetes (Windsor et al. 2004). However the effects were complicated with a dependence on age of onset and a clear benefit from heterozygosity. In unpublished studies, heterozygous carriage of the minor allele has shown weak association with survival in untreated Gambian HIV patients. Caucasian carriers of the minor allele were less likely to have extremely low CD4 T-cell counts (Price P, personal communication). These findings were consistent with a protective effect against HIV-SN and CMV retinitis in HIV-positive Caucasians on ART (Price et al. 2002; Cherry et al. 2008). Here no association was seen with either HIV-SN or pain (Table 5.5) including genotypic tests which assess the effects of heterozygosity (genotypic test Empirical p values; SN =0.59, presence of pain =1.00, pain intensity =0.35).

5.3.3.6 *IL18*

IL-18 protein has been associated with neuropathic pain in a rat nerve ligation model (Miyoshi et al. 2008), but showed no association with HIV-SN or pain in this cohort. High serum IL-18 levels have associated with AIDS dementia complex and high IL-

18 plasma concentrations with viral load and HIV progression (von Giesen et al. 2004; Wiercinska-Drapalo et al. 2004). The G allele of the *IL18* SNP assessed here (rs187238, IL18-137) associated with protection from lipodystrophy in Brazilians (Castelar et al. 2010), but with risk of HIV infection in North Indians (Sobti et al. 2011).

5.3.3.7 CCL5

Minor alleles of CCL5 SNPs rs2107535 and rs2280789 associated with HIV progression in African Americans, Caucasians and North Indians (An et al. 2002; Rathore et al. 2008) and the minor allele of rs2107538 was protective against type 1 diabetes (Zhernakova et al. 2006). The minor alleles of CCL5 associated with lower serum levels of CCL5 (An et al. 2002; Zhernakova et al. 2006) and elevated plasma CCL5 was seen in patients with HIV-SN (Chew et al. 2011) so implicating the major alleles in the pathogenesis of HIV-SN. However, no association was seen between CCL5 SNPs and presence of HIV-SN, or presence of intensity of HIV-SN related pain (Table 5.5).

5.4 Conclusions

This was the first assessment of cytokine and chemokine SNPs in an African population with HIV-SN. It was also the first assessment of cytokine and chemokine SNPs in relation to pain in HIV-SN. I identified associations with genes encoding the anti-inflammatory cytokines IL-4 and IL-10. These cytokines have mutually antagonistic relationships with TNFα, a cytokine long associated with HIV-SN pathogenesis, and so it is biologically plausible that they play a role. The major allele of an *IL4* SNP (rs2243250) associated with risk of HIV-SN. This allele has also associated with higher CD4 T-cell counts and additionally, high IL-4 levels have associated with painless neuropathy suggesting a potential role for IL-4 in early stage asymptomatic neuropathy. One *IL10* haplotype inferred protection against HIV-SN with another demonstrating a trend for risk of pain. These are the first human data implicating these cytokines in HIV-SN. Repetition in other cohorts and further

assessment using more SNPs to identify critical haplotypes in African populations is needed.

There were several limitations to this study. The selection of SNPs from the literature meant that gene coverage was limited and biased towards SNPs identified in other races. Negative data relating to the chosen SNPs in my cohort do not rule out associations between the related genes and HIV-SN or pain. Tag-SNPs generated from HapMap from the Yoruba population may provide better gene coverage. Another limitation was the small number of subjects in this cohort with non-painful neuropathy, so the study had limited power to detect weak effects. Recruitment and genotyping of more subjects with painless neuropathy would improve these analyses.

In conjunction with animal and autopsy studies (allowing study of tissues and related proteins that cannot be practically sampled in life e.g. DRG), genetic association studies assist in implicating proteins involved in disease. This is essential for the identification of drug targets. As there are no proven, commercially available drugs available to treat HIV-SN or the pain associated with it (Phillips et al. 2010), evidence that IL-4 and IL-10 influence SN is worthy of further investigation.

CHAPTER 6

ANALYSIS OF UNCOUPLING PROTEINS IN HIV-SN

Data presented in this chapter have been accepted for publication:

Wadley, A.L., Lombard, Z., Cherry, C.L., Price, P., Kamerman, P.R.

"Polymorphisms in uncoupling proteins genes *UCP2* and *UCP3* are not associated with HIV-associated sensory neuropathy in African individuals".

Journal of the Peripheral Nervous System, in press

And presented at a conference:

14th World Congress on Pain, Milan, Italy. 27th-31st August 2012

6.1 Introduction

Like diabetic neuropathy, HIV-SN is a small fibre, length-dependent neuropathy associated with mitochondrial dysfunction and associated calcium dysregulation (Verkhratsky et al. 2008; Hoke et al. 2009; Lehmann et al. 2011) which is affected by modulation of Sarco/Endoplasmic Reticulum Ca2+-ATPase (SERCA) (Zherebitskaya et al. 2012).

Uncoupling proteins 2 and 3 (UCP2 and UCP3) are implicated in neuropathy. These proteins are expressed in neurons and dorsal root ganglia (Fleury et al. 1999; Vincent et al. 2004) and are involved in regulating oxidative stress within cells (Mattson et al. 2003). Oxidative stress resulting from mitochondrial dysfunction can lead to the development of neuropathy, as shown in diabetes (Low et al. 1997). Additionally expression of UCP3 is proportionate to intracellular calcium modulated by SERCA (De Marchi et al. 2011). Minor alleles of single nucleotide polymorphisms (SNPs) in the genes encoding uncoupling proteins *UCP2* (rs659366, -866G>A) and *UCP3* (rs1800849, -55C>T) have associated independently with a reduced prevalence of symptomatic neuropathy in Caucasians with Type 1 diabetes (Rudofsky et al. 2006). The minor alleles of both SNPs associate with increased mRNA expression in adipose and skeletal tissue and the minor allele of *UCP2* associated with increased transcriptional activity in an adipocytes cell line (Schrauwen et al. 1999; Krempler et al. 2002). Both SNPs lie upstream of the genes in or near putative promoter sequences.

I investigated whether polymorphisms in *UCP2* and *UCP3,* previously associated with symptomatic diabetic neuropathy, also associate with the presence of symptomatic HIV-SN. The burden of disease was particularly high in this cohort with 57% suffering with HIV-SN (Chapter 3).

6.2 Methods

Methodology is as described in Chapter 2. The patients used in this analysis are described in Figure 6.1 and the SNPs assessed shown in Table 6.1.

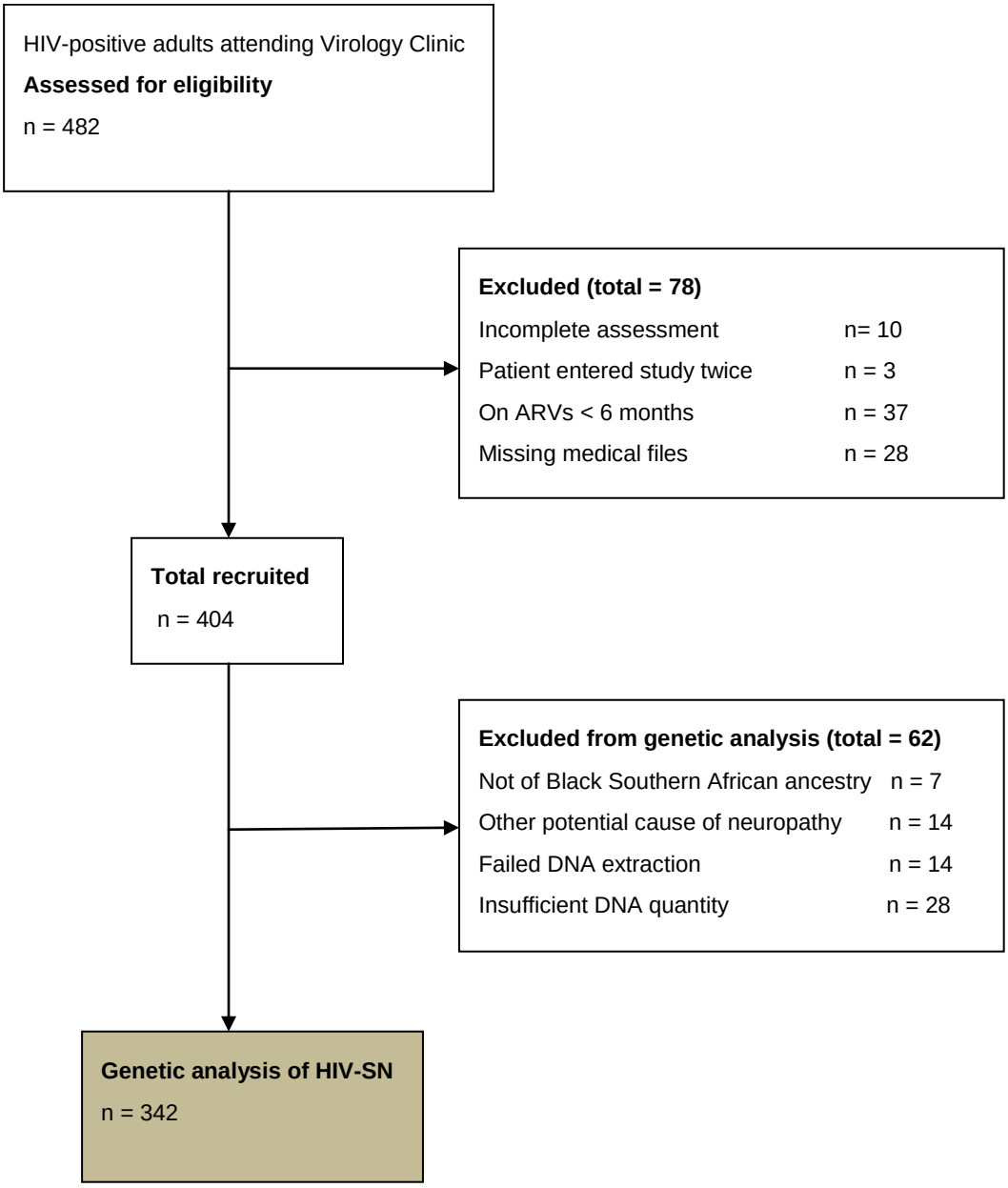


Figure 6.1 Patients included in the UCP analysis

Table 6.1 SNPs assessed on chromosome 11

Gene	SNP ID	Common name	Chromosomal position
UCP2	rs659366	G-866A	73372402
UCP3	rs1800849	C-55T	73397813

6.3 Results

Three hundred and thirty-five participants were successfully genotyped for these two UCP SNPs and were included in the analysis. Both polymorphisms were C>T SNPs, as described on the forward strand. Both SNPs were in Hardy-Weinberg equilibrium. The MAFs of the two SNPs (Table 6.2) were similar to those previously published in Caucasians [rs659366 = 0.38; rs1800849 = 0.10] (Rudofsky et al. 2006) [Chi^2; p>0.05 for both]. LD was not demonstrated between the two SNPs in Southern Africans (D'=0.17, R^2=0.005).

Table 6.2 MAFs of the UCP SNPs were similar in this cohort to those previously published in Caucasians

Gene	SNP ID	n	Alleles		Genotype frequency			MAF
			1	2	1,1	1,2	2,2	
UCP2	rs659366	335	C	T	127	140	68	0.41
UCP3	rs1800849	334	C	T	266	65	3	0.11

Univariate analyses

Table 6.2 shows univariate analysis of carriage of *UCP* SNPs and presence of HIV-SN with no significant association observed. Genotypic, dominant and recessive model tests for HIV-SN could not be run for rs1800849 as there were no homozygotes for the minor allele, however, these tests were run for rs659366 but did not show any association (genotypic, p=0.3; dominant, p=0.1; recessive, p=0.3). Due to the lack of association multivariate analysis was not carried out.

Table 6.3 Univariate analysis between UCP SNPs and presence of HIV-SN

SNPs	**Percentage with minor allele**		**Empirical p value**			
	HIV-SN n=186	No HIV-SN n=149	Allelic	Genotypic	Dominant	Recessive
Rs659366	38%	45%	0.14	0.29	0.16	0.32
Rs1800849	10%	11%	0.90	1.00	1.00	1.00

6.4 Discussion

I found no association between alleles of *UCP2* or *UCP3* and presence of symptomatic HIV-SN. In Caucasians, carriage of minor alleles of *UCP2* and *UCP3* associated with reduced risk of symptomatic diabetic neuropathy (Rudofsky et al. 2006), a disease that has similarities to HIV-SN. Whilst differences in MAFs between the Caucasian cohort (Rudofsky et al. 2006) and ours were not significant (p=0.07) the apparent discord in relation to our findings may reflect differing haplotypic associations in Caucasians and Africans (Tishkoff et al. 2002).

Despite the similarities in mitochondrial dysfunction and calcium dysregulation in both diabetic and HIV-SN it is possible that uncoupling proteins associate with another pathological aspect particular to diabetic neuropathy, for example hyperglycaemia. Overexpression of UCP2 in pancreatic β-cells impeded glucose stimulated insulin secretion (Dalgaard 2011) and the minor allele of *UCP2* associated with increased tissue mRNA expression (Krempler et al. 2002).

There were limitations to my study. Although I excluded diagnosed diabetic patients, I did not formally test glucose tolerance. However, the prevalence of diabetes in South African HIV-positive patients is approximately four percent (Levitt et al. 2006) only and so this should have had little impact. Secondly, ethnic heterogeneity may have influenced results. However, we reduced population stratification by only including local black Southern Africans who declared no Caucasian ancestry and whose local ethnic affiliation belonged to the Niger-Kordofanian language grouping. This grouping shows lower inter-heterogeneity than other African population groups (Campbell et al. 2008). A recent study of ancestry informative markers in black Africans living in a Johannesburg township found no significant substructure in this population (Lombard et al., unpublished results). Additionally another study found little variation between the southeastern Bantu speakers of South Africa (Lane et al. 2002); a sub grouping that incorporates the ethnic groups living in Johannesburg. Whilst the ethnic grouping used for the study does not preclude stratification, the effect is probably marginal.

In summary, there was no association between the 2 SNPs in uncoupling proteins 2 and 3 and HIV-SN in this black Southern African cohort. Haplotypic associations within the gene may vary with ethnicity. Additionally uncoupling proteins may be involved in another pathological aspect of diabetic neuropathy that HIV-SN does not have in common.

CHAPTER 7

ANALYSIS OF A PREVIOUSLY IDENTIFIED 'PAIN PROTECTIVE' HAPLOTYPE AND INDIVIDUAL POLYMORPHISMS IN THE *GCH1* GENE IN HIV-SN

Data presented in this chapter have been published:

WADLEY AL, LOMBARD Z, CHERRY CL, PRICE P, KAMERMAN PR

Analysis of a previously identified 'pain protective' haplotype and individual polymorphisms in the *GCH1* gene in Africans with HIV-associated sensory neuropathy: a genetic association study

Journal of Acquired Immune Deficiency Syndromes, 60: 20-23, 2012

And presented at a conference:

13th World Congress on Pain, Montreal, Canada. 29 August – 2 September 2010

Poster presentation: The role of GCH1 polymorphisms on pain perception in black Africans with HIV-associated sensory neuropathy

7.1 Introduction

As I described in Chapter 3 the most common symptom of HIV-SN is pain, which affected approximately 75% of individuals with HIV-SN; with three-quarters of those affected individuals experiencing pain of moderate to severe intensity. This level of neuropathic pain markedly increases the burden of disease in the community (Doth et al. 2010; Ellis et al. 2010), especially as the pain is resistant to most conventional treatments for painful neuropathy (Phillips et al. 2010). Investigations into the underlying mechanisms of the pain are warranted to improve diagnosis and treatment.

GCH1 and its role in pain sensitivity was introduced in Chapter 1, Section 1.3.3.1. Variations of the gene encoding GCH1, *GCH1,* were associated with reduced pain in humans, with a 15-single nucleotide polymorphism (SNP) haplotype and five individual SNPs within the haplotype being associated with reduced pain intensity following discectomy for persistent radicular low back pain (Tegeder et al. 2006; Tegeder et al. 2008). A subsequent study showed that this 15-SNP haplotype could be identified in Caucasians by genotyping for a smaller three-SNP haplotype (Lotsch et al. 2007).

However the results from subsequent *GCH1* pain association studies have been inconsistent. It has been suggested that population stratification may have contributed to the detection of the original associations or that the different haplotype structure in *GCH1* across ethnic groups may account for the inconsistency (Kim et al. 2007). Studies of different ethnic groups are now needed to determine whether published results may be generalised. Here I describe the first investigation into *GCH1* polymorphisms and pain associated with HIV-SN in an African population. I also examined linkage disequilibrium (LD) between the *GCH1* SNPs.

7.2 Methods

Methodology is as described in Chapter 2. The patients used in this analysis are described in Figure 7.1 and the SNPs assessed shown in Table 7.1. The larger cohort of 342 participants (with and without HIV-SN) was used to examine the linkage disequilibrium (LD) between the 6 SNPs using the confidence interval method in Haploview (Barrett et al. 2005). This was compared with the LD structure for Caucasians (CEU) assessed using data from HapMap (Genome Browser release#27).

Table 7.1 GCH1 SNPs assessed

Gene	SNP ID	Common name	Chromosomal position
GCH1	rs10483639	c.4279	54376206
	rs3783641	c.343+8900	54429888
	rs4411417	c.509+5836	54390312
	rs752688	c.627-708	54381318
	rs8007201	c.509+1551	54394597
	rs8007267	c.-9610	54448740

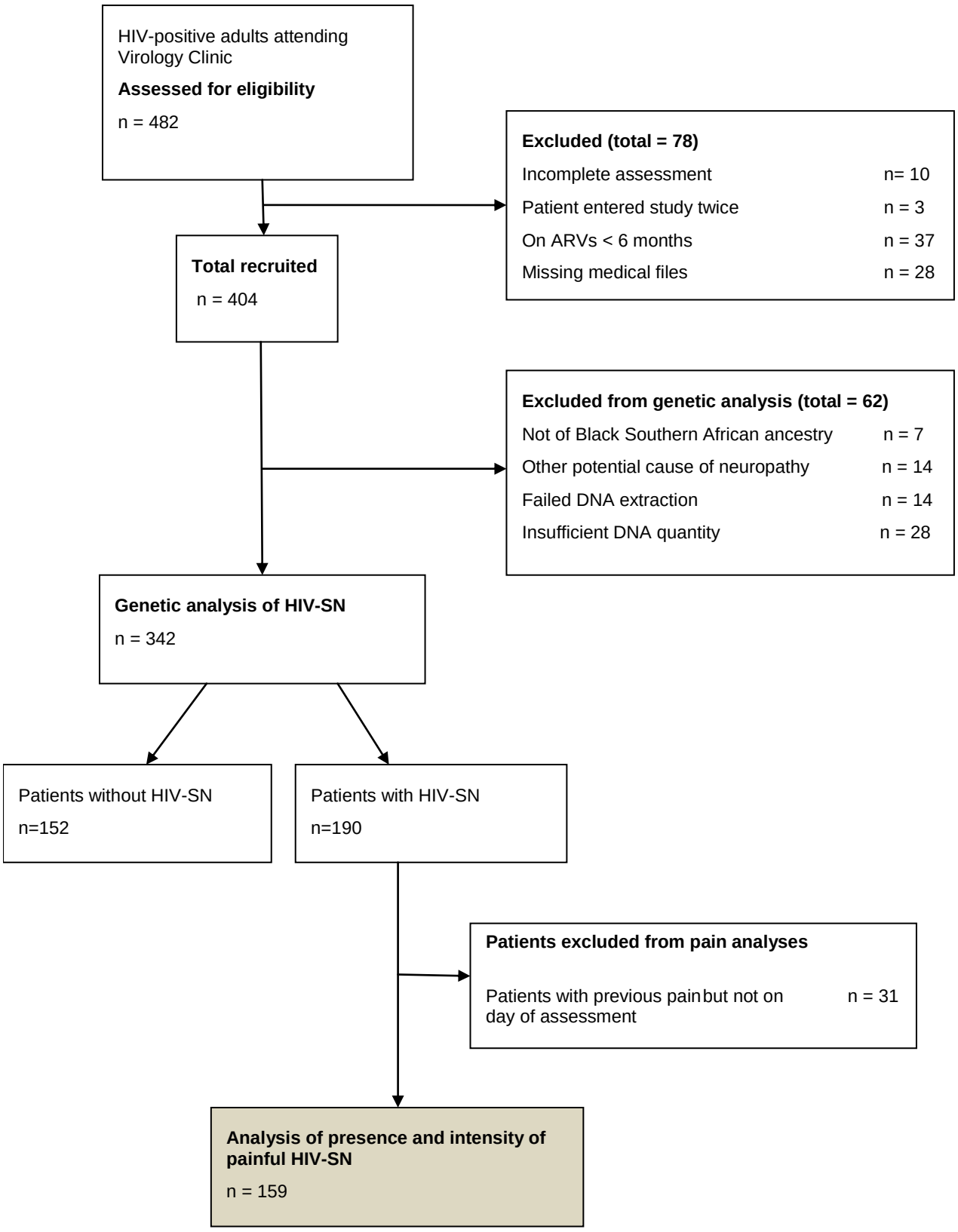


Figure 7.1 Individuals included in the GCH1 analysis

7.3 Results

The major and minor of alleles of rs8007201 and rs8007267 were reversed when our cohort was compared with a Caucasian population (CEU; HapMap). The SNPs were all in Hardy-Weinberg equilibrium and the MAFs are shown in Table 7.2. The SNP rs4411417 was excluded from the analysis due to a MAF <0.1.

Table 7.2 MAF of GCH1 SNPs

SNP name	n	Alleles		Genotype frequency			MAF
		1	**2**	**1,1**	**1,2**	**2,2**	
rs10483639	336	G	C	205	108	23	0.21
rs752688	335	C	T	264	61	10	0.10
rs4411417	**337**	T	C	265	65	7	**0.09**
rs8007201	336	G	A	180	132	24	0.25
rs3783641	331	T	A	170	128	33	0.30
rs8007267	336	T	C	138	146	52	0.35

rs4411417 was excluded due to a MAF<0.1 (in bold).

No individual SNPs associated with the presence or intensity of pain (Tables 7.3 and 7.4). We then addressed 3-SNP haplotypes, including the 'pain-protective' haplotype (Lotsch et al. 2007) that includes rs8007267 allele T (denoted CAT), and haplotypes of all 6 SNPs. The cohort had 5 of the 8 possible 3-SNP haplotypes and 8 of the 36 possible 6-SNP haplotypes. The 3-SNP haplotypes shown here accounted for 99% of the cohort and the 6-SNP haplotypes described accounted for 82% of the cohort. The 3-SNP 'pain protective' haplotype (CAT) and a 6-SNP haplotype including this motif (CTCGAT) were not associated with pain intensity but *were* associated with reduced presence of pain (Table 7.5). However, after correcting for factors that have previously been associated with pain (age, sex and CD4 T-cell count), the associations with the 'pain protective' 3-SNP haplotype (logistic regression; p=0.05)

and the 6-SNP haplotype (logistic regression; p=0.09) were no longer significant. An additional 3-SNP haplotype (GTC) was associated with increased risk of pain on univariate analysis (p=0.04) but the association was also no longer significant after correction for risk factors (logistic regression; p=0.08).

Table 7.3 Univariate analysis of association between GCH1 SNPs and presence of pain in HIV-SN

SNP	Percentage with minor allele		Allelic test	
	Pain n=144	No pain n=15	Empirical p value	Empirical p value adjusted for multiple comparisons
rs10483639	20%	33%	0.14	0.40
rs752688	9%	20%	0.07	0.25
rs8007201	26%	13%	0.12	0.45
rs3783641	28%	43%	0.10	0.38
rs8007267	36%	17%	0.05	0.13

Table 7.4 Univariate analysis between GCH1 SNPs and pain intensity

SNP	R^2	Empirical p value	Empirical p value adjusted for multiple comparisons
rs10483639	0.003	0.50	0.94
rs752688	0.007	0.30	0.77
rs8007201	8.61e-07	0.99	1.00
rs3783641	0.005	0.40	0.90
rs8007267	0.0006	0.77	0.99

Wald test

Table 7.5. Univariate analysis between the 3-SNP and 6-SNP haplotypes and presence of pain

3-SNP haplotypes				
	Frequency	Pain n=144	No pain n=15	Empirical p value
GTC	0.34	36%	17%	**0.04**
CAT	0.07	5%	17%	**0.02**
GAT	0.23	23%	27%	0.62
CTT	0.13	14%	17%	0.65
GTT	0.23	21%	23%	0.80
6-SNP haplotypes				
	Frequency	Pain n=144	No pain n=15	Empirical p value
GCTATC	0.24	26%	10%	0.06
GCTGTC	0.10	8%	0%	0.22
CTCGAT	0.06	5%	17%	**0.04**
GTCGAT	0.01	1%	0%	0.42
GCTGAT	0.22	21%	23%	0.54
CTCGTT	0.02	2%	0%	0.64
CCTGTT	0.12	11%	13%	0.77
GCTGTT	0.22	22%	27%	0.61

The 3-SNP haplotypes comprise rs10483639, rs3783641 and rs8007267, including the 'pain protective' 3-SNP haplotype as described by Lotsch et al (2007) in bold.

The 6-SNP haplotypes comprise rs10483639, rs752688, rs4411471, rs8007201 rs3783641, rs8007267. The 6-SNP haplotype highlighted in bold contains the 3-SNP 'pain protective' haplotype.

Linkage disequilibrium (LD) was mapped in all 342 patients recruited for this study (with and without HIV-SN) and a representative sample of Caucasians (Figure 7.2). Consistent with higher genomic variability within African populations, the LD between the 6 SNPs was lower in our cohort than in Caucasians.

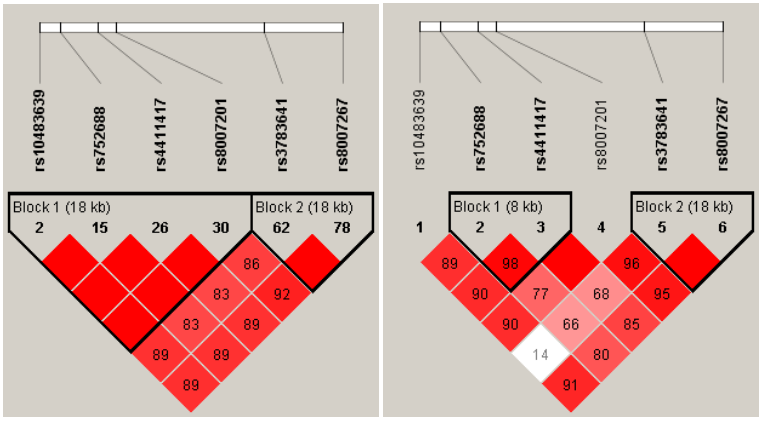


Figure 7.2 LD within GCH1 in Caucasians and our Southern African cohort
Caucasian (left), Southern African (right)

The two populations shared a haplotype block made up of two of the three SNPs belonging to the three-SNP haplotype. The three-SNP haplotype SNPs are rs10483639 (far left) and rs3783641 and rs8007267 (far right in LD).

The South African cohort contained both those with and without HIV-SN (n=342). The comparative Caucasian diagram was generated using the CEU population from HapMap (Genome Browser release #27) and the disease status of participants is therefore unknown. The diagrams were built in Haploview (Version 4.2, 15 September 2009) and use the confidence interval method.

7.4 Discussion

The protein encoded by *GCH1* has been implicated in neuropathic pain states in animals, and gene polymorphisms associate with reduced neuropathic pain in humans (Tegeder et al. 2006; Tegeder et al. 2008; Campbell et al. 2009). I undertook the first study to investigate associations between known genetic variants in *GCH1* with pain in Africans with HIV-SN. We found no independent association between individual *GCH1* SNPs and pain. However a previously identified 'pain protective' 3-SNP haplotype, and a 6-SNP haplotype including this motif were associated with reduced risk of pain on univariate analysis. An additional novel 3-SNP haplotype was associated with increased risk of pain. However, these associations were not apparent after adjustment for other risk factors for pain (age, sex and CD4 T-cell count).

Several factors may explain the absence of an independent association. Firstly, associations between genes and pain sensitivity may be specific to the cause of the pain (Edwards 2006; Kim et al. 2009). Indeed there are differences in the features and pathophysiology of radicular pain secondary to disc herniation, as addressed in the initial study of Tegeder et al (Tegeder et al. 2006), and the small fibre distal sensory polyneuropathy of HIV-SN. Polymorphisms of *GCH1* that associated with radicular pain were not associated with the intensity of dental (Kim et al. 2007), visceral (Lazarev et al. 2008) or chronic widespread pain (Holliday et al. 2009) in Caucasians.

Secondly, the African genome is more variable than the Caucasian (Tishkoff et al. 2002). The higher genomic variability within African populations was evident here as weaker LD and smaller LD blocks in our cohort than that seen in Caucasians (Figure 7.2). Thus the weak associations seen in our cohort may reflect differences in the relationship between marker and causative SNPs in *GCH1* between Caucasian and African populations. Indeed, the weak associations we detected between the 3 and 6-SNP haplotypes indicate that further analysis of the role of polymorphisms in *GCH1* in painful HIV-SN are warranted, but using population-appropriate tag-SNPs.

There is no universally accepted “gold standard” diagnostic test for HIV-SN. In this thesis I chose to use a simple but validated clinical screen. The data assessed with this tool were necessarily limited, but we chose this method of patient assessment because the ACTG BPNS was specifically designed for use by non-medical staff, it has been widely used to study HIV-SN, and has been shown to be valid for use in epidemiological studies, such as those performed here (Cherry et al. 2005). It also complies with the latest guidelines of assessment of neuropathic pain (Haanpaa et al. 2011). I relied on patient self report of their ethnicity, which may have introduced bias. Our cohort, recruited in South Africa, comprised 93% individuals from South Africa and 7% from Southern African countries who also fall under the umbrella of the Niger-Kordofanian ethno-linguistic grouping (Tishkoff et al. 2009) and within South Africa the ethnic groupings show very little variation (Lane et al. 2002). The Niger-Kordofanian grouping, showing similarity at 1327 polymorphic sites, and consistent with self-described ethnicity, served to control population stratification in our cohort; a criticism of previous *GCH1* studies (Kim et al. 2007; Kim et al. 2009; Tishkoff et al. 2009).

In conclusion, I found no independent association between *GCH1*, including a previously identified ‘pain protective’ haplotype, and the presence or intensity of pain in HIV-SN in Southern Africans. However, association between *GCH1* and pain associated with HIV-SN cannot be ruled out and more detailed study of *GCH1* in this painful condition is required.

CHAPTER 8

CONCLUSION

HIV-SN is one of the most common neurological complications of HIV, as its prevalence has increased since the introduction of ART (Sacktor 2002). The negative economic and social implications of this situation are particularly important in South Africa, which has the highest burden of HIV (UNAIDS 2011) in the world. Exposure to stavudine continues in resource-limited countries (WHO 2010a) despite published associations with neuropathy and lipodystrophy. However neuropathy is not restricted to patients receiving stavudine, as identical symptoms are seen in some untreated patients and some treated patients who have never been exposed to d4T (Cherry et al. 2009; Cherry et al. 2010; Ellis et al. 2010) . Assessment of cohorts exposed to ART, including stavudine, to determine risk factors and pathogenesis of the disease will assist diagnosis and treatment. My study is the first to assess both clinical and genetic risk factors for HIV-SN in a Southern African population.

I recruited 395 stavudine-treated patients in a South African Virology clinic. I found that 57% of individuals who had been on ART for at least six months had symptomatic HIV-SN, confirming the clinical importance of this condition. My data confirmed studies of Asian and Caucasian populations showing increasing age and height associated with risk of HIV-SN. However I found no associations between SN and disease-related factors such as current or nadir CD4 T-cell counts. In addition, I identified that those who were *both* older *and* taller were at greatest risk of HIV-SN. My data highlight the extent of the problem of HIV-SN in stavudine-exposed individuals and the need for an alternative regimen wherever possible, particularly for older and taller patients (Chapter 3).

To date the pathogenesis of HIV-SN has mainly been examined in animal and *in vitro* models, which fail to replicate the complexity of HIV disease. Genetic association studies can implicate proteins involved in the development of HIV-SN in humans. I completed the first genetic association study to address HIV-SN in an African population as summarised in Table 8.1. My genetic data support the role of inflammation in HIV-SN through associations with TNF block haplotypes (Chapter 4), *IL4* SNPs and *IL10* haplotypes (Chapter 5). My study builds on work of my

colleagues addressing TNF block haplotypes (Chew et al. 2010) and cytokine genotypes associated with HIV-SN (Cherry et al. 2008).

Table 8.1 Genetic associations with HIV-SN

Gene	SNPs associated with SN	Implications
*BAT1**	rs3130059, rs2523504	These genes form part of the TNF block and whilst not necessarily causative in the pathogenesis of HIV-SN themselves, the strong LD with other immune-related genes suggest an inflammatory etiology to HIV-SN
*ATP6V1G2**	rs2071594	
*NFKBIL1**	rs2071592, rs2071591	
*LTA**	rs1041981, rs909253	
*IL4**	rs2243250	Major allele associated with high levels of an anti-inflammatory cytokine IL-4 which may associate with SN in early stage HIV disease
IL10†	rs1800872, rs1800896, rs1800890	Haplotype associated with high IL-10 also associated with SN protection. Low IL10 haplotype associated with increased pain supporting anti-inflammatory role of IL-10

**Minor alleles of the SNPs provided relative protection against HIV-SN*

† The IL10 CGA haplotype associated with protection against HIV-SN and the AAT haplotype showed a trend for increased risk of pain.

An important finding is that SNPs in *UCP2* and *UCP3* genes previously associated with diabetic neuropathy did not associate with neuropathy in HIV patients (Chapter 6). Perhaps uncoupling proteins are involved in pathology particular to diabetic (and not HIV) neuropathy. Alternatively, LD may differ between the Caucasian and African populations. Comparing different models of neuropathic pain may shed light on the pathogenesis of the conditions.

I also addressed the frequency and extent of pain experienced by Southern African individuals with HIV-SN and factors associated with pain. My clinical study (Chapter

3) identified that three-quarters of individuals with HIV-SN experienced pain and three-quarters of these described their pain as moderate to severe. These data are concerning considering the lack of proven treatments for pain associated with HIV-SN (Phillips et al. 2010). My final genetic association study was with *GCH1,* a gene previously associated with intensity of neuropathic pain in other settings (Chapter 7). I found that a 'pain protective' 3-SNP haplotype, and a 6-SNP haplotype including this motif, were associated with reduced risk of pain on univariate analysis. An additional novel 3-SNP haplotype was associated with increased risk of pain, but the associations were not independent of age, sex and CD4 T-cell count. Whilst there have been several negative *GCH1* studies (Kim et al. 2007; Lazarev et al. 2008; Holliday et al. 2009), they have all addressed non-neuropathic models of pain. It is also possible that this apparent discrepancy stems from different LD between Caucasians and Africans.

Future recommendations

Despite cessation of stavudine-based ART in resource-rich countries a high prevalence of HIV-SN has continued even in patients never exposed to the drug and the risk factors for this are poorly understood (Smyth et al. 2007; Ellis et al. 2010). South Africa has now introduced tenofovir-based HIV treatment. Assessment of incidence and, in due course, prevalence of HIV-SN in Southern Africans on this new regimen is important and may predict future neurological consequences of ART. An extension of my study could describe the demographic, clinical and disease-related factors associated with incident HIV-SN in patients on tenofovir-based regimens. The large numbers of patients initiating HIV treatment in South Africa would make a large study like this feasible. Using a prospective design could identify risk factors rather than just *associations,* possible with the cross-sectional design used here.

My genetic studies incorporated Southern Africans who fell under the Niger-Kordofanian language grouping (Tishkoff et al. 2009). Previous genetic studies have used African Americans for their 'African' cohorts. African American ancestry is also primarily Niger-Kordofanian, but includes significant admixture from European and

other African populations (Tishkoff et al. 2009). Admixture leads to population stratification (Kim et al. 2007; Campbell et al. 2009), which can create false associations, so studies in African Africans are essential. Future African HIV-SN studies could include the three other language groupings within Africa that denote shared ancestry: Nilo-Saharan, Afroasiatic and Khoesan (Tishkoff et al. 2009).

Published genetic studies in HIV-SN have used genes and SNPs identified from the HIV and neurology literature, but African populations are poorly represented. The incorporation of African cohorts is important to ensure results are generalisable. In addition, the lower LD in African populations may narrow down regions of interest and so may assist in the identification of causative SNPs in multiple ethnicities. The next step will be to select tagSNPs from a larger pool of SNPs that have been genotyped in Africans which will allow maximal genomic coverage and reduced genotyping redundancy. This will ensure genotype data representative of the underlying genomic structure in Africans. Currently there are no genome-wide South African SNP datasets available that can be used for the purpose of picking tagSNPs and therefore the best proxy (note, not an exact proxy) is to use another sub-Saharan African population for picking informative markers – most often the Yoruba who have been extensively genotyped in both the HapMap and 1000-Genomes projects. Sub-Saharan African populations have shown *similar* genomic structure that can be informative in picking SNPs based on linkage disequilibrium. Picking SNPs in this manner will enable a more extensive study of genes associated with the pathogenesis of HIV-SN and related pain in populations most affected by HIV. Such a study might include genes related to the production of cytokines which demonstrated an association (*IL4* and *IL10*) and genes which were not well covered (*CCL2, CCR2, CCL5, UCP2, UCP3, GCH1*). An additional region of interest would be the L1c mitochondrial DNA subhaplogroup recently associated with HIV-SN in African Americans from the ACTG 384 study group (Canter et al. 2010).

I employed a targeted approach, which involved assessing candidate genes using SNPs identified in other population groups with HIV-SN or related neuropathic pains.

While this approch is a useful starting point for assessing previously associated SNPs with HIV-SN in a new population, the usefulness of this approach in the identification of novel loci is limited. Identification of novel disease-associated loci is best achieved through genome-wide association studies (GWAS) or candidate gene studies where the selection of markers is based on the underlying genetic architecture and no *a priori* assumtions are made about causality. However, the large number of SNPs assessed in such studies would require an extremely large sample size; in the region of 1000 to 10 000 individuals, depending on the number of SNPs genotyped, the LD stucture, the penetrance of the risk allele and the frequency of the risk allele. A collaborative approach is probably the only way to achieve these numbers. For such a study to be successful, strict phenotyping criteria for HIV-SN would be critical. In addition, attention would need to be paid to the ethnicity of the individuals, so results are not confounded by population stratification. Alternatively, next generation sequencing could determine the sequence of whole genes or even entire chromosomes to highlight regions of interest but currently the cost is prohibitive. Another technique which would be of great interest in this field would be epigenetic analysis, which assesses heritable changes in gene expression but which do not involve changes in the underlying DNA sequence. This approach could assess the effect of, for example, stavudine exposure on gene expression. This may be a route for future animal studies of HIV-SN.

I identified a high prevalence of neuropathy-related pain of moderate to severe intensity in Southern Africans attending an HIV clinic. Most affected individuals were not receiving analgesics and indeed there is a lack of proven analgesic therapies for HIV-SN pain (Phillips et al. 2010). More research is urgently required to identify potential treatment targets and genetic association studies are one way to achieve this. Greater *GCH1* coverage achieved by genotyping more African-appropriate SNPs would provide a better analysis of association between *GCH1* and HIV-SN pain. Assessment of other genes associated with increased neuropathic pain in humans is also warranted including alleles of:

- *KCNS1* which encodes the potassium channel subunit KCNS1 and affects neuronal excitability [rs734784*2, rs13043825*2] (Costigan et al. 2010)
- *CACNG2* which encodes a voltage-dependent calcium channel, stargazin and is also involved in neuronal excitability [rs4820242*A, rs2284015*C, rs2284017*C] (Nissenbaum et al. 2010)
- *TRPV1* [Met315Ile] referred to as the capsaicin receptor and is involved in nociception (Armero et al. 2012).

Additionally, of interest is an *IL6* haplotype [rs1800897*G, rs1800796*G, rs1800895*G, rs13306435*A] associated with longer duration and presence of sciatic nerve pain associated with intervertebral disc disease in Caucasians (Noponen-Hietala et al. 2005; Karppinen et al. 2008). These *IL6* haplotypes contain the 2 SNPs studied in my cohort (rs1800797 and rs1800795), which I was unable to assess due to low MAFs. This could be overcome in an African population by recruiting a larger sample or by identifying African-specific SNPs in LD with rs1800797 and rs1800795.

A more complete assessment of the genetic associations with pain among individuals with HIV-SN would necessitate the collection of data and DNA from additional subjects, including a larger number of controls (i.e. individuals with symptomatic HIV-SN who are affected only by symptoms other than pain, such as numbness or paraesthesias). This may require collaboration across several clinics to achieve this as we found the majority of individuals with symptomatic HIV-SN were experiencing pain.

Psychosocial factors such as depression and catastrophising have been demonstrated to affect an individual's pain response (Green et al. 2003; Rahim-Williams et al. 2007) but in the context of HIV-SN, psychosocial factors have only been studied in non-African populations (Wright et al. 2008; Lucey et al. 2011; Keltner et al. 2012). As cultural factors also affect the pain response (Green et al. 2003; Rahim-Williams et al. 2007), the results may not be generalised to Africans.

Future HIV-SN pain studies should include measures of depression, anxiety, catastrophising and quality of life.

In conclusion, HIV-SN is a major clinical problem in Southern Africa. There is much work still to be done to identify and manage this important condition. However, my study has made an important contribution.



APPENDIX 1

ETHICS CLEARANCE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Kamerman

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080220**
PROJECT	Predictors of risk for developing antiretro-viral toxic neuropathy in patients of African ancestry infected with the HIV virus
INVESTIGATORS	Dr P Kamerman
DEPARTMENT	School of Physiology
DATE CONSIDERED	08.02.29
DECISION OF THE COMMITTEE* +	Approved subject to: Getting permission from relevant authorities

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

DATE 08.03.10 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : n/a

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

APPENDIX 2

TABLE OF ALLELE FREQUENCIES OF ASSOCIATED SNPs IN THIS COHORT AND 2 OTHER SUB-SAHARAN AFRICAN POPULATIONS

Gene	SNP	Alleles		Sub-Saharan African				Southern African			
		Reference	Other	LWK		YRI		HIV-SN		HIV+, no SN	
BAT1	rs3130059	C	G	0.58	0.42	0.52	0.48	0.69	0.31	0.59	0.41
	rs2523504	C	T	0.79	0.21	0.82	0.18	0.88	0.12	0.83	0.17
ATP6V1G2	rs2071594	C	G	0.58	0.42	0.52	0.48	0.32	0.68	0.42	0.58
NFKBIL1	rs2071592	A	T	0.57	0.43	0.49	0.51	0.31	0.69	0.42	0.58
	rs2071591	A	G	0.59	0.41	0.51	0.49	0.32	0.68	0.40	0.60
LTA	rs1041981	A	C	0.59	0.41	0.51	0.49	0.67	0.33	0.59	0.41
	rs909253	A	G	0.41	0.59	0.49	0.51	0.34	0.66	0.42	0.58
IL4	rs2243250	T	C	0.80	0.20	0.81	0.19	0.77	0.23	0.67	0.33
IL10	rs1800872	G	T	0.60	0.40	0.52	0.48	0.60	0.40	0.66	0.34
	rs1800896	T	C	0.64	0.36	0.71	0.29	0.71	0.29	0.67	0.33
	rs1800890	A	T	0.76	0.24	0.81	0.19	0.22	0.78	0.26	0.74

LWK = Luhya in Webuye from Kenya; YRI = Yoruba from Nigeria

APPENDIX 3

Cherry, C.L., Affandi, J.S., Brew, B.J., Creighton, J., Hooker D.J., Imran, D., Kamarulzaman, A., Kamerman, P., McArthur, J.C., Price, P., Smyth, K., Tan, IL., Vaneer, S., Wadley, A., Wesselingh, S., Yunihastuti, E. (2010) "Hepatitis C seropositivity is not a risk factor for sensory neuropathy among HIV patients." Neurology, 74: 1538-42

Hepatitis C seropositivity is not a risk factor for sensory neuropathy among patients with HIV

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ABSTRACT

Background: Sensory neuropathy (SN) is common in patients with HIV. Hepatitis C (HCV) coinfection is often cited as an HIV-SN risk factor, but data to support this are lacking. This collaboration aimed to examine the association between HCV serostatus and SN risk among ambulatory HIV-positive patients.

Methods: Patients with HIV were assessed in cross-sectional studies in Baltimore, Jakarta, Johannesburg, Kuala Lumpur, Melbourne, and Sydney for SN (defined by both supportive symptoms and signs). HCV seropositivity was assessed as an SN risk using a χ^2 test, followed by logistic regression modeling to correct for treatment exposures and demographics.

Results: A total of 837 patients of African, Asian, and Caucasian descent were studied. HCV seroprevalence varied by site (Baltimore n = 104, 61% HCV+; Jakarta 96, 51%; Johannesburg 300, 1%; Kuala Lumpur 97, 10%; Melbourne 206, 16%; Sydney 34, 18%). HCV seropositivity was not associated with increased SN risk at any site, but was associated with reduced SN risk in Melbourne ($p = 0.003$). On multivariate analyses, the independent associations with SN were increasing age, height, and stavudine exposure. HCV seropositivity was not independently associated with an increased SN risk at any site, but associated independently with reduced SN risk in Baltimore ($p = 0.04$) and Melbourne ($p = 0.06$).

Conclusions: Hepatitis C (HCV) seropositivity was not associated with increased sensory neuropathy risk among HIV-positive patients at any site. While we were unable to assess HCV RNA or liver damage, the data suggest that HCV coinfection is not a major contributor to HIV-SN.
Neurology® 2010;74:1538–1542

GLOSSARY

HCV = hepatitis C; **SN** = sensory neuropathy.

Sensory neuropathy is a common complication of HIV infection and some HIV treatments.[1-4] Hepatitis C (HCV) can also be complicated by peripheral neuropathy, with and possibly without cryoglobulinemia.[5-7] Since HCV and HIV infection share risk factors and frequently coexist,[8] concerns of possible synergistic effects of these viruses on the peripheral nervous system have been raised.[3,9] However, this has not been evaluated systematically.[10]

We surveyed outpatients with HIV infection for the presence of neuropathy at 6 centers with varying HIV management practices and rates of HCV coinfection. Here we use data from all sites to address whether HCV seropositivity is associated with an increased risk for symptomatic neuropathy among ambulatory patients with HIV. We have recently developed algorithms for the

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Study funding: The data reported here were collected in studies related to HIV-associated neuropathy that were funded by the Australian Centre for HIV and Hepatitis Research, Australia's National Health and Medical Research Council, the National Institutes of Health (NS 26643), and the South African National Research Foundation.

Disclosure: Author disclosures are provided at the end of the article.

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evaluation of neuropathy risk.[11,12] Here we systematically evaluate whether HCV seropositivity should be considered in this context.

METHODS Cross-sectional data were analyzed from 7 studies of HIV-associated neuropathy performed at 6 sites in 5 countries. All studies were approved by the local Human Research and Ethics Committee and all participants gave written, informed consent to participate. All studies recruited ambulatory adults (aged at least 17 years) with HIV infection attending hospital clinics for HIV care.

Study sites involved in this work were as follows.

1. Moore Clinic, Johns Hopkins Hospital, Baltimore, MD. Patients were recruited in 2002–2003 onto an NIH-funded project examining the pathogenesis and risks for HIV-associated neuropathies.[4]
2. Pokdisus AIDS Clinic, Cipto Mangunkusumo Hospital/Faculty of Medicine University Indonesia, Jakarta, Indonesia. Patients were recruited in a neuropathy screening program in 2006.[11]
3. Virology Clinic, Charlotte Maxeke Johannesburg Academic Hospital, Johannesburg, South Africa. Patients were recruited in a neuropathy screening program in 2008–2009.[13]
4. Infectious Diseases Clinic, University of Malaya Medical Centre, Kuala Lumpur, Malaysia. Patients were recruited in a neuropathy screening program in 2005–2006.[11]
5. Infectious Diseases Clinic, The Alfred Hospital, Melbourne, Australia. Patients were recruited in 2001 and 2006 in 2 separate neuropathy screening programs.[14] Only data collected in 2006 are included for patients assessed in both studies (n = 34).
6. Immunology B (HIV) Clinic, St Vincent's Hospital, Sydney, Australia. Patients were recruited in a neuropathy screening program in 2009.

All patients were assessed for neuropathy using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen.[15] Neuropathy was defined as present if the individual had one or more of the lower limb neuropathic symptoms elicited using this tool (pain, aching or burning, pins and needles, or numbness) and one or more signs (absent ankle reflexes or reduced vibration sense at the great toe—vibration of a 128-Hz tuning fork felt for 10 seconds or less). Patient height was measured and data on other possible risk factors for neuropathy were collected from the medical file. HCV antibody status was assessed using commercially available assays performed in the hospital laboratory at each study site, as follows.

1. Baltimore: Abbott AxSYM HCV version 3.0 microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, IL).
2. Jakarta: ORTHO HCV version 3.0 ELISA (Ortho-Clinical Diagnostics, Raritan, NJ).
3. Johannesburg: Abbott AxSYM HCV version 3.0 microparticle enzyme immunoassay (Abbott Laboratories).
4. Kuala Lumpur: Abbott ARCHITECT chemiluminescent microparticle enzyme immunoassay (Abbott Laboratories).
5. Melbourne: Abbott AxSYM HCV version 3.0 microparticle enzyme immunoassay until 2004. From 2004, Abbott ARCHITECT chemiluminescent microparticle enzyme immunoassay (Abbott Laboratories).
6. Sydney: Abbott ARCHITECT chemiluminescent microparticle enzyme immunoassay (Abbott Laboratories).

Statistical analyses were performed using Stata 10.1 (StataCorp). Rates of HCV seropositivity were compared between patients with and without neuropathy using χ^2 tests. Univariate analyses used χ^2 tests (dichotomous variables), Wilcoxon rank sum test (non-normally distributed continuous variables), or unpaired t tests (normally distributed continuous variables) to determine other factors associated with neuropathy. Multivariate analyses of neuropathy risk factors within each cohort and overall were then undertaken using logistic regression modeling. This included HCV antibody status, factors previously associated with neuropathy risk (increasing age and stavudine exposure), and any factor with $p < 0.1$ on univariate analysis. Variables other than HCV antibody status were then removed in a reverse selection procedure to obtain the models shown.

RESULTS A total of 837 patients were surveyed at 6 sites between 2001 and 2009. The cohorts had different demographic profiles, rates of HCV seropositivity, and exposure to stavudine, reflecting patient populations at each site (table 1). On univariate analysis, HCV seropositivity was not associated with an

Table 1 Description of the cohorts surveyed[a]

	Baltimore	Jakarta	Johannesburg	Kuala Lumpur	Melbourne	Sydney
No.	104	96	300	97	206	34
Sensory neuropathy rate, %	62	34	62	19	39	62
Age, y	46 (27-66)	29 (17-56)	39 (19-69)	41 (23-75)	43 (19-75)	53 (35-77)
Height	176 (150-192)	168 (155-189)	159 (132-187)	167 (151-184)	175 (147-196)	175 (164-192)
Female gender, %	25	14	79	16	8	0
Hepatitis C antibody positive, %	61	51	1	10	16	18
Lowest CD4 T-cell count recorded, cells/μL	63 (0-958)	42 (1-633)[b]	97 (2-403)[b]	51 (0-530)	117 (0-796)	180 (14-680)
Stavudine ever, %	66	100	96	47	67	25
Ethnicity, %	88 African American	66 Malay; 30 Chinese	100 African (34 Zulu, 15 Sotho, 12 Xhosa)	70 Chinese; 15 Malay; 10 Indian	83 Caucasian	94 Caucasian

[a] Continuous variables are shown as median (range).
[b] Patients are not generally commenced on antiretroviral therapy at these sites until CD4 T-cell count declines below 200 cells/μL. The patients with higher counts who have received stavudine represent some who commenced treatment in the private sector before their first assessment at the hospital-based clinic and some who had an AIDS-defining illness at a higher CD4 T-cell count and were therefore commenced on treatment.

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Table 2 Proportion of patients who were hepatitis C antibody positive at each site by sensory neuropathy status

	Sensory neuropathy free (%)	Sensory neuropathy (%)	p (χ^2)
Baltimore (n = 104)	27/40 (68)	36/64 (56)	0.25
Jakarta (n = 96)	30/63 (48)	19/33 (58)	0.35
Johannesburg (n = 300)[a]	0/114 (0)	3/186 (2)	0.18
Kuala Lumpur (n = 97)	9/79 (11)	1/18 (6)	0.46
Melbourne (n = 206)	27/126 (21)	5/80 (6)	0.003[b]
Sydney (n = 34)	3/13 (23)	3/21 (13)	0.5

[a] Six patients in Johannesburg had indeterminate hepatitis C antibody tests. Five were sensory neuropathy free and 1 had a clinical diagnosis of sensory neuropathy. No association between hepatitis C and sensory neuropathy is seen if these are considered positive (5/114 vs 4/186, $p = 0.29$).
[b] Hepatitis C seropositivity was associated with a reduced neuropathy risk.

increased risk of neuropathy among patients at any site (table 2). In Melbourne, HCV seropositivity was associated with a reduced risk of neuropathy.

Logistic regression modeling was then used to correct for other risk factors that may have masked an association between HCV serostatus and neuropathy. HCV seropositivity was not independently associated with increased neuropathy risk in any cohort surveyed, nor in the combined data set. After correcting for patient age and stavudine use, an association between HCV antibody status and a reduced risk of neuropathy emerged in the Baltimore cohort, but became less apparent in Melbourne patients (table 3). Increasing age, a history of stavudine use, and increasing height were independently associated with neuropathy risk overall.

Table 3 Results of logistic regression modeling looking for independent associations between hepatitis C antibody status and neuropathy after correcting for factors independently associated with sensory neuropathy status in that cohort

Cohort	Odds ratio	95% Confidence interval	p Value	Covariates corrected for
Baltimore	0.36	0.13–0.94	0.04	Age, y (OR 1.1, $p = 0.02$) D4T use (OR 3.3, $p = 0.01$)
Jakarta	2.05	0.71–5.94	0.2	Age, y (OR 1.1, $p = 0.02$) Height (cm) (OR 1.1, $p = 0.02$) (all patients had used d4T)
Johannesburg	Not done[a]			
Kuala Lumpur	0.56	0.06–5.01	0.6	Age, y (OR 1.1, $p = 0.03$) Height (cm) (OR 1.1, $p = 0.1$)
Melbourne	0.30	0.08–1.04	0.06	Age, y (OR 1.1, $p < 0.001$) Height (cm) (OR 1.1, $p = 0.02$) D4T ever (OR 9.1, $p < 0.001$)
Sydney	Not done[b]			
Overall	0.75	0.51–1.11	0.2	Age, y (OR 1.1, $p < 0.001$) Height (cm) (OR 1.01, $p = 0.08$) D4T use (OR 5.1, $p < 0.001$)

[a] Analysis not performed because only 3 patients were hepatitis C antibody positive. All had used d4T and all had neuropathy.
[b] Multivariate analysis not performed due to the small number of participants at this site.

DISCUSSION We found no association between HCV seropositivity and increased neuropathy risk in any cohort of patients with HIV studied. These data represent more than 800 patients of African, Asian, and Caucasian descent in cohorts with varying HCV infection rates and antiretroviral treatment exposures. Differences in HCV seropositivity probably reflect the predominant mode of HIV infection, but definitive data are not available from the clinic populations sampled. The lack of an association with increased neuropathy risk in any cohort suggests that HCV coinfection is not a major risk factor for neuropathy among ambulatory patients with HIV.

The apparent association between HCV seropositivity and a reduced risk of neuropathy among patients with HIV in Melbourne raises the question of whether coinfected patients (often IV drug users) may have lower rates of antiretroviral drug use and hence exposure to potentially neurotoxic agents such as stavudine. However, 60% of HCV seropositive patients and 68% of HCV antibody negative patients in Melbourne had used stavudine ($p = 0.3$, χ^2 test). HCV coinfected patients were younger than HIV monoinfected patients in the Melbourne cohort (mean age 40 vs 45 years, $p = 0.008$, unpaired t test). However, multivariate analysis correcting for age and stavudine use (table 3) revealed a continuing trend associating HCV coinfection with a reduced risk of neuropathy. A similar association emerged in the Baltimore cohort.

A protective effect from HCV coinfection against neuropathy in patients with HIV is difficult to explain biologically. HCV can infect peripheral nerves[16,17] and the risk of neuropathy among monoinfected patients is well documented.[5-7] The association observed between HCV and a reduced rate of neuropathy likely reflects factors not measured in this study that may influence neuropathy risk. For example, patients surveyed in Jakarta and Johannesburg had similarly high rates of stavudine exposure. However, neuropathy was more common in Johannesburg, where few patients are HCV coinfected. The difference in neuropathy prevalence is only partly explained by the younger age of the Jakarta cohort, so other factors such as genetics[18-20] or other coinfections[21] may also play a role.

The limitations of this work include our inability to examine HCV RNA levels or evidence of liver damage as these are not routinely monitored at all sites. We therefore cannot exclude an association between neuropathy and HCV viremia or chronic liver disease. Similarly, we cannot exclude the possibility that some patients with HCV viremia may have tested antibody negative, as previously described in a small proportion of patients with HIV,[22] nor that

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some antibody-positive patients may have been aviremic. However, a recent study comparing neurocognitive function in patients with HIV with detectable vs undetectable HCV viral loads also showed no difference in neuropathy rates between these groups.[23]

PCR-based screening for HCV was not available in Johannesburg, but the observed low rate of HCV infection is consistent with other studies conducted in Southern Africa,[24-29] including a study of HIV-infected individuals[28] and a survey of HIV-infected patients presenting with liver disease.[29] Our data were obtained using the AxSYM 3.0 (Abbott) assay that detects antibodies to proteins that are conserved across HCV genotypes.[30] The manufacturers claim this includes genotype 5 (the most common type of HCV seen in South Africa[31,32]) but no genotype 5 sensitivity data are available for evaluation. The low prevalence of HCV in this cohort is attributed to HIV transmission via sexual contact (an uncommon route of HCV transmission) rather than through injecting drug use. It may also be significant that patients in this cohort were screened for HCV at the time of neuropathy assessments rather than at HIV diagnosis, so their HIV disease may have been more advanced. The 6 indeterminate results (table 2) may reflect patients with HCV infection but poor antibody responses due to immune suppression.[33] However, as 5 of these 6 patients were neuropathy free, their indeterminate status would not alter our finding that HCV seropositivity is not associated with an increased neuropathy risk in patients with HIV.

A more extensive examination of risk factors for neuropathy in HIV (including more detailed demographics, treatment exposures, clinical factors, viral studies, and host genetics) will be required to exclude a minor contribution from HCV status to the individual patient's risk of symptomatic neuropathy. We used a simple clinical definition of neuropathy. Although we have validated this definition against epidermal nerve fiber density and quantitative sensory threshold testing,[15] we cannot exclude associations between HCV coinfection and subclinical neuropathology or impaired sensory function in patients with HIV.

Despite these limitations, this work suggests that HCV coinfection does not increase neuropathy risks among ambulatory HIV-positive patients. This finding is strengthened by the inclusion of cohorts from settings with a range of HIV treatment practices, HCV seroprevalence, and ethnicity. While an individual coinfected patient may be at risk for peripheral nerve complications from HCV, these data suggest that HCV coinfection has minimal impact on the much greater neuropathy risk imposed by HIV and some HIV treatments.

AUTHOR CONTRIBUTIONS

Statistical analysis was conducted by Dr. C.L. Cherry.

ACKNOWLEDGMENT

The authors thank all the patients who volunteered to take part in this work and Yong Yean Kong for facilitating work in Kuala Lumpur.

DISCLOSURE

Dr. Cherry has served on scientific advisory boards for Roche and Gilead Sciences, Inc.; serves as Basic Science Editor for the *ASHM Journal Club* and on the editorial boards of *The Open AIDS Journal* and the *Journal of the International Association of Physicians in AIDS Care*; serves as a consultant to CNSBio; and has received research support from Roche and CNSBio. Dr. Affandi reports no disclosures. Dr. Brew serves on scientific advisory boards for Biogen Idec, GlaxoSmithKline, and Merck Serono; receives royalties from the publication of *HIV Neurology* (Oxford University Press, 2001) and *Palliative Neurology* (Cambridge University Press, 2005); has received speaker honoraria from GlaxoSmithKline, Boehringer Ingelheim, Tibotec Therapeutics, and Abbott; and receives research support from NHMRC (Australia). Dr. Creighton receives research support from the NIH (NIMH N01MH22005 [Research Coordinator] and NIMH 907647 [Research Coordinator]). Dr. Djauzi, Dr. Hooker, and Dr. Imran report no disclosures. Dr. Kamarulzaman serves on a scientific advisory board for Johnson & Johnson Asia Pacific; serves on the editorial board of *International Journal of Drug Policy*; and receives publishing royalties for contributions to *UpToDate*. Dr. Kamerman has received travel expenses for lectures and educational activities not funded by commercial entities; has received speaker honoraria from Merck & Co., Inc. and Novartis; has served as a consultant for Aspen Pharmacare Holdings Limited and Partners in Research; and receives research support from Adcock Ingram Pharmaceuticals, Aspen Pharmacare Holdings Limited (donations of antiretroviral drugs for investigator-initiated predinical research), the National Research Foundation (South Africa), and the Iris Ellen Hodges Trust of the University of the Witwatersrand. Dr. McArthur receives royalties from the publication of *Current Therapy in Neurologic Disease, 7th Edition* (Mosby, 2006); and receives research support from Biogen Idec, the NIH (R01 MH075673 [PI], RO1 NS44807 [PI], RO1 NS49465 [PI], and R01 MH067831 [Co-I]), the National Multiple Sclerosis Society, and from the Foundation for Peripheral Neuropathy. Dr. Moore serves on the editorial advisory boards of *HIV/AIDS–Research* and *Palliative Care*; has received speaker honoraria from Bristol-Myers Squibb and Merck Serono; serves as a consultant to Bristol-Myers Squibb; and receives research support from Pfizer Inc., the NIH (R01DA011602 [PI], U01AI069918 [PI], R01AA16893 [PI], and K24DA00432 [PI]), and the Agency for Healthcare Research and Quality (200600025 [PI]). Dr. Price has received research support from Australia's National Health and Medical Research Council. Dr. Smyth reports no disclosures. Dr. Tan has received funding for travel from Merck Serono and Biogen Idec. Dr. Vanar, Dr. Wadley, Dr. Wesselingh, and Dr. Yunihastuti report no disclosures.

Received October 20, 2009. Accepted in final form February 11, 2010.

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APPENDIX 4

AIDS CLINICAL TRIALS GROUP
BRIEF PERIPHERAL NEUROPATHY SCREEN

NEUROLOGICAL SCREENING TOOL

INSTRUCTIONS FOR RECORDING SUBJECTIVE ELICITED SYMPTOMS

Ask the subject to rate the severity of each symptom listed in question 1 on a scale of 01 (mild) to 10 (most severe) for right and left feet and legs Enter the score for each symptom in the column marked Presence/Severity. If a symptom has been present in the past, but not since the last visit, enter '00-Currently Absent' If the symptom has never been present, enter '11-Always Been Normal'

Always been normal	Currently absent	Mild									Severe
11	00	01	02	03	04	05	06	07	08	09	10

1. SYMPTOMS R L

   a. Pain, aching, or burning in feet, legs* ……. ☐ ☐

   b. "Pins and needles" in feet, legs ……. ☐ ☐

   c. Numbness (lack of feeling) in feet, legs ……. ☐ ☐

   * Option "a" refers to pain of any type, which is not described as "pins and needles" or "numb". If the patient chooses option "a" to describe their pain, please record what term(s) the patient used to describe the pain in the space below.

   _____

   _____

2. Location of symptoms

   Use Score of:

   0 = None
   1 = feet (or hands) only
   2 = extends to ankles (or wrists)
   3 = extends above ankle (wrist) but not to knee (elbow)
   4 = extends to knees (or elbows)
   5 = extends above knees (or elbows)

   R L

   ☐ ☐

a. Pain, aching, or burning in feet, legs …… | | |

b. "Pins and needles" in feet, legs …… | | |

c. Numbness (lack of feeling) in feet, legs …… | | |

INSTRUCTIONS FOR EVALUATING PERCEPTION OF VIBRATION

Strike the end of a 128 Hz tuning fork hard enough that the sides touch. Place the vibrating tuning fork on a bony prominence on the subject's wrist to be sure that they can recognise the vibration or "buzzing" quality of the tuning fork. Again, strike the ends of the tuning fork hard enough so that the sides touch. Immediately place the vibrating tuning fork gently but firmly on the top of the distal interphalangeal (DIP) joint of one great toe and begin counting the seconds. Instruct the subject to tell you when the "buzzing" stops. Repeat for the other great toe.

3. Vibration sense

R	L

a. Great toe DIP joint perception of vibration in seconds

INSTRUCTIONS FOR EVALUATING DEEP TENDON REFLEXES

With the subject seated, the examiner uses one hand to press upward on the ball of the foot, dorsiflexing the subject's ankle to 90 degrees. Using a reflex hammer, the examiner then strikes the Achilles tendon. The tendon reflex is felt by the examiner's hand as a plantar flexion of the foot, appearing after a slight delay from the time the Achilles tendon was struck. Use reinforcement, if necessary, by having subject clench fist.

<u>Ankle reflexes</u>

0 = absent
1 = Hypoactive
2 = Normal deep tendon reflexes
3 = Hyperactive (prominent spread)
4 = Clonus
8 = unable/did not assess

R	L

4. Ankle reflexes ……..

APPENDIX 5

Wadley, A.L., Cherry, C.L., Price, P., Kamerman, P.R. (2011) "HIV neuropathy risk factors and symptom characterization in stavudine-exposed South Africans." Journal of Pain and Symptom Management, 41: 700-706

Original Article

HIV Neuropathy Risk Factors and Symptom Characterization in Stavudine-Exposed South Africans

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Abstract

Context. HIV-associated sensory neuropathy (HIV-SN) is a frequent complication of both HIV and neurotoxic antiretroviral medications such as stavudine.

Objectives. To determine the prevalence, risk factors, and clinical characteristics of symptomatic HIV-SN in a Black South African cohort of patients exposed to stavudine.

Methods. HIV-positive Black South Africans ($n = 395$) who had received stavudine for at least six months were recruited at the Virology Clinic of the Charlotte Maxeke Academic Johannesburg Hospital, South Africa, and screened for neuropathy using the AIDS Clinical Trials Group neuropathy screening tool. HIV-SN was defined as present if the patient had both symptoms and signs of peripheral neuropathy. If present, the distribution and intensity of symptoms were recorded. In addition, anthropomorphic, demographic, and clinical information were recorded and analyzed as risk factors.

Results. The prevalence of symptomatic HIV-SN was 57% (226 of 395). Increasing age and height were independently associated with the development of SN among patients who had used stavudine. Pain was the primary symptom reported by participants with HIV-SN (76%, 172 of 226), followed by numbness (48%, 108 of 226), and pins and needles (46%, 105 of 226). About three-quarters of participants rated their symptoms as being of moderate to severe intensity. Symptoms were always present in the feet and only 23% experienced symptoms proximal to the feet.

Conclusion. HIV-SN was common in this population and frequently associated with moderate to severe pain in the feet. HIV-SN was significantly associated with increasing age and height, factors that could be measured at no added cost prior to stavudine prescription, allowing higher risk patients to be offered priority

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Accepted for publication: July 8, 2010.

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0885-3924/$ - see front matter
doi:10.1016/j.jpainsymman.2010.07.006

access to nonneurotoxic drugs. J Pain Symptom Manage 2011;41:700–706.
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Key Words
Risk factors in epidemiology, HIV, peripheral neuropathy, neuropathic pain

Introduction

HIV-associated sensory neuropathy (HIV-SN) affects quality of life and ability to work,[1] and is a well-documented complication of HIV infection.[2] Since the introduction of antiretroviral therapy (ART), the incidence and prevalence of HIV-SN has increased.[3] This increase has been linked to the use of ART regimens containing particular nucleoside reverse transcriptase inhibitors, notably stavudine.[4–8] Despite the World Health Organization recommending use of stavudine be phased out,[9] stavudine-based treatment programs continue to be introduced and expanded in many countries[10] because of lack of cost-effective alternatives,[11,12] so stavudine-related toxicities are expected to increase.

The aim of the study was to determine the risk factors for HIV-SN in a Black South African cohort with universal exposure to stavudine. We aimed to characterize the intensity and distribution of three common symptoms of peripheral neuropathy—pain, numbness, and pins and needles—to establish the symptom experience of HIV-SN. We also aimed to create a risk profile for individuals likely to develop HIV-SN after starting stavudine-based therapy to guide clinicians' prescription choices. This work is critical. Although access to alternative HIV treatments remains limited in this region, the only way to reduce the impact of stavudine toxicity is to understand which patients are at highest risk and offer these individuals priority access to alternative regimens.

Methods

Participants

HIV-positive adults who had used stavudine for at least six months were screened for neuropathy at the Virology Clinic of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, between July 2008 and April 2009. The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, South Africa (protocol number: M080220), and written, informed consent was obtained from all participants. An interpreter fluent in English and all local African languages facilitated consent and study procedures.

Procedures

Participants were screened using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen.[13] Participants were considered to have symptomatic HIV-SN if they had at least one symptom (pain, aching, burning, numbness, or pins and needles) and at least one clinical sign of neuropathy (reduced vibration sense or absent ankle reflexes) in each leg. Vibration sense was assessed using a 128 Hz tuning fork, which was placed on the interphalangeal joint of each great toe; vibration sense of 10 seconds or less was considered abnormal. If participants acknowledged the presence of symptoms, the anatomical distribution and intensity of the symptoms were recorded. Symptom intensity was rated on an 11-point rating scale anchored at "0" (no symptom experienced) to "10" (worst imaginable). Pain was classified as moderate to severe with a score of four or greater on this scale.

Demographic (age, gender, ethnicity) and clinical information (current and nadir CD4 T-cell counts, duration of HIV infection, AIDS-defining illnesses), antiretroviral treatment history, and other potential causes of neuropathy (diabetes mellitus, alcoholism, vitamin B12 deficiency, exposure to isoniazid and chemotherapy) were obtained through participant self-recall and their medical files. Participants' heights and weights were recorded and a venous blood sample was taken for hepatitis C serology (Abbott AxSYM HCV version 3.0 microparticle enzyme immunoassay, Abbott Laboratories, Abbott Park, IL).

Statistical Analyses

Normally distributed continuous data are presented as mean (SD), and nonparametric data as median (range). Univariate analyses of risk factors associated with neuropathy were undertaken using Chi-squared tests (dichotomous variables), unpaired *t*-tests (parametric continuous variables), and Mann-Whitney tests (nonparametric continuous variables). Multivariate analysis was performed using multiple logistic regression modeling with a reverse selection procedure. Variables were included in the model if they had previously been associated with HIV-SN or if they were associated with SN ($P < 0.1$) in the present study. The variable least strongly associated with SN was then removed in a stepwise fashion until the removal of any more variables substantially impaired the resulting model. Receiver operating characteristic (ROC) analyses were used to determine cutoff values for continuous variables that were associated with neuropathy in this cohort that provided optimum diagnostic efficiency of HIV-SN. That is, cutoffs that provided the best levels of sensitivity and specificity when categorizing individuals into higher and lower HIV-SN risk groups were chosen.

Results

Three hundred and ninety-five patients participated in the study, of whom 226 (57%) had a clinical diagnosis of symptomatic HIV-SN. All participants identified themselves as Black Africans, were 18 years or older, had a confirmed HIV infection, and had been on stavudine-based ART for at least six months.

Demographic and Clinical Data

Demographic and clinical data are shown in Table 1. Non-HIV-related neuropathy risk factors such as diabetes[14] were uncommon. Hepatitis C testing of the first 300 participants showed no association with HIV-SN.[15] Nadir CD4 T-cell counts were not available for 18 participants who had commenced antiretroviral treatment at other clinics. Although 24% ($n = 96$) of participants had been exposed to stavudine at a dose of 40 mg twice daily, no participant was receiving more than 30 mg stavudine orally twice daily at the time of screening. No patients had been prescribed a protease inhibitor or zalcitabine.

Table 1
Participant Demographic, Disease, and Treatment Characteristics

Variable	
Number of participants	395 (100%)
Female gender	295 (75%)
Age (years)	39 ± 8
Height (cm)	159 ± 8
Weight (kg)	69 ± 14
Current CD4 (cells/μL) ($n = 386$)	383 (27–1091)
Nadir CD4 (cells/μL)	94 (1–403)
Months since HIV diagnosis	45 (6–240)
History of an AIDS-defining illness	176 (45%)
Seropositive for hepatitis C	9 (2%)
History of tuberculosis[a]	160 (41%)
Diabetes	12 (3%)
Stavudine use ever/currently using	395 (100%)/115 (29%)
Didanosine use ever/current using	37 (9%)/25 (6%)

Dichotomous variables are presented as number (%), normally distributed continuous variables are presented as mean ± standard deviation, and nonnormally distributed continuous variables are presented as median (range).
[a]All patients with a history of tuberculosis had been treated with isoniazid.

Prevalence and Characteristics of HIV-SN

In addition to the 226 (57%) patients with symptomatic HIV-SN, 25% ($n = 97$) had either symptoms or signs of neuropathy, but not both. Pain was the symptom most often reported by participants diagnosed with symptomatic HIV-SN (76%, 172 of 226) (Table 2). Seventy-four percent (128 of 172) of participants with pain, 76% (82 of 108) of participants with pins and needles, and 77% (81 of 105) of participants with numbness reported experiencing the symptom at moderate to severe intensity. Thirty-nine percent (89 of 226) reported two or more symptoms. All participants with HIV-SN experienced symptoms in their feet. Pain proximal to the feet was reported by less than half of those with pain.

Table 2
Prevalence and Features of HIV-SN Observed in This Study

Feature	Number (%)
HIV-SN (symptoms and signs)	226 (57)
Symptom prevalence in HIV-SN	
Pain, aching, or burning	172 (76)
Pins and needles	105 (46)
Numbness	108 (48)
Sign prevalence in HIV-SN	
Reduced vibration sense	99 (44)
Absent ankle jerks	191 (85)

Risk Factors Associated with HIV-SN

Increasing age ($P < 0.001$) and increasing height ($P = 0.005$) were the only factors significantly associated with HIV-SN on univariate analyses (Table 3). Factors included in the multiple regression model were 1) age, height, didanosine exposure, and a history of an AIDS-defining illness (based on $P < 0.1$ on univariate analysis), and 2) a history of an AIDS-defining illness, nadir CD4, diabetes mellitus, or isoniazid use (based on associations with HIV-SN in other cohorts).[4,14,16] The only factors independently associated with neuropathy risk among stavudine-exposed African HIV patients were age and height (Table 4).

ROC analysis comparing age and neuropathy status in all 395 participants yielded an area under the curve (AUC) of 0.66 (95% confidence interval [CI]: 0.61−0.72). An age cutoff of ≥38 years had a sensitivity of 68% and a specificity of 63% for predicting neuropathy. When comparing height and the presence of neuropathy with ROC analysis, the AUC was 0.59 (95% CI: 0.53−0.65), and a height cutoff of ≥158 cm had a sensitivity of 64% and a specificity of 51% for predicting neuropathy. When age and height were combined, the prevalence of neuropathy was 35% in younger, shorter participants and 76% in older, taller participants (Table 5).

Discussion

We identified risk factors, created a risk profile, and described the symptom experience of symptomatic HIV-SN in a stavudine-exposed cohort of South African patients. Patients most likely to develop a symptomatic HIV-SN after stavudine exposure may now be identified and the expected severity of their symptoms better understood. The prevalence of symptomatic HIV-SN was 57%, showing this is a common problem in African patients exposed to stavudine-based ART. Importantly, three-quarters of individuals with HIV-SN reported moderate to severe pain demonstrating substantial suffering. Age and height were the only independent risk factors associated with HIV-SN in our cohort. Although these factors do not explain all the variation in SN status following stavudine exposure (model $r^2 = 0.07$), simple stratification of patients by age (≥38 years) and height (≥158 cm) can predict those at highest risk for HIV-SN (Table 5). HIV-SN is, therefore, a clinically important condition and efforts to reduce the incidence of new cases of HIV-SN are critical, particularly in clinics where stavudine use continues.

Routinely recording age and height are quick and easy tasks that can be part of a normal patient assessment, and our finding that risk of symptomatic HIV-SN increases with increasing age and height, across all ages and heights, has important clinical implications. In any setting where the use of stavudine is an economic necessity, we suggest that priority access to less toxic antiretroviral agents should be given to older and taller (and particularly both older and taller) patients. In this way, rates of SN are likely to be reduced. Improved patient and doctor awareness of HIV-SN and

Table 3
Univariate Associations Between HIV-SN Status and Demographic/Clinical Variables

Variable	SN Free ($n = 169$)	SN ($n = 226$)	P-value
Female gender	128 (76%)	167 (74%)	0.7
Age (years)	36.4 ± 7.6	40.8 ± 8.0	<0.0001
Height (cm)	157.6 ± 8	160.1 ± 8	0.003
Weight (kg)	68.2 ± 13	69.6 ± 14	0.3
Months since HIV diagnosis	45 (6−204)	46 (7−240)	0.4
Current CD4 T-cell count	386 (45−1079)	380 (27−1091)	1.0
Nadir CD4 T-cell count	96 (2−403)	91 (1−253)	0.7
AIDS-defining illness	68 (40%)	108 (48%)	0.1
Tuberculosis (isoniazid use)	65 (38%)	95 (42%)	0.5
Hepatitis C seropositivity	4 (2%)	5 (2%)	0.9
Didanosine ever	11 (7%)	26 (12%)	0.09
Diabetes mellitus	4 (2%)	8 (4%)	0.5

Dichotomous variables are shown as number (%) and compared with SN status using a Chi-squared test. Normally distributed variables are shown as mean ± standard deviation and compared with SN status using an unpaired t-test. Nonnormally distributed continuous variables are shown as median (range) and compared with SN status using a Mann-Whitney U test.

Table 4
Multiple Logistic Regression Model of Factors Associated with Prevalent HIV-SN in This Cohort

Variable	Odds Ratio	95% CI	*P*
Age (years)	1.08	1.05−1.11	<0.001
Height (cm)	1.04	1.01−1.07	0.005

Neuropathy risk increased with both increasing age and increasing height. Factors included in the analysis were those associated with HIV-SN on univariate analysis (age, height, having had an AIDS-defining illness, and exposure to didanosine) and factors that have previously been found to be risk factors for HIV-SN in other cohorts (nadir CD4 T-cell count, isoniazid exposure, and diabetes mellitus). The final model was obtained using a reverse selection procedure, and has model $r^2 = 0.07$ and model $P < 0.0001$.

increased frequency of follow-up of patients at risk of HIV-SN also may facilitate prompt regimen revision if neuropathy symptoms develop. Early switching off stavudine when neuropathy develops may improve the likelihood of symptom resolution.[17,18] However, although the cutoff values for age and height identified using ROC analysis provide the optimum predictive efficiency for HIV-SN in the cohort studied, precise cutoffs will vary by population[4] and they should not be adopted more broadly. Rather, they are provided to illustrate the important point that older, taller individuals are at increased risk of developing HIV-SN.

It is worth noting that 57% of patients presented with symptomatic HIV-SN despite most patients only ever having been exposed to twice daily 30 mg doses of stavudine. Treatment guidelines worldwide have changed to reflect the reduced toxicity of 30 mg rather than 40 mg stavudine doses.[19] However, our data show an unacceptably high prevalence of HIV-SN, even among patients exposed to this "less toxic" dose. Makinson et al.[20] recently reported the effectiveness of 20 mg doses of stavudine. Although stavudine use is due to be phased out,[9] this will take time as there are few cost-effective alternatives.[11] Should the effectiveness of 20 mg doses be confirmed and if the safety profile of this dosage is more acceptable, use of a 20 mg twice daily stavudine dose should be considered to reduce toxicities while stavudine is being phased out.

Table 5
Prevalence of HIV-SN by Age and Height Groups Among African Patients Who Had Been Treated with Stavudine

	Height < 158 cm (%)	Height ≥ 158 cm (%)
Age < 38 years	35	45
Age ≥ 38 years	64	76

Our findings that increasing age and height are associated with increased HIV-SN risk are consistent with several other studies.[4,21−24] Indeed, where height has been assessed and found not to be associated with HIV-SN, the diagnosis of "neuropathy" did not require the presence of symptoms.[25] We found an association between height and HIV-SN risk despite our cohort being 5 cm shorter on average than other ethnic groups studied,[4] and the height cutoff values identified in our cohort through ROC analysis were 12 cm lower. This finding, that height was a risk despite the shorter height of our cohort, may be explained by longer leg length relative to the trunk length in Black compared with White individuals.[26] Our findings also confirm that factors associated with HIV-SN in untreated HIV patients such as nadir CD4 T-cell count[3] are less important in the context of ART.[5,6,24] Lastly, we did not find an association between isoniazid use and HIV-SN in our cohort. Isoniazid is neurotoxic.[27] Although coadministration of pyridoxine (as provided to most patients with tuberculosis in our cohort) minimizes the risk of SN,[28] isoniazid/pyridoxine use was associated with moderate to severe neuropathy in another African HIV cohort.[29] However, in that study, the cohort had not been universally exposed to stavudine. It may be that the greater neurotoxicity of stavudine and the greater exposure to it in our cohort disguised the neurotoxicity of isoniazid/pyridoxine.

Symptoms of HIV-SN are described as following a "glove and stocking" distribution, with the feet being the primary site affected.[30−32] However, the distribution of HIV-SN symptoms is rarely described in the literature. Consistent with earlier reports on HIV-SN symptoms in the pre-HAART era,[30] all patients with HIV-SN in our study experienced symptoms in their feet, and over one-third of these patients felt symptoms only in their feet, usually the soles. However, the proximal extension of symptoms above the ankle was greater in our cohort than in untreated HIV-positive patients,[30] which may be related to our cohort being exposed to neurotoxic antiretroviral drugs.

The most common symptom experienced in our cohort was pain, with the majority (74%)

experiencing moderate to severe pain. This contrasts with early reports of HIV-SN in untreated patients, where symptoms "were usually mild" and "painful dysethesias were uncommon."[32] This extent and severity of pain observed in our cohort will have a major impact on quality of life and may reduce adherence to antiretroviral drugs.[1,33,34]

The limitations of this study include that it was cross-sectional. Although this provides an accurate picture of patients attending the clinic currently, we relied on patient recall and data recorded in the medical file to assess risk factors for HIV-SN rather than collecting data prospectively and monitoring for incident cases of HIV-SN. Furthermore, age and height did not fully explain the variation in neuropathy status in our cohort, and, therefore, other factors not measured here also must be important. For example, host genetics may influence HIV-SN risk in patients exposed to stavudine.[21,35,36] A study is underway to look at associations between cytokine polymorphisms and SN risk in this cohort. In addition, we used a relatively simple clinical tool to diagnose patients with symptomatic HIV-SN. We have validated this tool against objective measures in the context of HIV infection,[13] but it is possible that milder cases of HIV-SN were missed with our chosen diagnostic criteria. In addition, although this tool assesses the major symptoms that have been associated with HIV-SN (pain, pins and needles, and numbness),[22,32] our characterization of neuropathy symptoms among African patients exposed to stavudine is limited to the symptoms assessed by this tool.

In conclusion, HIV-SN is a common problem that frequently presents with moderate to severe pain. Patients at highest risk of HIV-SN following stavudine exposure can be identified prior to initiating ART at no extra cost by recording age and height. In resource-limited settings where stavudine frequently still forms the backbone of ART, prioritizing older and taller patients for access to alternative agents could be an effective, inexpensive way to reduce patient suffering.

Disclosures and Acknowledgments

This study was funded by the Medical Faculty Research Endowment Fund, University of the Witwatersrand. The authors declare no conflicts of interest.

The authors wish to thank the staff and patients of the Virology Clinic in the Charlotte Maxeke Johannesburg Academic Hospital, and Florence Mtsweni for acting as the interpreter for the study. They are also grateful for the assistance of Drs. Sonia Hitchcock and Lindi Mnguni of the University of Pretoria, and Drs. Jaya George and Noko Mphahlele of the University of the Witwatersrand.

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APPENDIX 6

Wadley, A.L., Lombard, Z., Cherry, C.L., Price, P., Kamerman, P.R. (2012) "Analysis of a previously identified 'pain protective' haplotype and individual polymorphisms in the *GCH1* gene in Africans with HIV-associated sensory neuropathy: a genetic association study." JAIDS 60: 20-23, 2012

Analysis of a Previously Identified "Pain-Protective" Haplotype and Individual Polymorphisms in the *GCH1* Gene in Africans With HIV-Associated Sensory Neuropathy: A Genetic Association Study

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Abstract: We analyzed GTP cyclohydrolase 1 in symptomatic HIV-associated sensory neuropathy in Southern Africans including a "pain-protective" 3-SNP haplotype and 6 SNPs, analyzed individually and in a 6-SNP haplotype. The "pain-protective" 3-SNP haplotype and a 6-SNP haplotype containing these alleles associated with a reduced risk of pain. Another 3-SNP haplotype associated with increased presence of pain. Associations were lost after correction for age, gender, and CD4 T-cell count. Linkage disequilibrium differed between our cohort and Caucasians suggesting that these SNPs may not be ideal markers in Africans. Subsequently, the role of GTP cyclohydrolase 1 in painful HIV-associated sensory neuropathy remains possible.

Key Words: GCH1, HIV, neuropathy, African

(*J Acquir Immune Defic Syndr* 2012;60:20–23)

INTRODUCTION

HIV-associated sensory neuropathy (HIV-SN) is a length-dependent peripheral neuropathy, which affects 34%–57% of HIV patients on antiretroviral therapy in resource-limited countries.[1–3] The most common symptom of HIV-SN is pain, which affects approximately 75% of individuals with HIV-SN; about two-thirds of affected individuals experiencing pain of moderate to severe intensity.[3–5] This level of neuropathic pain markedly increases the burden of disease in the community,[6] especially as it is resistant to most conventional treatments for painful neuropathy.[7] Investigations into the underlying mechanisms of the pain are warranted to improve diagnosis and treatment.

GTP cyclohydrolase 1 (GCH1) is the rate-limiting enzyme in the production of tetrahydrobiopterin, a cofactor required for the synthesis of algesic molecules nitric oxide, serotonin, and catecholamines.[8] Reduced GCH1 activity is associated with decreased nociceptive hypersensitivity in animal models of neuropathic pain.[9] Variations of the gene encoding GCH1, *GCH1*, were associated with reduced pain in humans, with a 15-single nucleotide polymorphism (SNP) haplotype and 5 individual SNPs within the haplotype being associated with reduced pain intensity after discectomy for persistent radicular low back pain.[9,10] A subsequent study showed that this 15-SNP haplotype could be identified in Caucasians by genotyping for a smaller 3-SNP haplotype.[11]

However, the results from subsequent *GCH1* pain association studies have been inconsistent. It has been suggested that population stratification may have contributed to the detection of the original associations or that the different haplotype structure in *GCH1* across ethnic groups may account for the inconsistency.[12] Studies of different ethnic groups are now needed to determine whether published results may be generalized. Here, we describe the first investigation into *GCH1* polymorphisms and pain associated with HIV-SN in an African population. We also examine linkage disequilibrium (LD) between the *GCH1* SNPs.

MATERIALS AND METHODS

Participants

The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, South Africa (protocol number: M080220), and written informed consent was obtained from all participants. Participants were HIV-positive adults and had been on highly active antiretroviral therapy for at least 6 months when they were screened for neuropathy at the Virology Clinic of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa, between July 2008 and April 2009. Participants were selected based on self-reported black African ethnicity. Individuals who identified themselves as Cape Mixed

Received for publication September 28, 2011; accepted January 18, 2012.

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Supported by Medical Research Endowment Fund, Faculty of Health Sciences, and the University Research Council of the, University of the Witwatersrand, South Africa. Funding also received from the National Research Foundation (South Africa) Rated Researchers Programme.

Presented at the 13th World Congress on Pain, Montreal, August 29, 2010, to September 2, 2010.

The authors have no conflicts of interest to disclose.

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Ancestry (mixed black African and Caucasian descent) were not included. In this clinic, first-line highly active antiretroviral therapy included the nucleotide reverse transcriptase inhibitor, stavudine (d4T), in accordance with national guidelines current at that time.[13] An interpreter fluent in English and local African languages facilitated consent and study procedures. Three hundred forty-two patients met the study criteria and were screened. Of these, 159 had HIV-SN and were included in analyses of associations between SNPs and the presence and intensity of pain. All 342 individuals were used to examine LD in the population.

Phenotyping Procedure

Patients were screened using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen, which is a validated tool for identifying symptomatic HIV-SN.[14] One hundred fifty-nine participants of the 342 screened were considered to have symptomatic HIV-SN and were included in the analysis of association between SNPs and haplotypes and the presence and intensity of pain. Symptomatic HIV-SN was diagnosed based on the bilateral presence of at least 1 symptom (pain, aching, burning, numbness, or pins-and-needles) and at least 1 clinical sign of neuropathy (reduced vibration sense or absent ankle reflexes). Vibration sense was assessed using a 128 Hz tuning fork, which was placed on the interphalangeal joint of each great toe; vibration sense of 10 seconds or less was considered abnormal. Pain intensity was rated on an 11-point rating scale anchored at "0" (no symptom experienced) to "10" (worst imaginable). Although previous symptoms count toward an HIV-SN diagnosis when using the tool in a clinical setting, participants who did not classify their pain as "current" were excluded from analyses relating to symptomatic HIV-SN in this study. Individuals with other potential causes of neuropathy (eg, diabetes mellitus, alcoholism, vitamin B12 deficiency, exposure to isoniazid and chemotherapy) also were excluded, as were individuals whose ethnicity could not be confirmed as black Southern African.

Genotyping

The initial 39 DNA samples were extracted from saliva, but thereafter the protocol was changed to DNA extraction from blood, for all following samples, after concerns over DNA quantity. DNA was extracted from the saliva samples using a QIAamp DNA mini kit (QIAGEN; Valencia, CA) and from the blood samples using the salting-out method[15] and stored at 4°C. All SNPs were submitted to Illumina Technical Support for evaluation by the Illumina assay design tool. All SNPs were scored (varying from 0 to 1) by assay design tool based on compatibility to successful GoldenGate genotyping. All 6 SNPs were deemed successful and were genotyped using the Goldengate assay on the Illumina BeadXpress genotyping platform. All genotyping and DNA extraction was carried out in the Division of Human Genetics, National Health Laboratory Services, and University of the Witwatersrand, Johannesburg, South Africa.

Statistical Analyses

Statistical analyses and haplotype reconstruction were carried out using PLINK.[16,17] Standard quality control filters were applied to the data as follows[18]: minor allele frequency >0.01, SNP missingness rate <0.05, individual missingness rate <0.2, and Hardy–Weinberg equilibrium $< 1 \times 10^{-4}$. An allelic (χ^2) test was used to detect for association between individual *GCH1* SNPs and the presence of pain. The Wald test was used to detect for association between individual SNPs and pain intensity. Univariate analyses of the reconstructed 3-SNP and 6-SNP haplotypes were performed to detect associations with pain intensity and presence of pain. Multiple regression was then carried out to correct for variables previously found to be associated with risk of pain or pain intensity, that is, age, gender, and CD4 T-cell count.[19–21] Significance was considered as $P < 0.05$.

The larger cohort of 342 participants (with and without HIV-SN) was used to examine the LD between the 6 SNPs using the confidence interval method in Haploview.[22] This was compared with the LD structure for Caucasian (CEU) assessed using data from HapMap (Genome Browser release#27).

RESULTS

Characteristics of the Cohort

The cohort was 78% (124 of 159) female, the mean age was 41 years (SD: 8), and median CD4 T-cell count was 399 (min/max range: 81–1091). Ninety one percent (91%, 144 of 159) reported a painful neuropathy and 68% of those (108 of 159) described their pain as moderate to severe (≥4 of 10 on the 11-point numerical pain rating scale). Patients describing pain were no more likely to be female [76% (110/144) of those with pain were female, vs. 93%(14 of 15) of those without, $P = 0.19$, Fisher exact]. We found no other associations between clinical factors (including CD4 T-cell count) and the presence of pain among patients with HIV-SN. All participants donated DNA.

Most participants were South African (94%, 150 of 159) with the remaining 6% (9 of 159) from other Southern African countries that are included in the Niger-Kordofanian ethnolinguistic grouping[23] (Zimbabwean n = 4, Mozambiquean n = 3, Malawi n = 1, Zambia n = 1). This grouping shows greater homogeneity than other African populations[24] and shows similarity at 1327 polymorphic sites that are consistent with self-described ethnicity.[23] A study from Soweto (a Johannesburg township) using 18 ancestry informative markers found no significant substructure in this African population (A. L. Wadley, BSc Hons, Z. Lombard, PhD, C. L. Cherry, PhD, P. Price, PhD, P. R. Kamerman, PhD, unpublished data, February 2012).

Allele Information

The median genotyping success rate was 92% (range 90%–92%). The major and minor of alleles of rs8007201 and rs8007267 were reversed when our cohort was compared with a Caucasian population (CEU; HapMap). The allele frequencies for all SNPs ranged from 9% to 35%, and all were in Hardy–Weinberg equilibrium.

Association Study

No individual SNPs were associated with the presence or intensity of pain ($P = 0.05$–0.99, data not shown). We then

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addressed 3-SNP haplotypes, including the "pain-protective" haplotype[11] that includes rs8007267 allele T (denoted CAT), and haplotypes of all 6 SNPs. The cohort had 5 of the 6 possible 3-SNP haplotypes and 8 of the 21 possible 6-SNP haplotypes. The 3-SNP haplotypes accounted for 99% of the cohort and the 6-SNP haplotypes accounted for 82% of the cohort. The 3-SNP "pain-protective" haplotype (CAT) and a 6-SNP haplotype including this motif (CTCGAT) were not associated with pain intensity but were associated with reduced presence of pain (Table 1). However, after correcting for factors that have previously been associated with pain (gender, age, and CD4 T-cell count), the associations with the "pain-protective" 3-SNP haplotype (logistic regression; $P = 0.05$) and the 6-SNP haplotype (logistic regression; $P = 0.09$) were no longer significant. An additional 3-SNP haplotype (GTC) was associated with increased risk of pain on univariate analysis ($P = 0.04$), but the association was also no longer significant after correction for risk factors (logistic regression; $P = 0.08$).

LD Structure

LD was mapped in all 342 patients recruited for this study (with and without HIV-SN) and a representative sample of Caucasians (Fig. 1). Consistent with higher genomic variability within African populations, the LD between the 6 SNPs was lower in our cohort than in Caucasians.

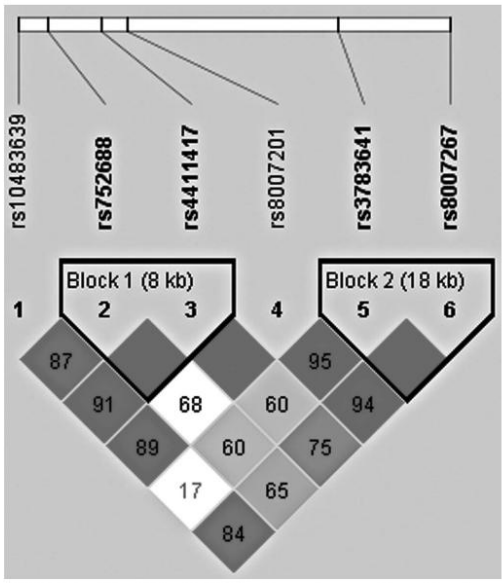

FIGURE 1. LD within GCH1 in Caucasians and our Southern African cohort.

DISCUSSION

The protein encoded by *GCH1* has been implicated in neuropathic pain states in animals and gene polymorphisms associate with reduced neuropathic pain in humans.[9,10,25] We describe the first study to associate known genetic variants in *GCH1* with pain in Africans with HIV-SN. We found no independent association between individual *GCH1* SNPs and pain. However, a previously identified "pain-protective" 3-SNP haplotype and a 6-SNP haplotype including this motif were associated with reduced risk of pain on univariate analysis. An additional novel 3-SNP haplotype was associated with increased risk of pain. However, these associations were not apparent after adjustment for other risk factors for pain (age, gender, and CD4 T-cell count).

Several factors may explain the absence of an independent association. First, associations between genes and pain sensitivity may be specific to the cause of the pain.[26,27] Indeed, there are differences in the features and pathophysiology of radicular pain secondary to disc herniation, as addressed in the initial study of Tegeder et al[9] and the small fibre distal sensory polyneuropathy of HIV-SN. Polymorphisms of *GCH1* that associated with radicular pain were not associated with the intensity of dental,[12] visceral,[28] or chronic widespread pain[29] in Caucasians.

Second, the African genome is more variable than the Caucasian.[30] The higher genomic variability within African populations was evident here as weaker LD and smaller LD blocks in our cohort than that seen in Caucasians (Fig. 1). Thus the weak associations seen in our cohort may reflect differences in the relationship between marker and causative SNPs in *GCH1* between Caucasian and African populations. Indeed, the weak associations we detected between the 3-SNP and 6-SNP haplotypes indicate that further analysis of the role

TABLE 1. Univariate Analysis Between the 3-SNP and 6-SNP Haplotypes and Presence of Pain

	Frequency	Pain, n = 144	No Pain, n = 15	*P*
3-SNP haplotypes				
GTC	0.34	36%	17%	**0.04**
CAT	0.07	5%	17%	**0.02**
GAT	0.23	23%	27%	0.62
CTT	0.13	14%	17%	0.65
GTT	0.23	21%	23%	0.80
6-SNP haplotypes				
GCTATC	0.24	26%	10%	0.06
GCTGTC	0.10	8%	0%	0.22
CTCGAT	0.06	5%	17%	**0.04**
GTCGAT	0.01	1%	0%	0.42
GCTGAT	0.22	21%	23%	0.54
CTCGTT	0.02	2%	0%	0.64
CCTGTT	0.12	11%	13%	0.77
GCTGTT	0.22	22%	27%	0.61

The 3-SNP haplotypes comprise rs10483639, rs3783641, and rs8007267, including the "pain-protective" 3-SNP haplotype as described by Lotsch et al (2007) in bold.

The 6-SNP haplotypes comprise rs10483639, rs752688, rs4411471, rs8007201, rs3783641, rs8007267. The 6-SNP haplotype highlighted in bold contains the 3-SNP "pain-protective" haplotype.

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of polymorphisms in *GCH1* in painful HIV-SN are warranted, but using population-appropriate tag-SNPs.

In conclusion, we found no independent association between *GCH1*, including a previously identified "pain-protective" haplotype, and the presence or intensity of pain in HIV-SN in Southern Africans. However, association between *GCH1* and pain associated with HIV-SN cannot be ruled out and more detailed study of *GCH1* in this painful condition is required.

ACKNOWLEDGMENTS

We wish to thank the staff and patients of the Virology Clinic in the Charlotte Maxeke Johannesburg Academic Hospital and Florence Mtsweni for acting as the interpreter for the study. In addition, we would like to thank Punita Pitamber for her assistance with genotyping and Constance Chew for her help with the genetic analysis.

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