Genetics of HIV-associated sensory neuropathy in black Southern Africans

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science (Medicine) in Human Genetics

Declaration

I, Liesl Mary Hendry, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science (Medicine) in Human Genetics to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Liesl Mary Hendry

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Date

This dissertation is dedicated to my family

Abstract

HIV-associated sensory neuropathy (HIV-SN) is a common complication associated with human immunodeficiency virus (HIV)-infection. A common symptom of HIV-SN is pain. Variation at specific loci within certain candidate genes has been suggested to alter susceptibility to developing HIV-SN as well as the susceptibility to developing pain and the intensity of the pain experienced. Few studies, however, have been conducted in individuals of black African ancestry. The aim of the current research was to conduct an in-depth study, in a black Southern African population, of genes previously associated with susceptibility to developing HIV-SN (*TNFA* and surrounding genes and *IL12B*) and variations in pain susceptibility and intensity (*GCH1, KCNS1, IL1B, IL6, CCL2* and *CCR2*) in other neuropathic pain states. Single nucleotide polymorphisms (SNPs) identified in the literature were supplemented with population appropriate tagSNPs to improve assessment of the genes in an African population. Genotyping of previously collected deoxyribonucleic acid (DNA) samples was carried out using a GoldenGate assay with VeraCode microbeads and data were read on an Illumina BeadXpress Reader. Data were statistically analysed to assess the association of the genetic variants with susceptibility to developing HIV-SN and pain and variability of pain intensity in those patients with painful HIV-SN. Although some SNPs and haplotypes in the genes investigated associated with HIV-SN susceptibility (*TNFA* region), pain susceptibility (*TNFA* region, *IL12B*, *KCNS1*, *IL6* and *CCR2*) or pain intensity (*TNFA* region, *KCNS1*, *IL1B* and *IL6*), none of the results were consistent with that which has been found in previous studies in non-African populations. Reasons for this may be that associations are population-specific or model-specific. Limitations of the study included the use of a relatively small sample, the method of sampling (convenience sampling), genotyping failure and tagging inefficiency in some instances, and the fact that there is no Southern

African population dataset to use for tagSNP selection. My findings emphasise the importance of conducting genetic association studies in separate ethnic groups.

Acknowledgements

I wish to express my sincere gratitude and appreciation to the following people and institutions:

- My supervisors, Zané and Peter, for their wonderful supervision. I am so fortunate to have had two such dedicated supervisors who were always willing to offer invaluable advice and guidance, who were always so prompt in their return of work and answers to queries/questions, and who were always willing to go the extra mile.

- Toni Wadley, for always being available to answer questions about and offer help with understanding the sample information, the use of gPLINK and paper submission and for always offering the help with friendliness and patience. Her initial collection of DNA samples and phenotype data is also much appreciated.

- Punita Pitamber, for her assistance and constant willingness to assist with the BeadXpress lab work and cleaning of the data and anything else I had trouble with in and out of the lab.

- TJ Naidoo, for his assistance with the TaqMan lab work.

- The students and staff in Human Genetics, for their friendliness and helping me to settle in when I was a new student, for making the working environment so pleasant, for help when it was needed, for their company at lunch time, and for all the enjoyable "outings".

- The Brain Function Research Group, for their friendliness and constant support.

- The Belgian Technical Cooperation, National Research Foundation, University of the Witwatersrand and National Health Laboratory Service Research Trust for financial support.

- My Mom (Gill), Dad (Keith) and brother (Neil) as well as other family and friends for their constant love, support and guidance in my studies and for always taking interest in what I was doing even if they didn't understand any of it.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Publications & presentations arising from this study

Publication:

HENDRY LM, LOMBARD Z, WADLEY AL, KAMERMAN PR

KCNS1, but not GCH1, is associated with pain intensity in a black Southern African population with HIV-associated sensory neuropathy: a genetic association study.

Journal of Acquired Immune Deficiency Syndromes

Presentations:

- **2012 PainSA Congress (Pretoria, 22-24 June 2012)** – Oral presentation entitled "Association of unique polymorphisms in KCNS1 with neuropathic pain sensitivity in African Individuals with HIV-associated sensory neuropathy"

- **14th World Congress on Pain (Milan, Italy, 27-31 August 2012)** – Poster presentation entitled "Association of unique polymorphisms in KCNS1 with neuropathic pain sensitivity in African Individuals with HIV-associated sensory neuropathy".

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Abbreviations

3’	three prime
5’	five prime
%	percentage
°C	degrees Celsius
µg	microgram
µl	microlitre
µM	micromolar
ADT	assay design tool
AIDS	acquired immunodeficiency syndrome
ARV	antiretroviral
ASW	African ancestry in Southwest USA
ATN	antiretroviral toxic neuropathy
ATP6V1G2	ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2 gene
BAT1	DEAD (Aspartic acid - Glutamic acid - Alanine - Aspartic acid) box polypeptide 39B gene
BDNF	brain-derived neurotrophic factor
BH_4	tetrahydrobiopterin
bp	base pairs
Bt20	Birth to Twenty
cAMP	cyclic adenosine monophosphate
cART	combination antiretroviral therapy
CCL2/*CCL2*	Chemokine (C-C motif) ligand 2/Chemokine (C-C motif) ligand 2 gene
CCR2/*CCR2*	C-C chemokine receptor type 2/ C-C chemokine receptor type 2 gene
CD4	cluster of differentiation 4

CEU	Utah residents with Northern and Western European ancestry
cm	centimetre
CXCR4	C-X-C chemokine receptor type 4
d4T	stavudine
ddC	zalcitabine
ddH_2O	double-distilled, deionised water
ddI	didanosine
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DOPA	3,4-dihydroxyphenylalanine
DRG	dorsal root ganglia
EDTA	Ethylenediaminetetraacetic acid
g	gram
GCH1	guanosine-5'-triphosphate cyclohydrolase 1 gene
gp120	envelope glycoprotein 120
GTP	guanosine-5'-triphosphate
HFE	hemochromatosis
HIV	human immunodeficiency virus
HIV-DSP	HIV-distal symmetrical polyneuropathy
HIV-SN	HIV-associated sensory neuropathy
HWE	Hardy-Weinberg equilibrium
IL-1	interleukin-1
IL-12β/*IL12B*	interleukin-12-beta/interleukin-12-beta gene
IL-1β/*IL1B*	interleukin-1-beta/interleukin-1-beta gene
IL-6/*IL6*	interleukin-6/interleukin-6 gene

IQR	interquartile range
kb/kbp	kilo base pair(s)
KCNS1	potassium voltage-gated channel subfamily S member 1 gene
KOH	potassium hydroxide
LD	linkage disequilibrium
LST/LST1	leukocyte specific transcript/ leukocyte specific transcript 1
LTA	lymphotoxin-alpha gene
LTB	lymphotoxin-beta gene
LWK	Luhya in Webuye, Kenya
MAF	minor allele frequency
MCCD1	mitochondrial coiled-coil domain 1 gene
MGB	minor groove binder
$MgCl_2$	magnesium chloride
MHC	major histocompatibility complex
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NCR3	natural cytotoxicity triggering receptor 3 gene
NFKBIL1	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 gene
NF-κB	nuclear factor-kappa B
ng	nanogram
nm	nanometre

NRTIs	nucleoside reverse transcriptase inhibitors
OR	odds ratio
P	previously associated SNP
p38 MAPK	p38 mitogen activated protein kinase
PCR	polymerase chain reaction
P_{EMP1}	pointwise empirical p-value
P_{EMP2}	Family-wise correction for multiple comparisons
RANTES	regulated upon activation, normal T-cell expressed and secreted
SD	standard deviation
SDS	Sequence Detection System
SNP	single nucleotide polymorphism
T	tagSNP
TE	Tris/EDTA
TNF	tumour necrosis factor
TNF-α/*TNFA*	tumour necrosis factor-alpha/tumour necrosis factor-alpha gene
UCSC	University of California, Santa Cruz
UNAFF	unaffected individuals
w/v	weight per volume
YRI	Yoruba in Ibadan, Nigeria

CHAPTER 1

INTRODUCTION

1.1 HIV-associated sensory neuropathy (HIV-SN)

Human immunodeficiency virus (HIV) infection is frequently associated with disorders affecting the central and peripheral nervous systems, with over 50% of infected individuals developing a neurological disorder during the course of the infection (Power et al., 2009). A common neurological disorder in HIV infection is peripheral neuropathy, with HIV-associated sensory neuropathy (HIV-SN) affecting about 20 to almost 60% of individuals with access to combination antiretroviral therapy (cART) (Kamerman et al., 2012a). The prevalence of HIV-SN is believed to be increasing due to the longer life-span of HIV-infected individuals and the continued use of neurotoxic antiretroviral (ARV) drugs (Ferrari et al., 2006).

HIV-SN, characterized by length-dependent nerve damage to the peripheral nervous system, can be divided into two subtypes: HIV-distal symmetrical polyneuropathy (HIV-DSP), which results from immune dysregulation and direct HIV-induced neuronal damage, and antiretroviral toxic neuropathy (ATN), which involves the additional insult of neurotoxic ARV drugs on nerve fibres (Kamerman et al., 2012b). Significant contributors to the toxicity are the dideoxynucleoside analogues of the nucleoside reverse transcriptase inhibitors (NRTIs), such as didanosine (ddI), zalcitabine (ddC) and stavudine (d4T) (Verma and Simpson, 2007).

1.1.1 Epidemiology and mechanisms of HIV-SN

The pathophysiology of HIV-DSP and ATN involve three mechanisms – the “dying back” or length dependent, “Wallerian-like” degeneration of long, peripheral axons (Pardo et al., 2001,

Keswani et al., 2002); the loss of unmyelinated fibres (Pardo et al., 2001); and an infiltration of activated macrophages in the peripheral nerves and dorsal root ganglia (DRG) (Kallianpur et al., 2006, Pardo et al., 2001).

1.1.1.1 HIV-DSP

HIV-DSP is common in individuals with severe immunosuppression (Nicholas et al., 2007), and is likely to result from neuronal damage caused by exposure to certain HIV proteins (Cornblath and Hoke, 2006). For example, exposure of DRG sensory neurons and Schwann cells to envelope glycoprotein (gp120), an HIV envelope protein which can act via the C-X-C chemokine receptor type 4 (CXCR4), leads to an upregulation of RANTES (regulated upon activation, normal T-cell expressed and secreted). This in turn leads to an upregulation of tumour necrosis factor-alpha (TNF-α) in the neurons, which has been linked to apoptotic cell death in the neurons (Keswani et al., 2003).

1.1.1.2 ATN

A possible mechanism of ARV toxicity is the inhibition of mitochondrial deoxyribonucleic acid (DNA) polymerase (Lewis and Dalakas, 1995), leading to an interruption of mitochondrial replication which ultimately results in impaired oxidative phosphorylation. Disruption of oxidative phosphorylation results in impaired energy production and an excess of free radicals being produced and subsequently oxidative stress. Studies conducted in cell lines, animal models and humans have shown changes in the morphology, quantity and function of mitochondria following exposure to ARVs (Kallianpur and Hulgan, 2009).

HIV-DSP and ATN are often difficult to distinguish, making diagnosis challenging. Both forms of HIV-SN affect the distal extremities. Key clinical signs of HIV-SN include reduced vibration sense, pin-prick sensitivity and absent ankle reflexes (Tagliati et al., 1999, Wadley et al., 2011). Common symptoms associated with HIV-SN include pain, numbness, aching, burning, and pins and needles (Tagliati et al., 1999, Wadley et al., 2011). The two entities are differentiated based on the onset and progression of the disorder; ATN is associated with starting ART and while ATN develops quite rapidly, HIV-DSP takes weeks or months to develop (Nicholas et al., 2007). ATN may also be more painful than HIV-DSP (Dieterich, 2003).

1.1.2 Non-genetic risk factors for HIV-SN

Risk factors for the development of HIV-SN differ among individuals depending on whether they have an untreated HIV-infection (HIV-DSP) or are on ARVs (ATN). Risk factors associated with HIV-DSP include a more advanced stage of HIV-infection and a longer duration of illness. In ATN, exposure to the neurotoxic ARV drugs is one of the greatest risk factors in itself, as is increasing age. Other risk factors include a prior tendency to developing peripheral neuropathy, such as having diabetes, consumption of alcohol and increased height. Several other factors may be associated, but the data are inconsistent. These include, among others, ethnicity. It is suggested that individuals of African ancestry are at a greater risk of developing HIV-SN than individuals of other ethnicities (Reviewed in Kamerman et al., 2012a).

1.1.3 Genetic risk factors for HIV-SN

In addition to the above risk factors, a genetic susceptibility to the development of HIV-SN has been suggested, particularly related to polymorphisms in genes involved in mitochondrial function and the inflammatory response (Kamerman et al., 2012a).

1.1.3.1 Genes associated with mitochondrial function

Genes associated with mitochondrial function are important candidates for HIV-SN risk, particularly ATN, due to the fact that the toxic ARVs cause a disruption in mitochondrial function (Kamerman et al., 2012a). Although the genes related to mitochondrial function were not investigated in the current study, reported associations are outlined briefly below.

- **Mitochondrial DNA**

Polymorphisms in mitochondrial DNA have been associated with HIV-SN risk. More specifically, polymorphisms in haplotype T (Canter et al., 2008, Hulgan et al., 2005) and haplotype L1c (Canter et al., 2010) in European and African-Americans, respectively, have been associated with an increased risk. As these two haplotypes are specific to the respective populations, this highlights the differences in genetic susceptibility between population groups and the fact that the genes associated with HIV-SN risk in separate ethnic groups may not be the same. Genetic variation in different ethnic groups will be discussed later in section 1.3.

- **Nuclear DNA**

Polymorphisms in certain genes associated with mitochondrial function in the nuclear DNA have also been identified to be associated with HIV-SN risk. In particular a polymorphism in the hemochromatosis (HFE) gene, which encodes a membrane protein that regulates iron absorption, an important element required for mitochondrial electron transport chain function, was associated with a decreased risk of developing HIV-SN (Kallianpur et al., 2006).

1.1.3.2 Genes associated with the inflammatory response

Damage to tissue is associated with an inflammatory response which involves an infiltration of immune cells and a release of several chemical mediators, such as prostaglandins, histamine, serotonin, bradykinin, protons, nerve growth factor and cytokines (Abbadie, 2005). Cytokines, in particular, have been linked to injury to nerve tissue and the development of neuropathic pain conditions (Sommer, 2006).

The role of pro-inflammatory cytokines in nerve injury and neuropathic pain has been demonstrated in several studies. Studies conducted on animal nerve injury models and later in humans showed an alteration in cytokine levels in the central and peripheral nervous systems. In particular, in rat and mouse sciatic nerve injury models, increased levels of the messenger ribonucleic acid (mRNA) and protein forms of TNF-α, interleukin-1β (IL-1β) and interleukin-6 (IL-6) have been seen localized to dedifferentiating Schwann cells and macrophages (Sommer and Schäfers, 2004). In a study in adult rats, TNF-α and IL-1β were upregulated following peripheral nerve injury (DeLeo et al., 1997, Sweitzer et al., 1999). Neuropathic symptoms were also present following central administration of the two

cytokines (Oka et al., 1995, Oka et al., 1996) and inhibition of the cytokines reduced the symptoms (Schafers et al., 2003, Sommer et al., 1999).

The pro-inflammatory cytokines are involved in enhancing excitability following nerve injury and facilitating the development of allodynia (pain resulting from a stimulus which does not normally cause pain) and hyperalgesia (an increased response to a stimulus that is normally painful) (White and Miller, 2010). Cytokines may act directly on nerve fibres or they may act indirectly via the release of prostaglandin E2 or nitric oxide (McMahon et al., 2005). In addition, cytokines may initiate the further release of additional mediators, such as chemokines (White and Miller, 2010).

The genes that were investigated mainly for their role in HIV-SN susceptibility (although also included in pain susceptibility and pain intensity analyses) – tumour necrosis factor-alpha gene *(TNFA)* and interleukin-12-beta gene *(IL12B)* – are discussed in further detail below. The cytokines and chemokines which were investigated only for their roles in pain susceptibility and pain intensity – interleukin-1-beta gene *(IL1B)*, interleukin-6 gene *(IL6)* and chemokine (C-C motif) ligand 2 gene *(CCL2)* and C-C chemokine receptor type 2 gene *(CCR2)* – will be discussed in further detail in section 1.2.2 (b).

- ***TNFA* region**

One cytokine significantly associated with nerve injury and neuropathy is tumour necrosis factor-α (TNF-α). TNF -α is a pro-inflammatory cytokine and exists in a transmembrane or secreted form (Sommer, 2006). Although an essential part of the immune system, TNF-α may be harmful if in excess (Ackerman et al., 2003).

Role of TNF-α in neuropathy and neuropathic pain

TNF-α has been shown to play a significant role in development of painful neuropathy and is thought to be a key modulator in pain-processing mechanisms through the initiation of an "inflammatory" cascade (Bhangoo et al., 2009). TNF-α is capable of initiating the production and release of other cytokines, including interleukin-1 (IL-1) and IL-6, from macrophages and Schwann cells (Bolin et al., 1995, Sommer et al., 1997, Wagner and Myers, 1996).

The expression of TNF-α is upregulated in Schwann cells in patients with painful neuropathy (Empl et al., 2001) and early release of TNF-α from macrophages and Schwann cells is seen following nerve injury (George et al., 1999, McMahon et al., 2005). In addition, elevated serum levels of TNF-α (Ludwig et al., 2008) and an increased number of TNF receptors (Empl et al., 2001) were seen in patients with mechanical allodynia, compared to those without.

Polymorphisms in TNFA and surrounding genes

The *TNFA* gene is located on the short arm of chromosome 6 and falls under the major histocompatibility complex (MHC) class III region (**Figure 1.1**). Numerous genes are densely contained within this MHC region and many of these genes have been linked to inflammation and the susceptibility to developing autoimmune and immunopathological diseases (Allcock et al., 2004, The MHC Sequencing Consortium, 1999). Strong linkage disequilibrium (LD) exists across the MHC, however, making the identification of the exact genes involved in disease susceptibility difficult (Price et al., 1999, Undlien et al., 2001).

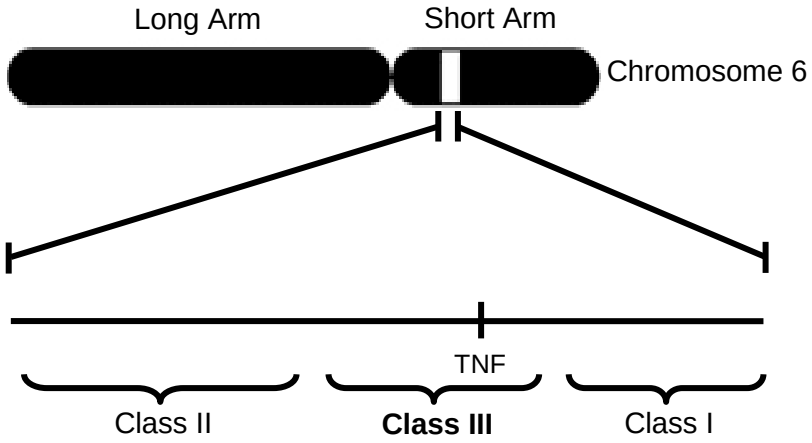

Figure 1.1. Chromosome six and the positions of the MHC class III region. (Modified from Schram and Warshaw, 2007)

Common polymorphisms associated with TNF-related pathologies are generally located in the promoter region of the gene, rather than the coding region (Richardson et al., 2001). Some of these single nucleotide polymorphisms (SNPs) have been linked to infectious disease susceptibility in Africa (Ackerman et al., 2003).

A study by Cherry et al. (2008) in Australian, predominantly Caucasian, HIV patients exposed to ARVs showed an association between a polymorphism in the *TNFA* gene and the presence of ATN. Carriage of the minor allele of the TNFA-1031 allele (TNFA-1031*2, rs1799964) correlated with an increased risk for ATN as its presence was higher in patients with ATN than those without. Cherry et al. further stated that it is uncertain whether the presence of this allele affects the production of TNF-α. They did, however, suggest that it is biologically plausible for there to be an association between the presence of the allele and ATN development. The positive association of this *TNFA* allele and ATN risk was replicated in Indonesian HIV patients exposed to d4T (Affandi et al., 2008).

It has been suggested that *TNFA* SNPs found to have an association with disease susceptibility may in fact not be primarily responsible for the association, but that SNPs far away in flanking genes may contribute to the association. Some SNPs, although not possessing LD with markers close by, exhibit high LD with markers further away (Ackerman et al., 2003). Chew et al. (2010) suggested that TNFA-1031*2 alone does not play a role in neuropathy susceptibility, and that the allele could be in LD with several SNPs around the *TNFA* gene. Carriage of a haplotype consisting of SNPs within the DEAD (Aspartic acid - Glutamic acid - Alanine - Aspartic acid) box polypeptide 39B (*BAT1)* promoter (rs2523506, rs2251824), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 (*NFKBIL1)* promoter (rs3219186), *TNF* promoter (rs2857713, rs1799964, rs4248158) and leukocyte specific transcript *(LST)* exon 5 (rs1052248) regions of the central MHC of chromosome 6 was associated with an increased risk of ATN in Chinese, Malay and Caucasian individuals treated with stavudine (Chew et al., 2010). The location of the *BAT1*, *NFKBIL1*, *TNF* and *LST* genes in relation to each other and associated genes is shown in **Figure 1.2**.

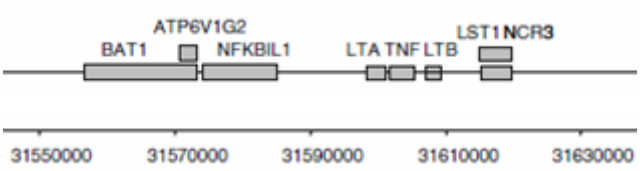

Figure 1.2. An approximate 80 kilo base pair (kbp) region of chromosome 6 showing the location of the *BAT1, NFKBIL1, TNF* and *LST* genes in relation to each other and associated genes. (Modified from Dunstan et al., 2007)

Functions of some important genes in the TNFA region

Important genes in the *TNFA* region include lymphotoxin-alpha (*LTA), BAT1*, *NFKBIL1* and mitochondrial coiled-coil domain 1 gene (*MCCD1). LTA* is a gene encoding lymphotoxin

alpha, a cytokine which is produced by lymphocytes and which is involved in a variety of immune responses. *BAT1* is a gene encoding an RNA-dependent ATPase which helps to direct ATP hydrolysis during pre-mRNA splicing. *NFKBIL1* encodes a member if the I-kappa-B family of proteins, although its exact function has not been determined. *MCCD1* encodes a mitochondrial protein (http://www.ncbi.nlm.nih.gov/gene).

- ***IL12B***

In the same study by Cherry et al. (2008) in the Australian cohort, an assessment was carried out on a polymorphism in *IL12B.* Carriage of the minor allele of a polymorphism in the 3' untranslated region (IL12B (3'UTR)*2) correlated with a decreased risk to the development of ATN. The minor allele was found to be more common in HIV-SN resistant individuals compared to HIV-negative controls and individuals with HIV-SN, which suggested the protective role that the minor allele plays. The finding could not be replicated in the Indonesian cohort (Affandi et al., 2008).

1.2 Painful HIV-SN

Pain is reported as being the most common symptom of HIV-SN, with it commonly occurring in the feet (Cherry et al., 2012, Wadley et al., 2011). Phillips et al. (2010) reported that pain is experienced in up to 90% of patients with acquired immunodeficiency syndrome (AIDS) defining conditions. Additionally, pain is the most common symptom associated with HIV-SN in black Southern Africans, with approximately 76% of individuals with HIV-SN reporting pain as a symptom, and most having moderate-to-severe pain (Wadley et al., 2011).

This pain, like other types of neuropathic pain (Bouhassira et al., 2004), is frequently described as burning or hot in quality (Shaikh, 2011).

1.2.1 Non-genetic risk factors for painful HIV-SN

Risk factors for the intensity of pain experienced in painful HIV-SN include the severity of the disease; decreased nerve fibre density; and psychosocial aspects (Kamerman et al., 2012a). In addition, the magnitude of the pain associated with HIV-SN tends to vary extensively between individuals and it may be that, as has been suggested for other types of pain, the differences in pain sensitivity among individuals with HIV-SN is also linked to genetic variability at several loci (Lacroix-Fralish and Mogil, 2009). Genetic risk factors for painful HIV-SN are discussed below.

1.2.2 Genetic risk factors for painful HIV-SN

Several genes have been identified to be associated with pain, and are discussed in more detail below. It is important to note that very few of these genetic associations have been studied in the context of HIV-SN or in African individuals.

1.2.2.1 *GCH1* and *KCNS1*

- ***GCH1*, its function and role in neuropathic pain**

The guanosine-5'-triphosphate cyclohydrolase 1 gene (*GCH1)* encodes guanosine-5'-triphosphate (GTP) cyclohydrolase, the rate-limiting enzyme in the de novo biosynthesis of tetrahydrobiopterin (BH_4) (**Figure 1.3**) (Tegeder et al., 2006).

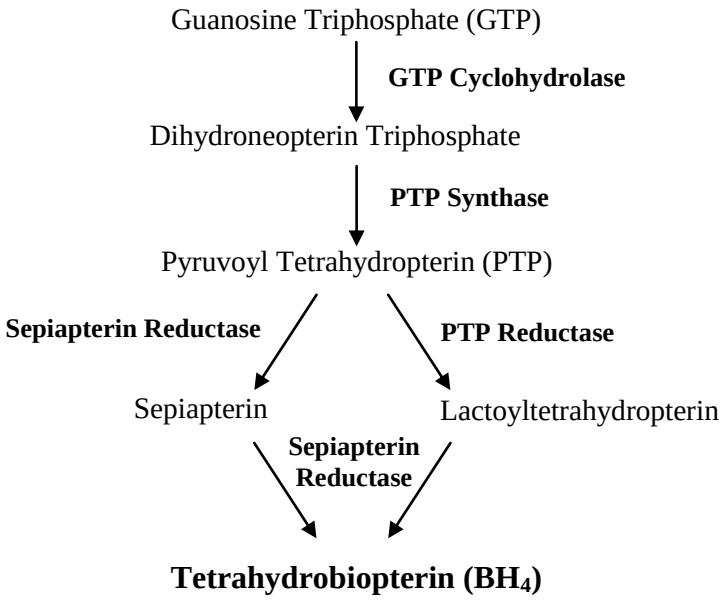

Figure 1.3. De novo biosynthetic pathway of BH_4. (Obtained from: http://www.uic.edu /classes/phar/phar332/Clinical_Cases/aa%20metab%20cases/ PKU%20Cases/bioh4.htm)

BH_4 is an essential cofactor (**Figure 1.4**) for several enzymes in the cells and tissues of higher organisms. Included in these enzymes are the three isoforms of nitric oxide synthase, which catalyze nitric oxide synthesis; tyrosine hydroxylase, which catalyzes the synthesis of biogenic amines, such as noradrenaline and dopamine; and tryptophan hydroxylase, implicated in the synthesis of serotonin (Lotsch et al., 2010, Thony et al., 2000).

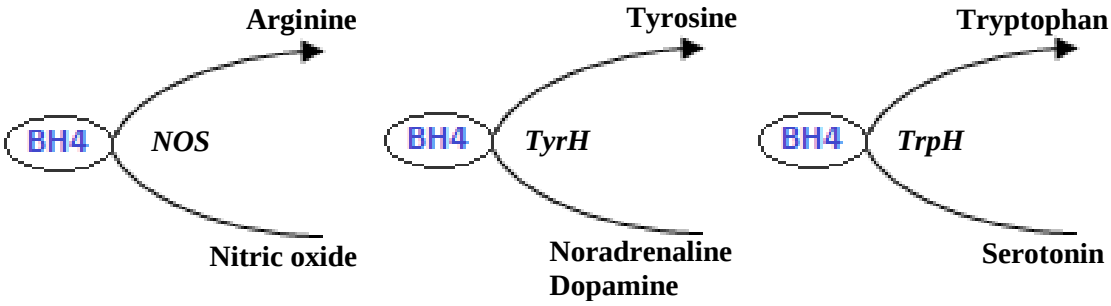

Figure 1.4. Role of BH_4 as a cofactor. (Lotsch et al., 2010)

GCH1 was named as a "key modulator of peripheral neuropathic and inflammatory pain" by Tegeder and co-workers (2006). Expression of *GCH1* is upregulated in sensory neurons following peripheral nerve injury (Lotsch et al., 2007) which leads to an increased synthesis of BH_4. Increased transcription of *GCH1* in the DRG has been associated with the development and persistency of pain (Kim et al., 2009). Additionally, increased concentrations of BH_4 have been linked to peripheral neuropathic and inflammatory pain (Burton, 2006), possibly due to an increase in nitric oxide synthesis (Foulkes and Wood, 2008).

Experiments conducted by Tegeder et al. (2006) in a spared nerve injury peripheral neuropathic pain rat model revealed that inhibition of BH_4 synthesis lead to a reduction in neuropathic pain; and that BH_4 induces neuropathic pain hypersensitivity, as demonstrated by the intrathecal injection of an active enantiomer of BH_4 (Tegeder et al., 2006). Down-regulation of *GCH1* could therefore be an effective means of pain relief (Kim et al., 2009).

GCH1 polymorphisms and differences in pain perception

GCH1 is highly polymorphic (Doehring et al., 2008, Thony and Blau, 2006), with 1222 SNPs having been identified in the human gene (http://www.ncbi.nlm.nih.gov/SNP). An assessment

by Tegeder and co-workers (2006), in 168 Caucasian individuals, of 15 SNPs located in non-coding and non-splicing regions and evenly spaced in the *GCH1* gene revealed a particular haplotype that was associated with decreased pain and improved outcome following a discectomy for lower back pain (**Figure 1.5**). This haplotype - termed the "pain-protective" haplotype - had a frequency of 15.4% in the studied group and five of the 15 SNPs were significantly associated with low pain scores for leg pain (Tegeder et al., 2006). It is believed that SNPs located in non-coding regions are likely to affect the transcription of *GCH1* (Lotsch et al., 2007). In this case, the haplotype may have altered *GCH1* mRNA and protein stability and caused a reduction in *GCH1* upregulation (Tegeder et al., 2006).

In a separate assessment of experimental pain in healthy individuals, Tegeder et al. (2006) found that those individuals homozygous for the haplotype appeared to be less sensitive to mechanical, heat and ischemic pain (Tegeder et al., 2006). Foulkes et al. (2008) later reported that individuals homozygous and heterozygous for the haplotype showed a reduced *GCH1* upregulation in response to cyclic adenosine monophosphate (cAMP).

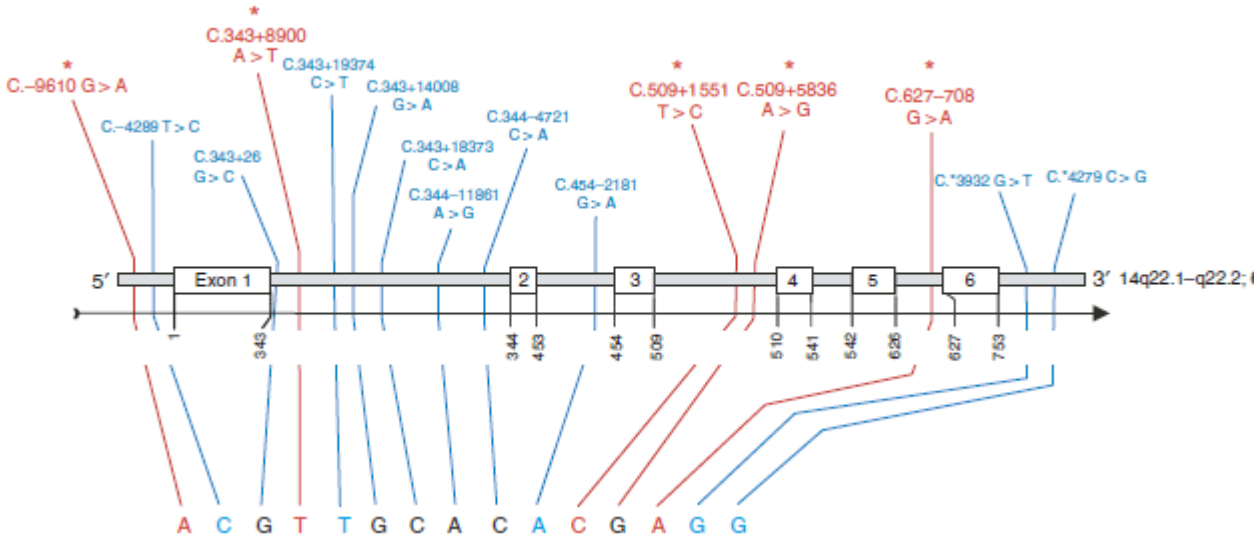

Figure 1.5. Locations of the 15 SNPs of the "pain-protective" haplotype on the coding DNA strand of *GCH1*. *[The 5 SNPs significantly associated with low pain scores are indicated in red. The common "pain-protective" haplotype which corresponded to the lowest leg pain scores is indicated.]* (Modified from Tegeder et al., 2006)

In a second study by Tegeder et al. (2008), they were able to verify the effect of the "pain-protective" haplotype and further assess the function of the haplotype at a biochemical level. They did this by demonstrating a reduction in GCH1 upregulation and BH_4 production. They also suggested that the haplotype only causes this when the GCH1 system is stimulated in some way (e.g., following injury) and when not stimulated has little effect (Tegeder et al., 2008).

In a study in 2007, in Caucasian individuals, it was reported that the "pain-protective" haplotype could be defined by three SNPs alone - rs8007267, rs3783641 and rs10483639 - due to the LD between SNPs in *GCH1* (Lotsch et al., 2007). In a later study, they reported that individuals who are homozygous for these SNPs took a lot longer to develop cancer-related pain (Lotsch et al., 2010).

Subsequent studies also revealed associations between polymorphisms in *GCH1* and pain. Three of the five SNPs significantly associated with lower pain scores in the study by Tegeder et al. (2006) were also associated with lower pain scores when 10% capsaicin was applied to the skin of healthy individuals of European American, African American and Asian American ethnicity (Campbell et al., 2009). In combination, these three SNPs accounted for 35% of the variance in pain scores (Campbell et al., 2009). Kim et al. (2010) reported that the T allele of SNP rs998259, part of the "pain-protective" haplotype, and a particular 12-SNP haplotype associated with an improved outcome following lumbar degenerative disc disease surgery.

Despite several positive findings, Kim and Dionne (2007) could not replicate the association with SNPs in *GCH1* in an assessment of clinical as well as thermal and cold pain in a sample of healthy individuals and individuals undergoing dental surgery, respectively. The sample

consisted of individuals of several ethnicities, namely European Americans, African Americans, Hispanics and Asian Americans and each group was analyzed separately. Kim and Dionne (2007) suggested that the difference in the results could be due to the different samples used and different methods of interpretation and analysis – they suggested that the study by Tegeder et al. (2006) may have used a mixed population instead of the European population that was reported. Similarly, Lazarev et al. (2008) found no association between the presence of the "pain-protective" haplotype and the occurrence or severity of recurrent acute pancreatitis and chronic pancreatitis.

A recent study, and the first in an indigenous African population, in HIV-positive individuals with HIV-SN investigated the association between six SNPs from the "pain-protective" haplotype and pain susceptibility and intensity (Wadley et al., 2012). No significant association was found between the SNPs individually and three- and six-SNP haplotypes and pain susceptibility and pain intensity after correcting for age, gender and CD4 T-cell count (Wadley et al., 2012). It was suggested that the role of *GCH1* could not be ruled out entirely, as the SNPs investigated were only those identified in a previous study in Caucasians, and that a more detailed study needs to be carried out using population-specific tagSNPs (Wadley et al., 2012).

Link with "non-pain" conditions

GCH1 has also been seen to be associated with "non-pain" conditions. Mutations in the coding and splicing regions of *GCH1* have been associated with neurological disorders such as 3,4-dihydroxyphenylalanine (DOPA)-responsive dystonia (a condition characterized by abnormal muscle tone resulting in muscular spasm and abnormal posture) and atypical

phenylketonuria (an autosomal recessive disorder characterised by the inability to metabolize phenylalanine, which results in brain and nerve damage if left untreated), which result from a deficiency of neurotransmitters (Blau et al., 2001, Ichinose et al., 1994) or accumulation of neurotoxic metabolites (Niederwieser et al., 1986) due to a lack of BH_4, respectively. Additionally, the T allele of rs841, a SNP in the non-coding region, was reportedly associated with cardiovascular conditions, namely increased heart rate and blood pressure (Zhang et al., 2007b). Doehring et al. (2008) stated that individuals who carry this particular SNP are also likely to carry the "pain-protective" haplotype as both are associated with similar changes in the amount of BH_4 and rs841 is in strong LD with the three SNPs (Lotsch et al., 2007) believed to define the "pain-protective" haplotype.

- ***KCNS1***

Prior to the identification by Costigan et al. (2010) of the potassium voltage-gated channel subfamily S member 1 gene *(KCNS1)* as a "pain gene", the gene had not previously been studied in the context of pain. *KCNS1* is a gene encoding a potassium ion channel subunit which is involved in excitation in sensory neurons. It was selected for genetic association studies based on an investigation of gene expression profiles in three different rodent nerve injury models (Costigan et al., 2010). A total of 124 genes were found to be co-regulated in all three models. Ten of these genes were analysed further as they were believed to be involved in neurotransmission and excitability in the neurons (Costigan et al., 2010). *KCNS1* fell into this group and was identified as being expressed in the DRG of sensory neurons under normal conditions, but was downregulated following nerve injury (Costigan et al., 2010). *KCNS1* was of particular interest among the ten genes as it was seen to be downregulated the most in comparison to the other genes (Costigan et al., 2010).

An assessment of 7 SNPs in a 15kb region encompassing *KCNS1* revealed a particular SNP (rs734784) associated with pain sensitivity in individuals with chronic pain in five of six independent non-African cohorts investigated. The rs734784 polymorphism, which is a missense mutation resulting in substitution of Valine for Isoleucine, was found to be present in patients with greater lumbar back pain, radicular pain, and pain following limb amputation (Costigan et al., 2010). In addition, the polymorphism was associated with greater sensitivity to experimentally applied painful stimuli in healthy individuals (Costigan et al., 2010).

1.2.2.2 Other cytokines and chemokines

- ***IL1B***

Wolf et al. (2006) cited several studies suggesting that IL-1, secreted by spinal glial cells, is believed to play a role in the development and maintenance of neuropathic pain . Elevated levels of IL-1 have been reported following peripheral nerve injury. Wolf and colleagues demonstrated the role of IL-1 in neuropathic pain in mice with impaired IL-1 signaling. Compared to wild-type mice who developed mechanical allodynia, thermal hyperalgesia and ectopic neuronal activity in the DRG, the knockout and transgenic mice (expressing an excess of IL-1 receptor antagonist) only presented with minor neuropathic symptoms, following peripheral nerve injury. In addition, the impaired mice exhibited a decrease in pain sensitivity. Previous studies have shown that peripheral or central administration of IL-1β leads to the development of hyperalgesia (Ferreira et al., 1988, Oka et al., 1995, Watkins et al., 1994) and spinal administration leads to the development of mechanical allodynia and thermal hyperalgesia (Falchi et al., 2001, Reeve et al., 2000, Tadano et al., 1999). In addition, other studies have shown that blockage of IL-1 at the spinal level prevents the development

of hyperalgesia (Milligan et al., 2001, Samad et al., 2001, Watkins et al., 1997, Watkins et al., 1994).

Additional studies have confirmed the role of IL-1 in neuropathic pain development and maintenance. Following partial sciatic nerve ligation in mice, an upregulation in the expression of the IL-1β precursor protein and the IL-1β mRNA was seen approximately 1-7 days following the ligation (Kiguchi et al., 2010).

The exact mechanism by which IL-1 is involved in neuropathic pain is unknown, although its action is thought to be mediated either through the p38 mitogen activated protein kinase (p38 MAPK) (Jin et al., 2003, Schafers et al., 2003, Tsuda et al., 2004) or nuclear factor-kappa B (NF-κB) (Bethea et al., 1998), which are activated following peripheral nerve injury . IL-1 causes the release of several other neuropathic pain mediators, including nitric oxide, bradykinin, prostaglandins or nerve growth factor (Wolf et al., 2006). IL-1β is able to act directly and indirectly on nociceptors (Moalem and Tracey, 2006).

- ***IL6***

IL-6 is absent from the peripheral nervous system in adults, but is upregulated in neurons and Schwann cells following nerve injury (McMahon et al., 2005). A possible mechanism by which IL-6 acts could be via upregulating brain-derived neurotrophic factor (BDNF) (Murphy et al., 2000), galanin and substance P (Sun and Zigmond, 1996). These molecules are all involved in sensory processing (McMahon et al., 2005).

- ***CCL2* and *CCR2***

Chemokines, a subfamily of cytokines which are chemotactic for leukocytes (Gao and Ji, 2010), are also upregulated and released following nerve injury (White and Miller, 2010). Chemokines are important for the recruitment and migration of leukocytes to the site of injury (Moalem and Tracey, 2006). Chemokines are believed to be expressed and secreted by many types of cells in the central nervous system, including microglia, astrocytes, endothelial cells and neurons (Gao and Ji, 2010). Some chemokines are constitutively expressed, although most are upregulated during pathological or neurodegenerative disease conditions (Gao and Ji, 2010).

Upregulation of chemokines following nerve injury contributes to the development and maintenance of chronic and acute pain conditions (White and Miller, 2010). Chemokines increase pain sensitivity or produce spontaneous pain by directly activating nociceptors or modifying their response to other algogenic substances (Marchand et al., 2005, Scholz and Woolf, 2002). They induce the migration of astrocytes and immune cells, such as leukocytes, as well as the proliferation of microglial cells (Abbadie, 2005). It has also been suggested that chemokines are directly involved in hypernociception (increased response to potentially noxious stimuli) in an animal model of HIV-induced hypernociception (Oh et al., 2001).

Chemokines cause a change in expression of genes in sensory neurons which leads to altered electrical activity and the development of neuropathic pain (McMahon et al., 2005). Chemokines and their receptors also seem to play a role in the pathogenesis of HIV and alterations in the expression of chemokines or chemokine receptors due to polymorphisms

within the genes have been linked to differences in disease progression related to HIV (Michael, 1999, Paxton and Kang, 1998).

A particular chemokine (CCL2) and its receptor (CCR2) have been implicated in neuropathic pain. Both are absent in the DRG neurons under normal conditions, but, following peripheral nerve injury, are unregulated in the DRG, in the central nervous system and at the site of injury (Abbadie, 2005, Jung and Miller, 2008). CCL2 plays a role in the recruitment of macrophages and microglia to the site of injury (McMahon et al., 2005, Thacker et al., 2009). The microglia then release several mediators involved in neuropathic pain (Thacker et al., 2009). Activation of CCR2 by CCL2 has also been linked with enhanced sensitivity to pain (Abbadie et al., 2009, Jung et al., 2009). Proof for the role of CCL2 and CCR2 in neuropathic pain has been demonstrated in several studies. A lack of CCR2 in mice correlated to decreased mechanical allodynia (Abbadie et al., 2003, Zhang et al., 2007a). Injection of a CCR2 antagonist into the spinal cord reduced mechanical allodynia associated with neuropathic pain (Bhangoo et al., 2007). A similar finding was reported when injecting CCL2-neutralizing antibodies into the spine (Gao et al., 2009, Thacker et al., 2009).

In addition to the role of CCL2 and CCR2 in neuropathic pain, the chemokine and its receptor have been linked to the development of HIV-1 (Michael, 1999, Paxton and Kang, 1998).

1.3 Significance of the study

Assessment of the genetic structure across different populations has shown that African individuals are genetically more diverse than individuals from non-African populations

(Campbell and Tishkoff, 2010) and that the level of genetic diversity is greatly decreased as one moves further away from Africa (Tishkoff et al., 2009). This is due to the founder effect or bottleneck event which occurs as groups leave Africa taking only a fraction of the genetic variation along with them (Tishkoff and Williams, 2002). Genetic diversity can be observed in the LD and haplotype structure. The pattern and level of LD is different among populations (Tishkoff and Verrelli, 2003), with African populations having lower levels of LD (Campbell and Tishkoff, 2008). This is due to the fact that recombination over time causes the LD structure to deteriorate (Tishkoff and Verrelli, 2003). As modern humans originated in Africa over 150 000 years ago and only started to migrate to other regions about 60 000 years ago, the African population is older than other continental populations. They have also not been as affected by population bottlenecks (a reduction in the variation of the genepool due to a reduction in the population size) as recently as other ethnic groups and there has been more time for this recombination to occur (Campbell and Tishkoff, 2008). There is also a resultant difference in haplotype block structure among populations. Whereas in non-African populations the haplotypes extend over a much longer genomic distance, haplotype blocks in Africans are considerably shorter and less uniform (Tishkoff and Verrelli, 2003, Campbell and Tishkoff, 2008). Studies have also suggested that there is great genetic diversity between subgroups within the African population itself (Tishkoff et al., 2009).

Despite the clear genetic differences among populations, genetic association studies have focused mainly on Caucasian, Asian or mixed populations and very few have studied native/indigenous African populations. Campbell and Tishkoff (2008) stated that there has in fact been an under-representation of Africans in genetic studies. Genetic association studies involving African individuals require a very different study design, with more specific and a larger density of SNPs required to obtain the same amount of haplotype coverage (Tishkoff

and Verrelli, 2003, Campbell and Tishkoff, 2008). Genetic association studies may involve the identification of either a SNP that has a direct association with the phenotype in question (i.e. it is the causal variant) or a SNP that has an indirect association and is in LD with the causal variant (Lewis and Knight, 2012). Due to the differences in LD structure between African and other populations and the fact that different SNPs may be in LD with the causal SNP in the different populations, markers identified in other populations as being associated with disease susceptibility may in fact have no association in an African population. This outlines the importance of carrying out genetic association studies in a diversity of populations, as is the case in this study where an African sample is investigated.

In addition, there are currently no treatment options available for the regeneration of neurons in HIV-SN, with current treatment options only slowing disease progression or offering symptom control (Kallianpur and Hulgan, 2009, Phillips et al., 2010). The main form of treatment is removal of the drug causing the neural damage (Verma and Simpson, 2007), although other options include the selection of a less toxic drug (Laurent et al., 2008, Moyle, 2000) or the reduction in the dose of the drug administered to the patient (Hill et al., 2007). The removal of the neurotoxic drug or the selection of a less toxic drug is not always possible though, especially in resource-limited settings where the less toxic antiretrovirals are not readily available (Kallianpur and Hulgan, 2009).

Identifying individuals who are at a greater risk for developing HIV-SN and pain associated with the neuropathy can result in these individuals being administered less toxic antiretrovirals in the future. These individuals can be identified by undertaking research into the genetics of the susceptibility to developing HIV-SN and pain and the variability in pain intensity in individuals with HIV-SN. Not only will this research allow us to identify the

"high-risk" individuals, but, more immediately, it will also allow us to gain a better understanding of the biology of this significant cause of long-term morbidity in HIV-positive South Africans, providing a basis for further research on possible treatment targets. Research of this nature will hopefully ultimately allow for an improvement in the overall well-being of these HIV-positive individuals and a reduction in the incidence of HIV-SN.

1.4 Research question, aims and objectives of study

Studies into the genetics of HIV-SN susceptibility and pain susceptibility and intensity have already been conducted in several non-African cohorts. An important question to ask is "Are the same genes and polymorphisms that are involved in HIV-SN or neuropathic pain and associated with HIV-SN susceptibility and pain susceptibility and intensity in non-African populations, also associated with HIV-SN susceptibility and neuropathic pain susceptibility and intensity in African individuals?"

The current study aimed to address this question by investigating:

1. The role of genetics in the susceptibility for HIV-SN in HIV-positive individuals of black Southern African ancestry.
2. The role of genetics in the variability of pain susceptibility and intensity in HIV-positive individuals of black Southern African ancestry with HIV-SN.

These aims have been met by:

1. Identifying, through literature search, candidate genes and SNPs or haplotypes within genes that have previously been involved in HIV-SN and neuropathic pain and linked to susceptibility for HIV-SN as well as with variability in pain susceptibility and intensity.
2. Identifying tagSNPs within these candidate genes that were representative for an African population, to ensure that all known common polymorphisms were either directly assayed or are associated with a tagSNP.
3. Genotyping the selected SNPs using a VeraCode assay on the Illumina BeadXpress platform.
4. Statistically assessing the association of genotypes with neuropathy development and pain susceptibility and intensity.

All materials and methods are presented in one chapter (Chapter 2). The results and relevant discussions are presented over three chapters with each chapter containing the results and discussion of specific genes. Chapter 3 is an investigation into the *TNFA* region and *IL12B* and involves HIV-SN susceptibility and pain analyses. Chapter 4 is an investigation into *GCH1* and *KCNS1* and involves only pain analyses. Chapter 5 is an investigation into *IL1B*, *IL6*, *CCL2* and *CCR2* and also involves only pain analyses. General conclusions are presented in Chapter 6.

CHAPTER 2

MATERIALS AND METHODS

2.1 Population and DNA samples

Blood samples were collected and DNA extracted by Mrs A Wadley (Brain Function Research Group, School of Physiology, University of the Witwatersrand) as part of her PhD study (Wadley, 2013) (Human Research Ethics Committee (Medical), University of the Witwatersrand, protocol no. M080220). Written informed consent, for the participation in studies investigating the predictors (genetic and non-genetic) of HIV-SN risk, was obtained from all participants prior to sampling. All samples were obtained from a convenience sample of black Southern Africans of 18 years of age or older recruited from the Virology Clinic of the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), South Africa. All participants had a confirmed HIV infection and had been on ARV therapy for at least six months, and had been exposed to stavudine. Ethical approval for the current genetic study was also obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (protocol no. M110754) (**Appendix A**).

DNA was extracted using a salting-out method (Miller et al., 1988) and was stored in the Division of Human Genetics Biobank. Absorbance-values at 260nm were measured and concentration calculated using the Tecan Infinite® 200 PRO NanoQuant and Freedom EVO® (All details of equipment, kits and reagents used are in **Appendix B**). All samples were normalized to 50 $ng.\mu l^{-1}$ with 1 X Tris/EDTA (TE) buffer (1 mM EDTA in 10 mM Tris-Cl, pH 8.0).

All participants were assessed by Mrs A Wadley at the time of blood sample collection. Information collected included demographic information (age, sex and self-reported ethnicity); patients' heights and weights; disease information (current and nadir CD4 T-cell

counts, time since HIV diagnosis and AIDS-defining illnesses); ARV treatment history; and other possible causes of neuropathy (diabetes mellitus, alcoholism, vitamin B12 deficiency and exposure to isoniazid and chemotherapy) (Wadley et al., 2011). Participants were screened for neuropathy using the AIDS Clinical Trials Group neuropathy screening tool (Cherry et al., 2005); a tool validated for the screening of HIV-associated sensory neuropathy (HIV-SN). Individuals were recorded as having symptomatic HIV-SN if they had bilateral presence of at least one symptom (pain, aching, burning, numbness or pins-and-needles) and at least one sign (reduced vibration sense or absent ankle reflexes) of neuropathy (Wadley et al., 2011). The distribution and intensity of pain was recorded in those individuals experiencing the symptom. Pain intensity was rated on an 11-point numeric pain rating scale where '0' represents no pain and '10' represents the worst pain possible. Mild pain was classified as a pain score of 1 to 3; moderate, 4 to 6; and severe, 7 to 10.

The total number of individuals initially assessed for possible inclusion in the study was 482. Of these, only 404 were recruited. A further 64 individuals were excluded from this cohort of 404, so the final number of individuals used in the genetic analysis was 340. Details of the reasons for exclusion and the numbers excluded are provided in **Figure 2.1**. Of the 340 participants, 56% (n = 189) were clinically diagnosed with HIV-SN; those individuals not clinically diagnosed with HIV-SN served as controls (n = 151) for my investigation into susceptibility to developing the neuropathy. Of those diagnosed with HIV-SN, 92% (n = 174) reported pain as a symptom. Of the 174 individuals, 31 reported that they already had pain prior to diagnosis – these individuals were excluded from all pain analyses. Those individuals clinically diagnosed with HIV-SN, but not experiencing pain served as controls for my investigation into pain susceptibility (n = 15). Descriptive statistics for the cohorts used in this study are provided in **Table 2.1**.

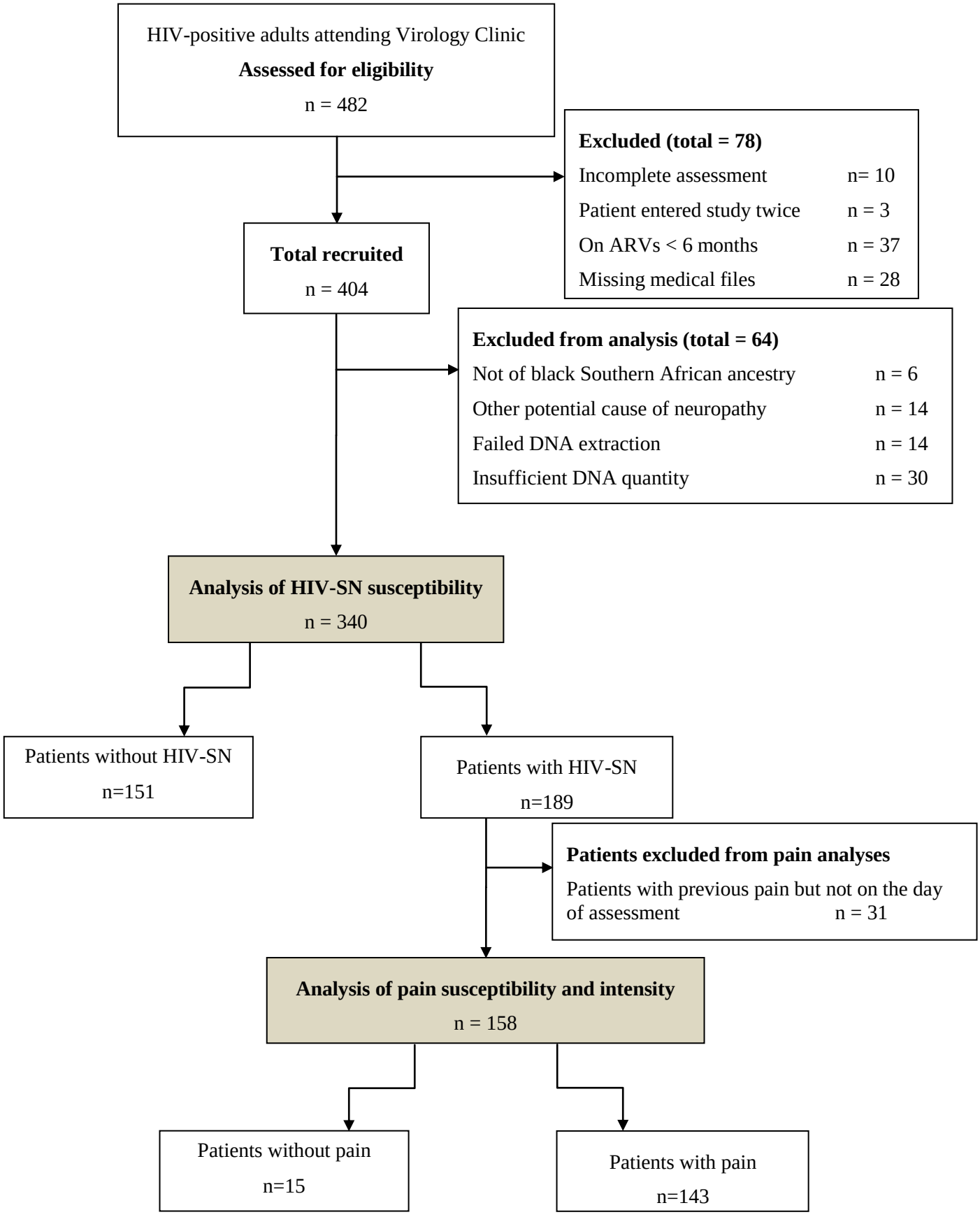

Figure 2.1. Patient recruitment and exclusion diagram.

Table 2.1. Sample descriptive statistics

	HIV-SN analysis individuals (n=340)	Pain analysis individuals (n=158)
Pain Score Mean (SD)	-	5.2 (3.2)
Females %	75.0	77.8
Age (years) Mean (SD)	38.9 (7.9)	40.9 (8.1)
Height (cm) Mean (SD)	158.9 (8.2)	159.6 (8.3)
CD4 T-cell Count (cells.mm^{-3}) Median (IQR)	390.0 (279-552)	400.0 (291-557)

2.2 Candidate gene and SNP selection

Candidate genes (*TNFA* and surrounding genes, *IL12B*, *GCH1*, *KCNS1*, *IL1B*, *IL6*, *CCL2* and *CCR2*) were identified following a literature review. SNP selection in these genes was based on those SNPs identified in the literature to have been previously associated with HIV-SN or pain, and supplemented with tagSNPs appropriate for an African population. SNPs are usually perfectly correlated to several nearby SNPs. Because of this, testing one SNP will produce the same outcome as testing the SNP(s) that it is perfectly correlated to. tagSNPs are those SNPs which are genotyped in a study and allows for any redundancy to be eliminated (The International HapMap Consortium, 2005) .

As there is no dataset for South(ern) Africans currently available, use was made of the Yoruba in Ibadan, Nigeria (YRI) dataset, which is considered the best-matched dataset available, for tagSNP selection. The YRI and Southern African populations are both Bantu-

speaking populations. One would thus expect them to have similar genetic architecture, due to the recent expansion (5000 years) not allowing enough time for gross changes to have occurred (Tishkoff et al., 2009). However this stays an assumption until both populations have had their genetic structure studied in detail.

All SNP information available for the YRI population, from the International HapMap dataset (HapMap Data Release 27, Phase II+III, February 2009, on the National Center for Biotechnology Information (NCBI) B36 assembly, dbSNP b126) (The International HapMap Consortium, 2003), was scored and a final list of tagSNPs was selected using the software program Haploview (version4.2) (Barrett et al., 2005). Scoring and finalization of the SNP list involved an iterative process of submitting candidate SNPs to Illumina for assay design tool (ADT) evaluation (Illumina, Technical Note: DNA Analysis). SNPs were scored based on their expected assay design success. SNPs were excluded if they obtained a designability rank of 0.5 or less or a failure code. Common failure codes included those indicating that a particular SNP was less than 61 nucleotides away from another SNP on the list (a prerequisite for the allele-specific probe binding nature of the protocol); or that degenerate nucleotide(s) were present in the assay design region. The tagSNPs were selected using a pairwise approach at $r^2 = 1.0$ and with minor allele frequency (MAF) > 0.01. All alleles are designated respective to the forward (+) strand of the genome assembly, and were obtained using BioMart (Ensembl release 67, May 2012) (Flicek et al., 2012, Kinsella et al., 2011).

Ancestry informative markers (AIMs), used to assess population substructure and control for admixture, were not included in the set of SNPs to be genotyped. In a previous study using the Birth to Twenty (Bt20) cohort (a Sowetan population similar to the sample used in the current study) (Richter et al., 2007), population substructure was assessed using 18 AIMs and

no significant admixture with European or Asian populations was found (Lombard et al., 2012). A significantly larger number of AIMs (93 in total) would be required to assess admixture between African groups, due to the similarity between the groups (Nassir et al., 2009), but this falls outside the scope of the current study and was therefore not carried out.

2.3 Genotyping

2.3.1 Genotyping using the BeadXpress system

Initial genotyping was carried out using a high-throughput genotyping method. A GoldenGate® genotyping assay with VeraCode® microbeads was employed and data was read on an Illumina BeadXpress® Reader (Lin et al., 2009). A total of 250 ng of each DNA sample (5 µl of 50 ng.µl^{-1} DNA) was used for BeadXpress genotyping.

2.3.1.1 Principles behind the assay

The assay allows for genotyping of up to 384 SNPs per DNA sample and displays a high consistency, reproducibility and success rate (Lin et al., 2009). In brief, genomic DNA is biotinylated and immobilized to streptavidin-coated paramagnetic beads. Two allele-specific and one locus-specific oligonucleotide primers are annealed to the biotinylated DNA which acts as a template for allele-specific extension and ligation reactions of the oligonucleotides. The reaction products are eluted from the immobilized genomic DNA and are amplified by universal polymerase chain reaction (PCR) using fluorophore-labeled universal primers. The non-fluorescent strand of the PCR product is removed to generate single-stranded DNA to which the VeraCode beads, each inscribed with a unique code, are hybridized via a specific

address sequence. The beads are scanned by the BeadXpress Reader, after which data is analyzed. (Lin et al., 2009) The process is outlined in **Figure 2.2**.

2.3.1.2 Preparation of BeadXpress machine before use

The system was initialized and the fluidics primed. The reagent bottles were filled with their respective solutions (double-distilled, deionised water (ddH_2O); absolute ethanol and 1 X v/v VeraCode Read buffer (200 ml 10 X VeraCode Read Buffer in 1800 ml ddH_2O)). The bead cell was reconditioned in a solution of 10% w/v potassium hydroxide (KOH) (50 g KOH pellets in 500 ml ddH_2O). A test and calibration strip was run to ensure the instrument was operating to correct specifications.

2.3.1.3 Pre-analysis data quality control

Following scanning of the plate, data was loaded into BeadStudio Data Analysis Software from Illumina, Inc (Version 3.1.3.0, genotyping module version 3.2.32). Samples with a zero or lower (<0.9) call rate were excluded. Internal control primers that assess each step in the protocol (which include allele specific extension, PCR uniformity, extension gap, first hybridization and second hybridization) are included in the assay (**Figure 2.3**). Samples that failed generally two or more controls were excluded. All samples were also assessed for contamination (**Figure 2.4**). SNPs were excluded if the SNP graph scatter plots indicated poor separation, poor clustering or low intensity (**Figure 2.5**).

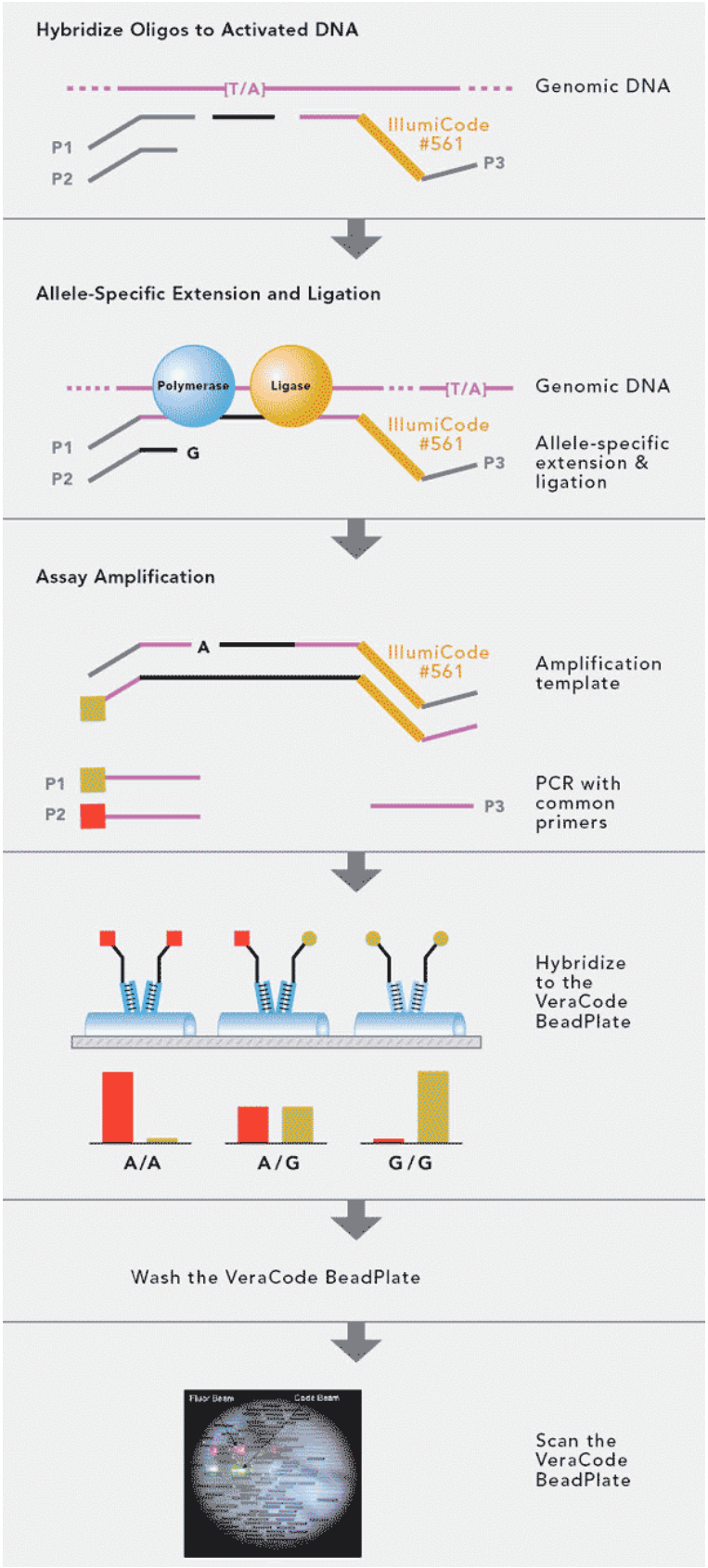

Figure 2.2. VeraCode GoldenGate assay process.
(http://www.illumina.com/technology/veracode_goldengate_assay.ilmn)

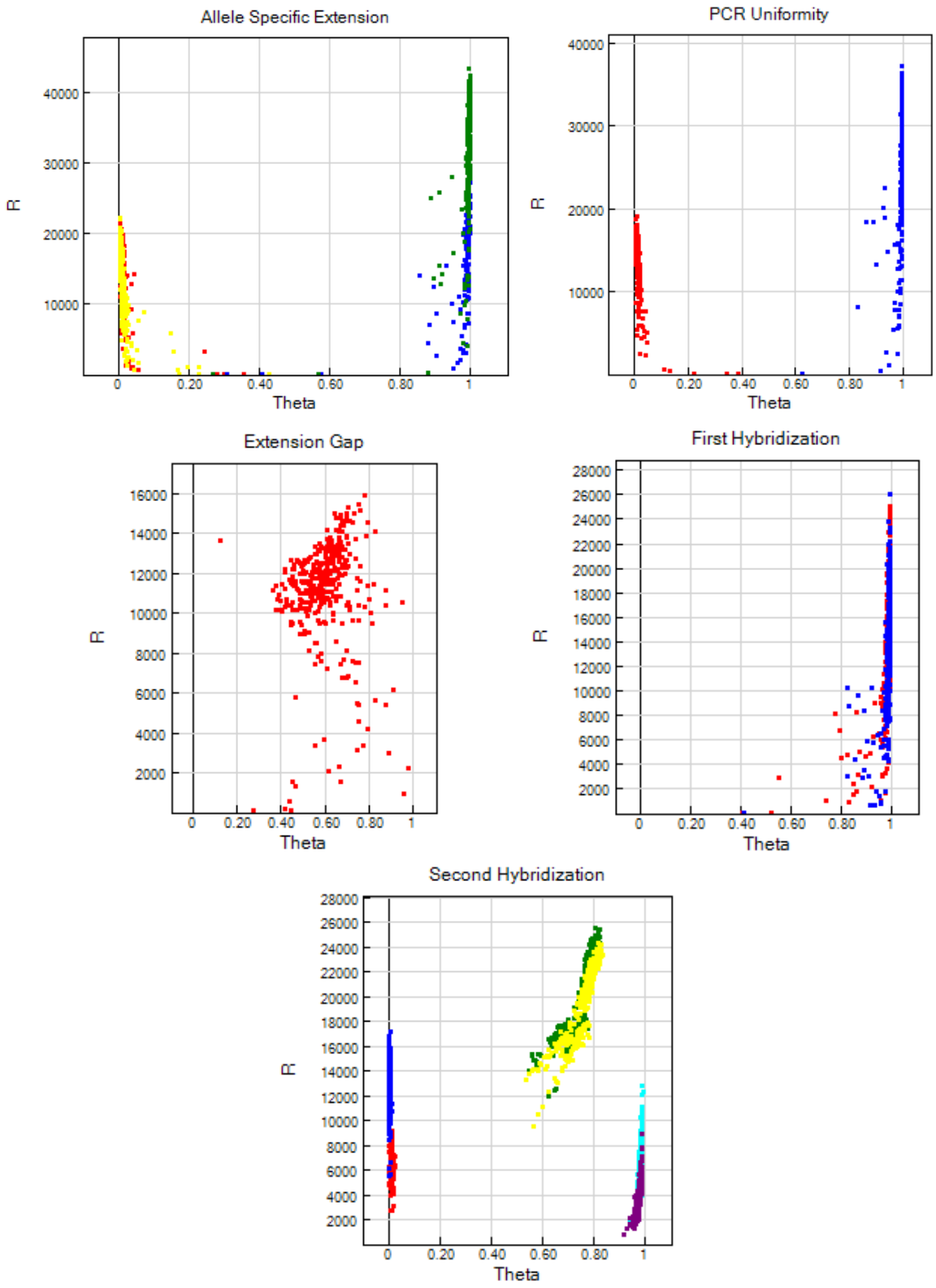

Figure 2.3. BeadStudio control graphs. *[Samples with theta values between 0.2 and 0.8 for the allele specific extension and PCR uniformity should be considered for exclusion. Samples with R less than 1000 for the extension gap and first and second hybridizations should be considered for exclusion.]*

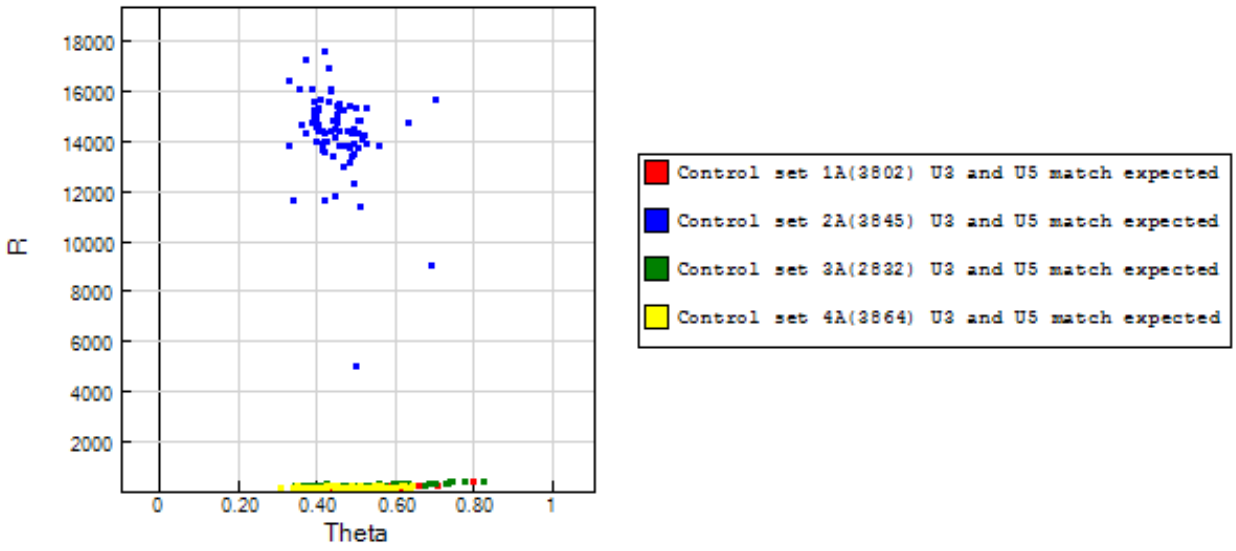

Figure 2.4. BeadStudio contamination graph. *[One coloured cluster amplified at a time indicates no contamination.]*

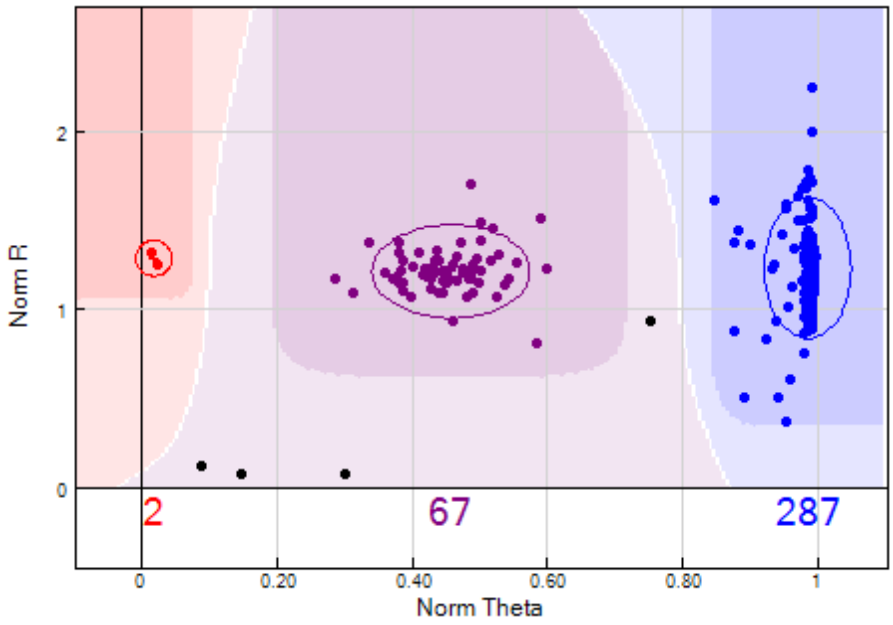

Figure 2.5. BeadStudio SNP graph. *[Samples (dots) are clustered according to their genotype. Genotypes are called according to their signal intensity (Norm R) and allele frequency (Norm Theta) relative to established cluster positions for a given SNP marker. Purple represents the heterozygous genotype and red and blue represent the two homozygous genotypes. Samples indicated in black have not been called as the genotype is uncertain.]*

2.3.2 Genotyping using a TaqMan® assay

One SNP, that failed genotyping using BeadXpress, was chosen to be re-genotyped by allelic discrimination using a TaqMan® SNP genotyping assay. The SNP was selected for re-genotyping as it is part of the *TNFA* region, an important region included in HIV-SN susceptibility analysis in this study. Four samples were sequenced prior to running the assay to obtain positive controls. The detailed sequencing protocol can be seen in **Appendix C**.

2.3.2.1 Sequencing

- **PCR and PCR clean-up**

Primers were designed using Primer3 (v 0.4.0) (Rozen and Skaletsky, 2000). Product size range was specified as 300-400 base pairs (bp) and all other conditions were left as default settings. The theoretical PCR result of the selected primers was examined using University of California, Santa Cruz (UCSC) In-Silico PCR of the UCSC Genome Browser (Assembly: Feb. 2009 (GRCh37/hg19)) (Kent et al., 2002). Primers were ordered from Inqaba Biotechnical Industries (Pty) Ltd and were resuspended in 1X TE buffer.

DNA was amplified according to the mastermix in **Table 2.2**.

Table 2.2. PCR mastermix

Component	[Stock]	[Required]	Volume (1x) (µl)
PCR Buffer	10 x	1 x	2.5
Primer – Forward	10 µM	0.4 µM	1
Primer – Reverse	10 µM	0.4 µM	1
deoxyribonucleoside triphosphates (dNTPs)	1.25 mM	125 µM each	2.5
magnesium chloride ($MgCl_{2)}$	25 mM	2.5 mM	2.5
Taq Polymerase (AmpliTaq GOLD)	5 $U.\mu l^{-1}$	0.04 $U.\mu l^{-1}$	0.2
DNA	50 $ng.\mu l^{-1}$	~10 ng-1 µg	1
dH_2O	-	-	14.3
		Total	25

The reaction was carried out in an Applied Biosystems 2720 Thermal Cycler under the conditions shown in **Figure 2.6**.

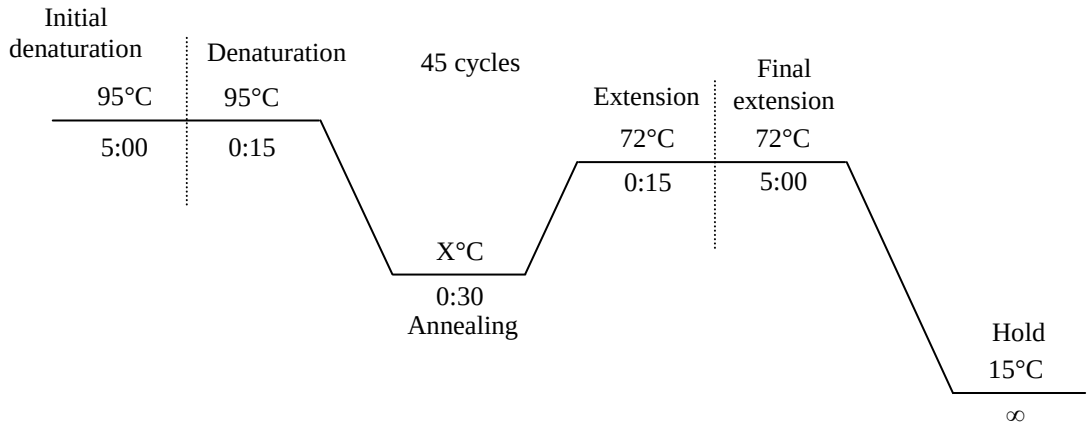

Figure 2.6. PCR thermal profile. *[The X refers to the annealing temperature specific to the primers used. In this case it is 58°C]*

The resultant PCR product was run on a 2% agarose gel to check if the DNA was amplified. The gel was viewed in a G:BOX Gel Documentation System using GeneSnap v6.08 software (Synoptics Ltd., Syngene, UK).

PCR clean-up was performed using a Nucleospin® Extract II Kit. This step eliminates excess dNTPs, Taq polymerase and primers from the reaction mixture.

- **Cycle sequencing PCR and cycle sequencing clean-up**

Cycle sequencing PCR was carried out using a BigDye® Terminator v3.1 Cycle Sequencing Kit. Separate forward and reverse reactions were set up according to the mastermix in **Table 2.3**.

Table 2.3. Cycle sequencing PCR mastermix

Component	[Stock]	Volume (1x) (µl)
Cleaned PCR product	-	2
BigDye v3.1	2.5 x	1
BigDye Sequencing Buffer	5 x	1.5
Primer (forward or reverse)	3 µM	1
ddH_2O	-	4.5
	Total	10

The reaction was carried out under the conditions shown in **Figure 2.7**.

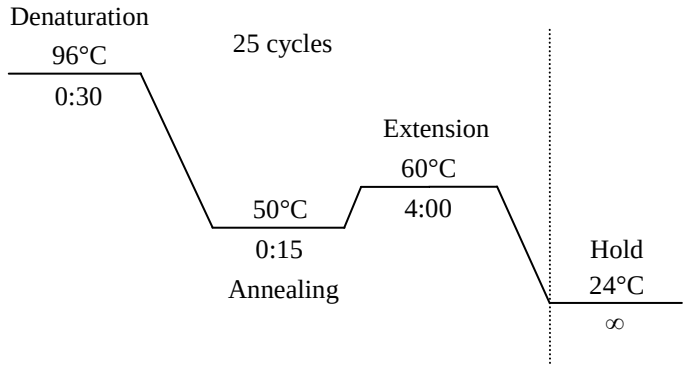

Figure 2.7. Cycle sequencing PCR thermal profile.

Cycle sequencing product clean-up was carried out using a DyeEx™ 2.0 Spin Kit. This step removes excess reagents before sequencing.

- **Sequencing**

The cleaned cycle sequencing products were resuspended in HiDye Formamide. Sequencing was performed on a 3130xl Genetic Analyzer. Data was collected using Applied Biosystems Data Collection Software v3.0 and base calling was performed using Applied Biosystems Sequencing Analysis Software v5.3.1. Sequence analysis was carried out using Applied Biosystems SeqScape® Software v2.5.

2.3.2.2 TaqMan® assay – allelic discrimination

- **Principles behind the assay**

The assay makes use of oligonucleotide probes with a reporter dye (VIC® or FAM™) attached to the 5' end and a quencher dye, with minor groove binder (MGB) technology, at the 3' end. Two probes are included in the PCR reaction mixture - one for each of the possible alleles at the SNP region - and are complementary to a short region encompassing the SNP. The probe specific to the allele that is present in the DNA anneals to the region between the forward and reverse primers during PCR. The polymerase performs its role of primer extension allowing replication of the DNA template. At the same time, 5' exonuclease activity of the polymerase leads to cleavage of the probe causing the reporter dye to be released and move away from the quencher, allowing a subsequent increase in the intensity of the fluorescence from the dye. The colour of the fluorescence seen corresponds to the dye that was present on the

original probe that bound to the DNA at the SNP location. (Premier Biosoft, Technical Note: TaqMan® Probes and Life Technologies, Product Bulletin, TaqMan® SNP Genotyping Assays) The mechanism behind the TaqMan® assay can be seen in **Figure 2.8**.

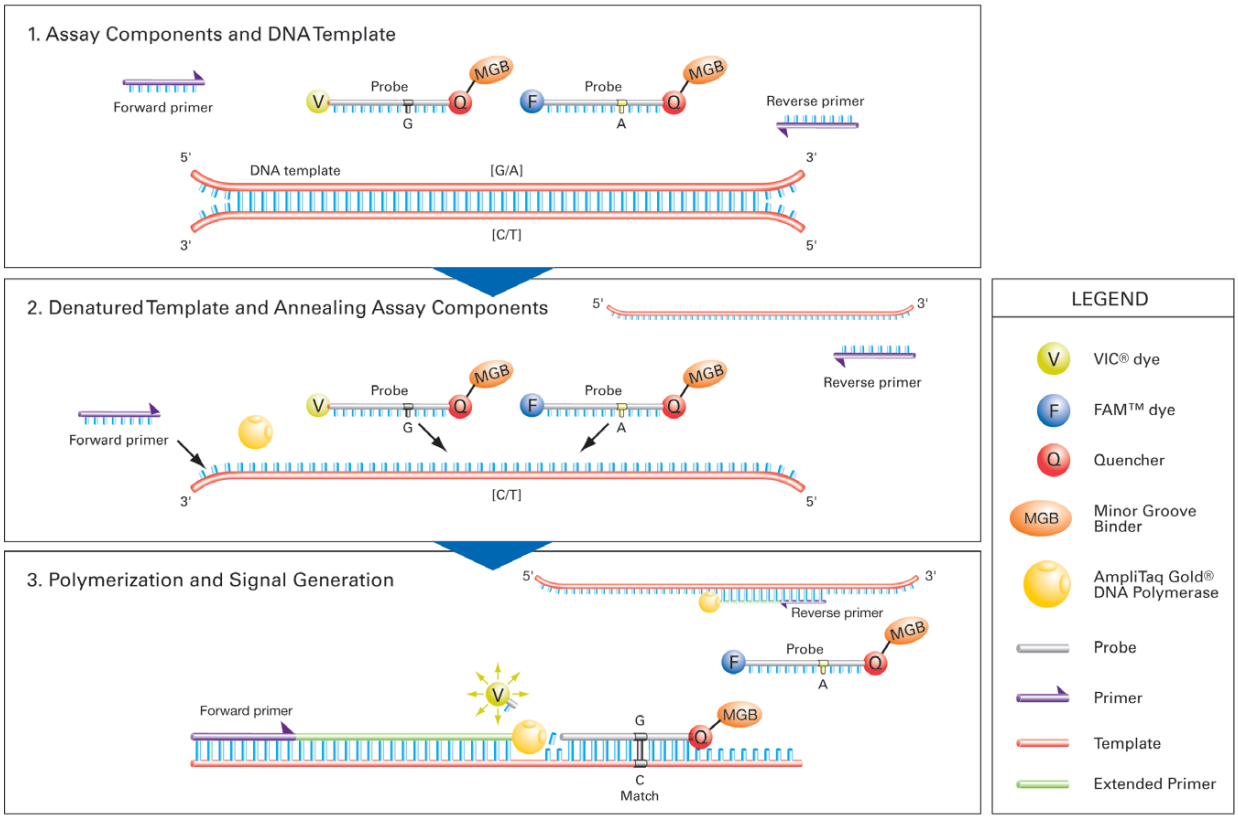

Figure 2.8. Mechanism behind the TaqMan® Assay (Life Technologies, Product Bulletin, TaqMan® SNP Genotyping Assays)

- **Protocol**

The DNA samples were diluted to 10 ng.µl^{-1} in 1 X TE buffer. Exact concentrations were ensured by quantifying the samples using a Quantifiler™ Human DNA Quantification Kit on a 7900HT Fast Real-Time PCR System. 384-well plates containing approximately 20 ng DNA per well were prepared by pipetting 2 µl of DNA into each well and allowing the samples to dry overnight by evaporation at room temperature.

5 µl of the mastermix in **Table 2.4** was added to each well.

Table 2.4. TaqMan® mastermix (384-well plate, dry-down DNA method)

Component	Volume (1x) (µl)
TaqMan Universal PCR Master Mix	2.5
20 x working stock of SNP genotyping assay	0.25
ddH_2O	2.25
Total	5

PCR amplification was performed on a 7900HT Fast Real-Time PCR System according to the standard protocol (**Figure 2.9**) after which an endpoint plate read was performed using Applied Biosystems Sequence Detection System (SDS) Software. Allelic discrimination was according to fluorescence values obtained during the read.

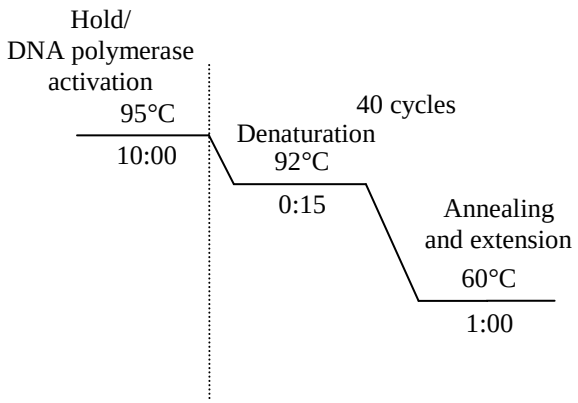

Figure 2.9. TaqMan® PCR amplification thermal profile.

2.4 Data analysis

Statistical analysis was performed using gPLINK software (version 2.050) for association analysis (Purcell et al., 2007).

2.4.1 Descriptive statistics

Analysis included basic descriptive statistics, including MAF and Hardy-Weinberg equilibrium (HWE). SNPs were assessed as having deviated from HWE, and were excluded from association analysis, if $p<0.01$ for the whole cohort (n=158) for the pain intensity analysis (a quantitative variable) and in controls only (unaffected individuals) for the HIV-SN susceptibility (n=151) and pain susceptibility (n=15) analyses (dichotomous variables) (Lunetta, 2008).

2.4.2 Single SNP association analysis (Clarke et al., 2011)

2.4.2.1 HIV-SN and pain susceptibility analysis

Univariate single SNP association analysis of HIV-SN and pain susceptibility involved performing chi-square tests using four models: allelic, genotypic, dominant and recessive. The models were all important for inclusion in analysis as they are standard models for disease penetrance (Clarke et al., 2011). The allelic model assessed the association between the presence of HIV-SN or pain and the alleles present (**Figure 2.10a**). The genotypic model assessed the association between the presence of HIV-SN or pain and the genotypes present (**Figure 2.10b**). The dominant model assessed the presence of HIV-SN or pain and having at least one minor allele versus not having any minor allele (**Figure 2.10c**). The recessive model assessed the presence of HIV-SN or pain and having two minor alleles versus having at least one major allele (**Figure 2.10d**). When there were five or less observations in a category, the Fisher's exact test (Freeman-Halton test for the 2x3 tables) was employed, as the chi-square test is not accurate in these cases.

(a)

		Allele	
		a	A
Presence of HIV-SN/Pain	Yes		
	No		

(b)

		Genotype		
		Aa	Aa	AA
Presence of HIV-SN/Pain	Yes			
	No			

(c)

		Genotype	
		Aa/ aa	AA
Presence of HIV-SN/Pain	Yes		
	No		

(d)

		Genotype	
		Aa/AA	Aa
Presence of HIV-SN/Pain	Yes		
	No		

Figure 2.10. Contingency tables of models used in single SNP association analysis for pain and HIV-SN susceptibility analyses. (a)Allelic model; (b)Genotypic model; (c)Dominant model; (d)Recessive model. *[A represents the dominant or major allele; a represents the recessive or minor allele; AA represents the homozygous dominant genotype, Aa represents the heterozygous genotype; and aa represents the homozygous recessive genotype]*

Multivariate analysis was performed using logistic regression. Age and height were found to be major risk factors for neuropathy in HIV patients treated with stavudine (Cherry et al., 2009). These two factors were thus corrected for in HIV-SN susceptibility multivariate analysis. Age, gender and CD4 T-cell count were chosen as factors to be corrected for in the pain susceptibility analysis to keep consistency with a recent study by Wadley et al. (2012) wherein *GCH1* was investigated for its role in painful HIV-SN in black Southern Africans. The factors were selected as they had previously been associated with risk of pain or pain intensity (Aouizerat et al., 2010, Dieleman et al., 2008).

2.4.2.2 Pain intensity analysis

Univariate single SNP association analysis of pain intensity involved performing a two-sample t-test to assess the association between the alleles present and reported pain intensity. Multivariate analysis correcting for age, gender and CD4 T-cell count was performed using linear regression. The rationale for the factors chosen are as stated above for the pain susceptibility analysis.

2.4.3 Haplotype association analysis

Haplotypes were constructed randomly or using the sliding window approach in gPLINK (to generate two- and three-SNP haplotypes along the gene), or were based on previously associated haplotypes.

Univariate and multivariate (correcting for the same covariates as in the single SNP association analyses) haplotype association tests were conducted using linear and logistic regression for the pain intensity and HIV-SN and pain susceptibility analyses, respectively.

2.4.4 Analysis strategy

Analysis was carried out according to the strategy outlined in **Figure 2.11**. A pointwise empirical p-value (P_{EMP1}; based on 1000 iterations) was calculated for all statistical comparisons. SNPs and haplotypes were only carried forward to multivariate analysis if $P_{EMP1}<0.05$. Significant findings were those with $P_{EMP1}<0.05$ at both univariate and multivariate levels. Family-wise correction for multiple comparisons (P_{EMP2}) was also done in each case and very significant findings were considered those with P_{EMP1} *and* $P_{EMP2}<0.05$. A MAF threshold of 0.01 was used throughout the analysis. BeadStudio SNP graph scatter plots were reviewed when a positive association was found, to ensure that findings weren't due to genotyping error. The effect (odds ratio (OR) or beta) presented in the results is with respect to the minor allele in the sample for individual SNP association analysis. An OR of greater than one indicates that the minor allele increases the risk of developing HIV-SN or pain relative to the major allele, and *vice versa*. A positive beta value indicates that the presence of the minor allele is associated with an increased risk of developing a higher pain intensity compared to if the major allele was present, and *vice versa.* For haplotype association analysis, the OR and beta values are with respect to the alleles indicated.

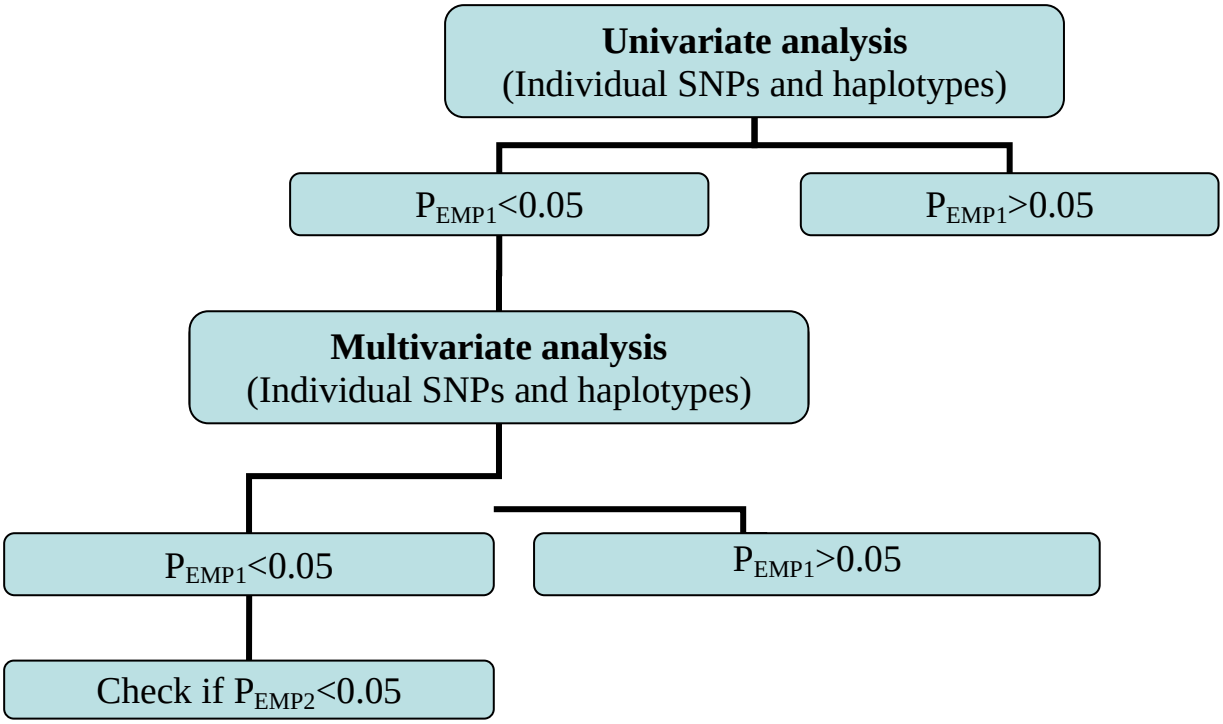

Figure 2.11. Data analysis strategy

2.5 Post-analysis assessment of significant findings

The allele frequencies of the healthy controls were compared to allele frequencies of three African populations (Yoruba in Ibadan, Nigeria (YRI), African ancestry in Southwest USA (ASW) and Luhya in Webuye, Kenya (LWK)) and one European population (Utah residents with Northern and Western European ancestry (CEU)) in SNPs that were found to be significantly associated with the phenotype in question. The allele frequencies for the African and European populations were calculated using SPSmart ENGINES (v5.1.1) (Amigo et al., 2008). The tagger function in Haploview (using settings of MAF<0.01 and r^2=1) was used to assess which SNPs are tagged by the significantly associated SNPs in the YRI and CEU populations. Significantly associated SNPs were inserted into RegulomeDB (Boyle et al., 2012), a database used to identify functional variants and the regulatory potential of variants, to look for any potential functional or regulatory role of the associated SNPs.

CHAPTER 3

SNPs in the *TNFA* region strongly associate with HIV-SN susceptibility, while *IL12B* associates with pain susceptibility

3.1 Background

As described in Chapter 1, polymorphisms in the *TNFA* and surrounding genes have been associated with the susceptibility to developing HIV-SN (Cherry et al., 2008, Chew et al., 2010). Similarly, a polymorphism was identified in the 3' untranslated region (IL12B (3'UTR)*2) which correlated with a decreased risk for developing HIV-SN (Cherry et al., 2008).

The objective of this section of the research was to carry out a fairly detailed investigation into *TNFA,* and some surrounding genes in chromosome six, as well as an investigation into *IL12B* in a black Southern African population. The main part of the analysis in both genes involved an investigation of whether the previously associated SNPs as well as population specific tagSNPs were associated with HIV-SN susceptibility in the black Southern African population. Analysis was also carried out on the association of the genes with pain susceptibility and pain intensity. The methodology used is described in detail in **Chapter 2**.

3.2 Results

3.2.1 *TNFA* region

The seven SNPs making up the haplotype found to be associated with HIV-SN in Asian and Caucasian individuals (Chew et al., 2010) were considered for inclusion in the *TNFA* region SNP list. Two of the SNPs (rs2523506 and rs3219186) failed ADT evaluation when developing the SNP list and were thus not included. The SNP previously identified as rs2857713 merged into rs2229094 and is thus now known by this SNP ID. The remaining

four SNPs (rs2251824, rs1799964, rs4248158 and rs1052248) passed ADT evaluation and were genotyped. tagSNP selection was carried out in a 60000 to 65000 bp region encompassing the genes which contain the previously associated SNPs as well as a few additional genes in the same region. tagSNP selection produced a further 42 SNPs for genotyping and analysis. Initial genotyping of three of the SNPs (rs3179003, rs2516478 and rs2523502) failed – the former was re-genotyped using the TaqMan® SNP genotyping assay and the latter two were excluded from analysis. Two SNPs (rs2239707 and rs3093982) were shown to be monomorphic after assessment of the BeadStudio SNP graphs and were thus excluded from analysis. Several SNPs (including one of the previously associated SNPs, rs4248158) were excluded from analysis as the minor allele frequency fell below the threshold of 0.01 (indicated in Table 3.1 with a superscript 'i'). The layout of the *TNFA* region referred to in this study is shown in **Figure 3.1**. The SNPs (and their relevant genes) included in the analysis (and those excluded due to MAF<0.01) are shown in **Figures 3.2a-d** (The region is broken up into four groups of genes for easier representation) with further details presented in **Table 3.1**.

For HIV-SN susceptibility analysis, two SNPs (rs2079170 and rs3093544) failed HWE and were thus excluded from analysis. For pain susceptibility analysis, all SNPs were in HWE and therefore no SNPs were excluded from analysis. For pain intensity analysis, two SNPs (rs2079170 and rs3093544) failed HWE and were excluded from analysis.

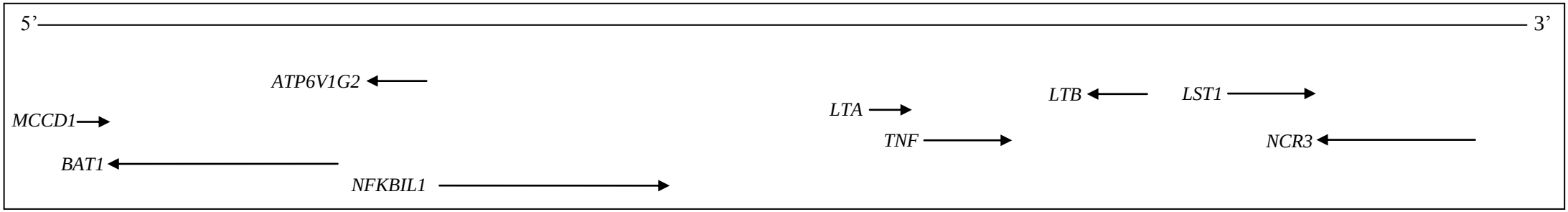

Figure 3.1. Layout of the *TNFA* region from 31496739bp (5’ end) to 31560762bp (3’ end) showing the approximate positions of the genes in the region relative to each other. *[MCCD1 - mitochondrial coiled-coil domain 1 gene; BAT1 - DEAD (Aspartic acid - Glutamic acid - Alanine - Aspartic acid) box polypeptide 39B; ATP6V1G2 - ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2 gene; NFKBIL1 - nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1 gene; LTA - lymphotoxin-alpha gene; TNF - tumour necrosis factor gene; LTB - lymphotoxin-beta gene; LST1 - leukocyte specific transcript 1 gene; NCR3 - natural cytotoxicity triggering receptor 3 gene]*

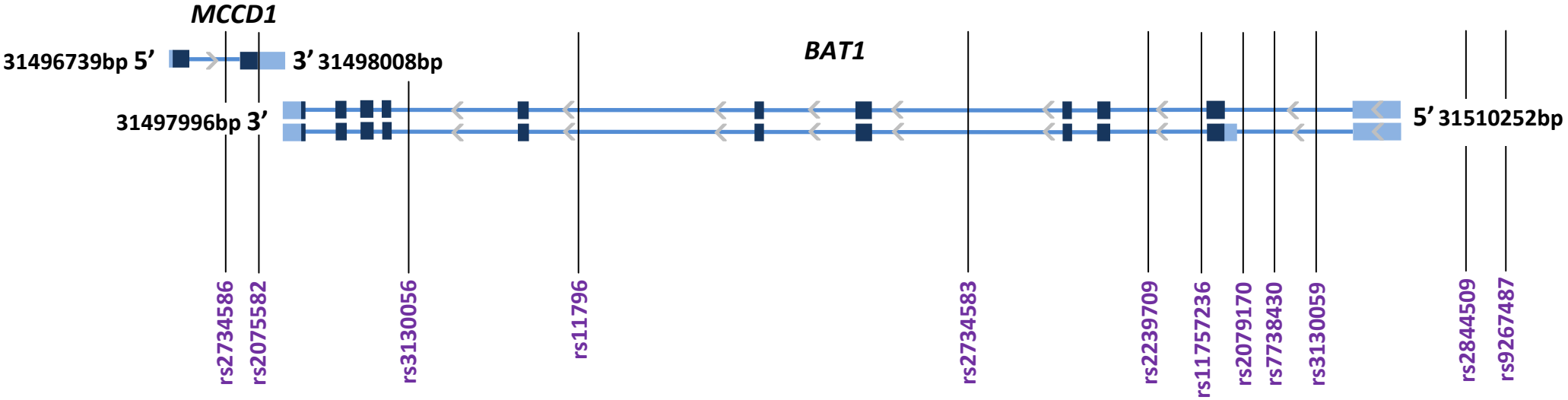

Figure 3.2a. *MCCD1* and *BAT1* gene diagrams of the *TNFA* region with the SNPs chosen for analysis annotated on the diagrams. *[SNPs in purple indicate the tagSNPs. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The National Center for Biotechnology Information (NCBI) Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

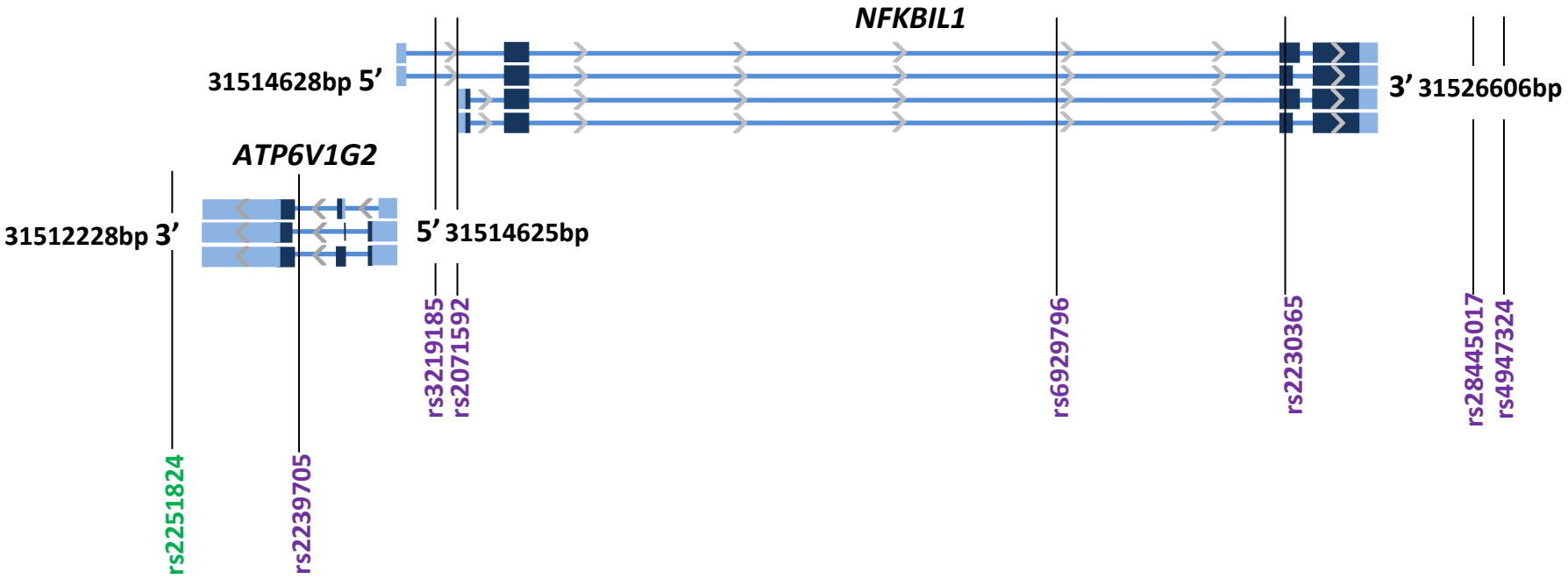

Figure 3.2b. *ATP6V1G2* and *NFKBIL1* gene diagrams of the *TNFA* region with the SNPs chosen for analysis annotated on the diagrams. *[SNPs in purple indicate the tagSNPs; and the SNP in green indicates a SNP that was both previously associated and resulted from tagSNP selection. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The National Center for Biotechnology Information (NCBI) Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

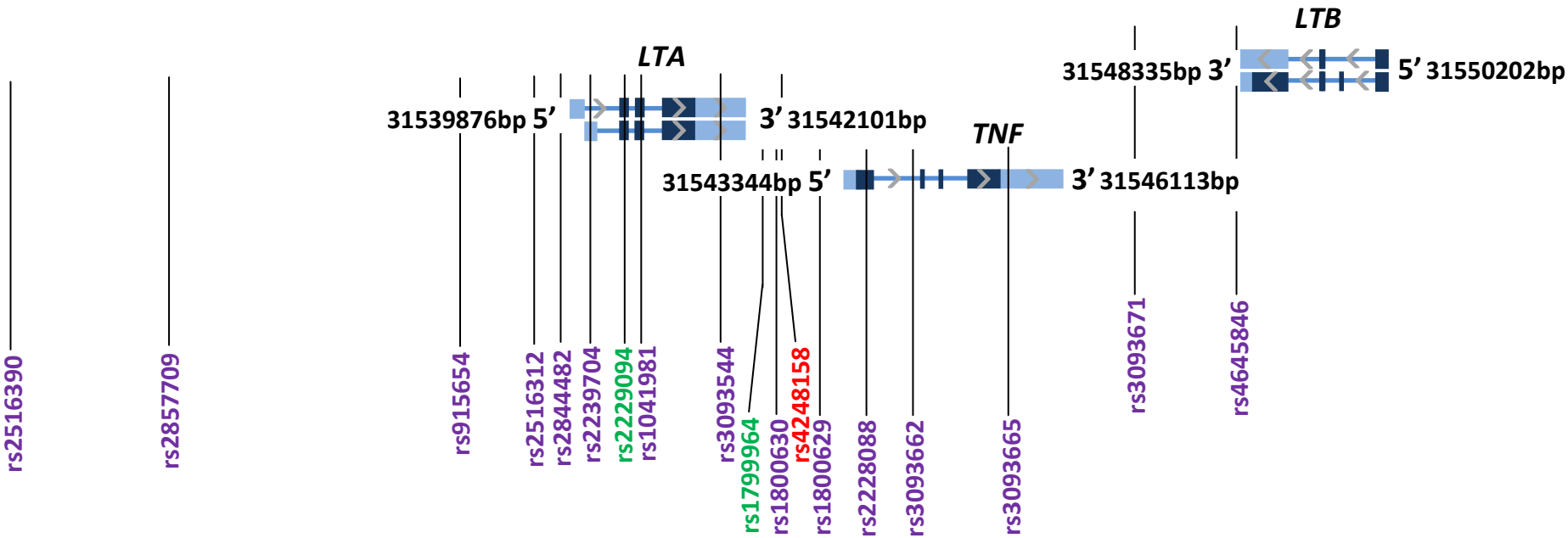

Figure 3.2c. *LTA*, *TNF* and *LTB* gene diagrams of the *TNFA* region with the SNPs chosen for analysis annotated on the diagrams. *[The SNP in red indicates a previously associated SNP; SNPs in purple indicate the tagSNPs; and the SNPs in green indicate SNPs that were both previously associated and resulted from tagSNP selection. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The National Center for Biotechnology Information (NCBI) Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

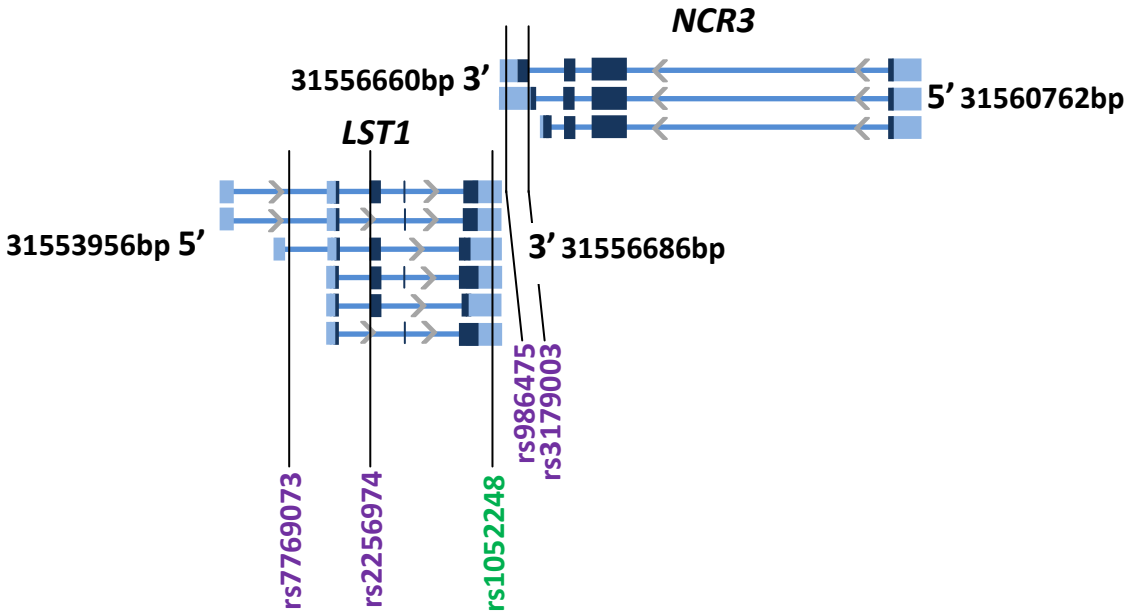

Figure 3.2d. *LST1* and *NCR3* gene diagrams of the *TNFA* region with the SNPs chosen for analysis annotated on the diagrams. *[SNPs in purple indicate the tagSNPs; and the SNP in green indicates a SNP that was both previously associated and resulted from tagSNP selection. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The National Center for Biotechnology Information (NCBI) Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 3.1. Details and summary statistics of the *TNFA* region SNPs included in analysis

SNP ID	Gene	T/P [a]	Position (bp) [b]	Allele 1 [c]	Allele 2 [c]	Minor allele (sample)	SN MAF (sample) [d]	Pain/PI MAF (sample) [e]	HWE (UNAFF - SN) [f]	HWE (UNAFF - PAIN) [g]	HWE (ALL) [h]
rs4248158 [i]	*LTA*	P	31542533	C	T	T	<0.01	<0.01	1.00	1.00	1.00
rs1041981	*LTA*	T	31540784	C	A	C	0.37	0.32	0.22	0.07	0.46
rs11757236	*BAT1*	T	31508029	T	C	T	0.02	0.02	1.00	1.00	1.00
rs11796	*BAT1*	T	31501212	A	T	A	0.36	0.32	0.50	0.06	0.46
rs1800629	*TNF*	T	31543031	G	A	A	0.17	0.18	0.34	0.20	0.29
rs1800630	*TNF /LTA*	T	31542476	A	C	A	0.10	0.10	0.17	1.00	1.00
rs2071592	*NFKBIL1*	T	31515340	T	A	A	0.36	0.30	0.17	0.12	0.71
rs2075582	*MCCD1*	T	31497706	T	C	C	0.10	0.09	0.27	1.00	0.61
rs2079170	*BAT1*	T	31508493	C	G	C	0.03	0.03	<0.01	1.00	<0.01
rs2228088	*TNF*	T	31543605	T	G	T	0.10	0.09	0.67	1.00	0.61
rs2230365	*NFKBIL1*	T	31525448	T	C	T	0.03	0.03	1.00	1.00	1.00
rs2239704	*LTA*	T	31540141	C	A	A	0.09	0.07	0.37	1.00	0.55
rs2239705	*ATP6V1G2*	T	31513402	A	G	A	0.03	0.02	1.00	1.00	0.07
rs2239709	*BAT1*	T	31507447	T	C	T	0.06	0.05	1.00	1.00	1.00
rs2256974	*LST1*	T	31555392	A	C	A	0.45	0.48	0.86	0.62	0.87
rs2516312	*LTA*	T	31539435	A	G	G	0.04	0.03	1.00	1.00	1.00
rs2516390	*LTA*	T	31529883	T	C	C	0.09	0.08	0.22	1.00	1.00
rs2734583	*BAT1*	T	31505480	A	G	G	0.14	0.15	0.07	1.00	1.00
rs2734586	*MCCD1*	T	31497342	C	G	G	0.13	0.13	0.71	1.00	1.00
rs2844482	*LTA*	T	31539767	T	C	T	0.14	0.13	0.53	1.00	1.00
rs28445017	*NFKBIL1*	T	31527756	A	G	A	0.03	0.04	1.00	1.00	1.00
rs2844509	*BAT1*	T	31510924	A	G	G	0.14	0.10	0.77	1.00	0.66
rs2857709 [i]	*LTA*	T	31532814	A	G	A	<0.01	<0.01	1.00	1.00	1.00
rs3093544	*LTA*	T	31541779	A	G	G	0.04	0.02	<0.01	1.00	<0.01
rs3093662	*TNF*	T	31544189	A	G	G	0.09	0.06	0.37	1.00	0.44
rs3093665	*TNF*	T	31545391	A	C	C	0.04	0.02	1.00	1.00	0.05
rs3093671	*LTB /TNF*	T	31546980	A	G	A	0.09	0.08	1.00	1.00	1.00
rs3130056	*BAT1*	T	31499354	T	C	C	0.05	0.05	1.00	0.10	0.37

rs3130059	*BAT1*	T	31509284	G	C	G	0.35	0.30	0.23	0.12	0.45
rs3179003	*NCR3*	T	31556928	C	A	A	0.11	0.11	0.65	1.00	0.64
rs3219185	*NFKBIL1*	T	31515078	C	G	C	0.08	0.07	0.27	1.00	1.00
rs4645846 [i]	*TNF/LTB*	T	31548266	C	G	C	<0.01	<0.01	1.00	1.00	1.00
rs4947324	*NFKBIL1*	T	31528130	C	T	T	0.14	0.10	0.01	1.00	0.65
rs6929796	*NFKBIL1*	T	31522669	A	G	A	0.26	0.25	1.00	0.46	0.53
rs7738430 [i]	*BAT1*	T	31508836	T	C	C	0.01	<0.01	1.00	1.00	0.01
rs7769073	*LST1*	T	31554607	A	T	A	0.09	0.09	1.00	1.00	1.00
rs915654	*LTA*	T	31538497	A	T	T	0.43	0.40	0.61	1.00	0.23
rs9267487	*ATP6V1G2 /BAT1*	T	31511350	T	C	C	0.08	0.07	0.61	1.00	0.55
rs986475 [i]	*LST1 /NCR3*	T	31556709	A	G	G	<0.01	0.00	1.00	1.00	1.00
rs1052248	*LST1*	T and P	31556581	T	A	A	0.28	0.27	1.00	1.00	0.16
rs1799964	*LTA /TNF*	T and P	31542308	T	C	C	0.20	0.19	1.00	0.33	0.79
rs2229094	*LTA*	T and P	31540556	T	C	C	0.29	0.24	0.45	1.00	0.51
rs2251824	*BAT1 /ATP6V1G2*	T and P	31511857	G	A	A	0.12	0.11	1.00	1.00	1.00

[a] T=tagSNP; P=Previously associated SNP

[b] Position on chromosome 6

[c] BioMart assigned alleles

[d] Minor allele frequency of the SNP in the entire sample used for HIV-SN susceptibility association analysis (case-control analysis – HIV-SN+ vs. HIV-SN-)

[e] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis

[f] Used to determine exclusion of SNPs due to deviation from HWE in the HIV-SN susceptibility (case-control) analysis. Individuals included in calculation are all individuals without HIV-SN (n=151)

[g] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)

[h] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

[i] The SNPs was excluded from HIV-SN and pain analyses as the minor allele frequency fell below the threshold of 0.01

Only significant results are shown in this chapter. The full results set appears in **Appendix D**.

3.2.1.1 Single SNP association analysis for HIV-SN susceptibility

Table 3.2. *TNFA* region HIV-SN susceptibility SNP association results

MODEL	SNP	UNIVARIATE		MULTIVARIATE			RISK OF HIV-SN
		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
Allelic	**rs1041981**	0.033	0.71	0.079	0.241	0.75	↓
	rs11796	0.027	0.70	0.049*	0.166	0.72	↓
	rs2071592	0.008	0.64	0.011*	0.040	0.65	↓
	rs2075582	0.028	0.58	0.028*	0.087	0.54	↓
	rs3130059	0.018	0.67	0.028*	0.101	0.69	↓
	rs4947324	0.047	0.65	0.143	0.372	0.70	↓
Genotypic	**rs1041981**	0.040	-	0.430	0.671	-	-
	rs2071592	0.008	-	0.137	0.189	-	-
	rs2075582	0.032	-	0.874	1.000	-	-
	rs3130059	0.019	-	0.264	0.416	-	-
	rs4947324	0.004	-	0.747	1.000	-	-
Dominant	**rs1041981**	0.017	-	0.015*	0.030	0.56	↓
	rs11796	0.020	-	0.021*	0.047	0.58	↓
	rs2071592	0.004	-	0.003*	0.004	0.49	↓
	rs3130059	0.008	-	0.004*	0.008	0.53	↓
	rs4947324	0.014	-	0.011*	0.040	0.54	↓
Recessive	**rs2075582**	0.002	-	0.871	1.000	<0.01	↓
	rs2228088	0.024	-	0.512	1.000	<0.01	↓
	rs915654	0.044	-	0.088	0.088	0.56	↓

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

Allelic model:

Six SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the allelic model and were thus taken through to multivariate analysis. Four of these SNPs showed a significant association with HIV-SN susceptibility after correcting for age and height, and one of the significantly associated SNPs, rs2071592, passed the family-wise correction for multiple comparisons ($P_{EMP2}<0.05$). The results indicate that carriage of the minor allele of each

significantly associated SNP is associated with a decreased risk of developing HIV-SN relative to the major allele (OR<1.0). There is thus a significant association between the allele present at four loci and the presence or absence of HIV-SN.

Genotypic model:

Five SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the genotypic model and were thus taken through to multivariate analysis. None of the SNPs were significantly associated with HIV-SN susceptibility after correcting for age and height. There is thus no significant independent association between the genotype present at the various loci and the presence or absence of HIV-SN.

Dominant model:

Five SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the dominant model and were thus taken through to multivariate analysis. All of the SNPs were also significantly associated with HIV-SN susceptibility after correcting for age and height and all passed the family-wise correction for multiple comparisons ($P_{EMP2}<0.05$ in all cases). rs2071592 and rs3130059 had highly significant P_{EMP1} and P_{EMP2} values. The results again indicate that carriage of the minor allele of each significantly associated SNP is associated with a decreased risk of developing HIV-SN relative to the major allele (OR<1.0). There is thus a significant association between the presence of HIV-SN and having at least one minor allele versus not having any minor allele at these five SNPs.

Recessive model:

Three SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the recessive model and were thus taken through to multivariate analysis. None of the SNPs were significantly

associated with HIV-SN susceptibility after correcting for age and height. There is thus no significant independent association between the presence or absence of HIV-SN and having two minor alleles versus having at least one major allele at any of the loci.

Six SNPs – rs1041981, rs11796, rs2071592, rs2075582, rs3130059 and rs4947324 - stand out as being of particular significance across all models. An assessment of the BeadStudio SNP graph scatter plots showed distinct clusters for four of the SNPs. In these cases, we can rule out genotyping errors and spurious associations. The SNP graph for rs2071592 reveals that, although clear clusters can be seen, the heterozygous cluster is in quite close proximity to one of the homozygous clusters and the results for this SNP should perhaps be treated with caution (**Appendix E**). The clusters in the rs1041981 SNP graph are sitting at a fairly low intensity and the results for this SNP should also be treated with caution (**Appendix E**).

The previously identified SNP (rs1799964) reported to be associated with an increased risk of developing HIV-SN in Caucasian (Cherry et al., 2008) and Indonesian (Affandi et al., 2008) populations did not pass the univariate analysis threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant association between the presence or absence of pain and the allele or genotype present; having at least one minor allele versus not having any minor allele; and having two minor alleles versus having at least one major allele at this locus in the *TNFA* region in the Southern African population.

- **Post-analysis assessment of significant findings – HIV-SN susceptibility**

The population allele frequencies for the six positively associated SNPs in the five populations are shown in **Figure 3.3**.

Three of the significantly associated SNPs (rs11796, rs2071592 and rs4947324) are not tagged along with any other SNPs in the YRI population. Two of these SNPs (rs2071592 and rs4947324) were also not tagged along with any other SNPs in the CEU population. All other SNPs were tagged along with one or more SNP(s) in the YRI and CEU population. The SNPs tagged along with each of the significantly associated SNPs are presented in **Table 3.3**.

Table 3.3. SNPs tagged along with the SNPs significantly associated with HIV-SN susceptibility

	SNP					
Population	**rs1041981**	**rs11796**	**rs2071592**	**rs2075582**	**rs3130059**	**rs4947324**
YRI	rs1041981 rs909253 rs2071591	rs11796	rs2071592	rs2523507 rs2075580 rs2075582 rs3130055 rs1055388 rs2239528 rs933208 rs2523504	rs3130059 rs2071594	rs4947324
CEU	rs909253 rs1041981	rs2239527 rs2071591 rs3093974 rs11796 rs2071594 rs3130059	rs2071592	rs2523507 rs3130055 rs2239528 rs933208 rs1055388 rs2075580 rs2523504 rs2075582	rs2239527 rs2071591 rs3093974 rs11796 rs2071594 rs3130059	rs4947324

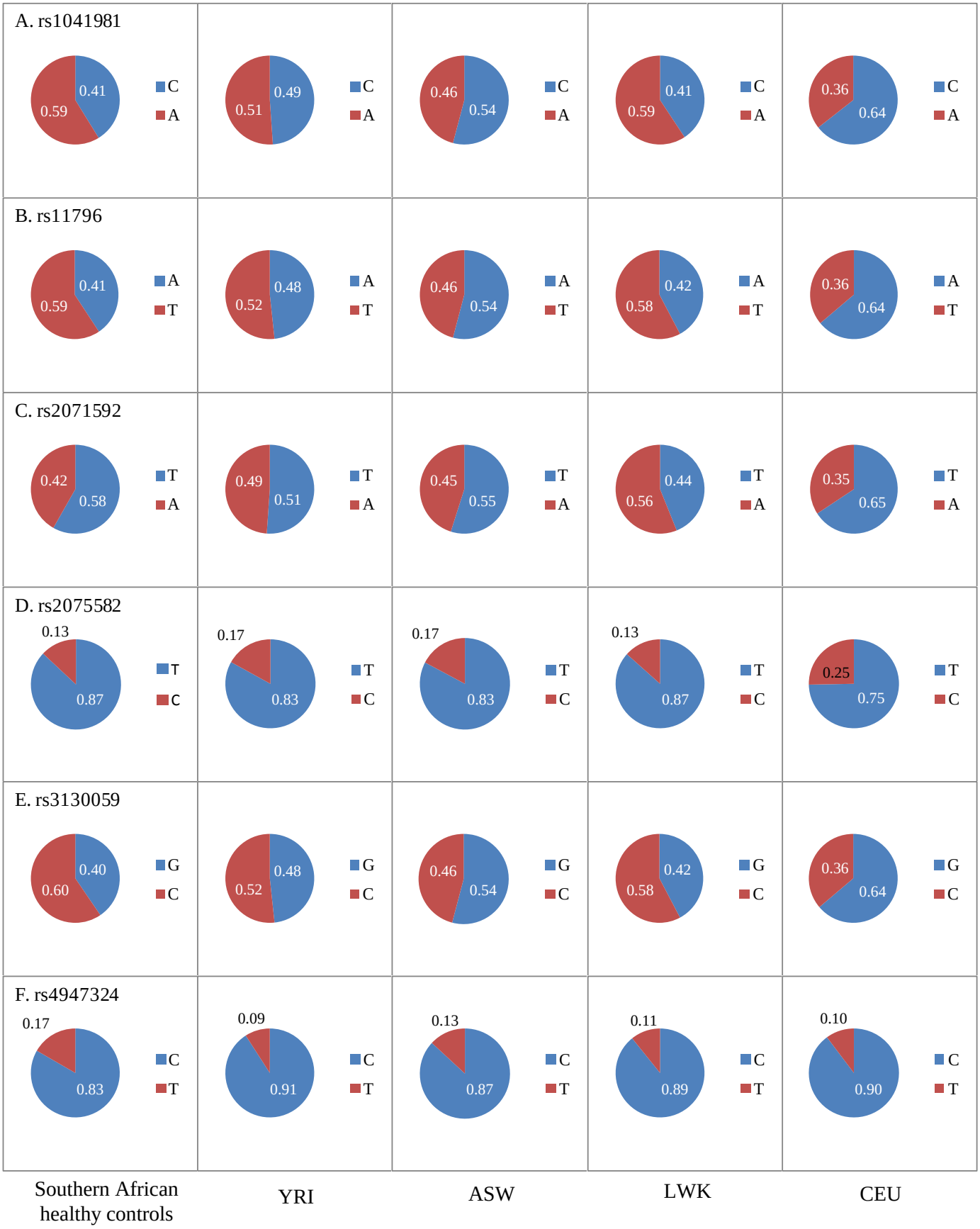

Figure 3.3. Allele frequency comparison of the six significantly associated SNPs (A-F) among five populations.

Input of rs1041981 and rs4947324 into RegulomeDB reveals that they have minimal binding evidence (i.e. evidence is lacking to suggest that the SNPs disrupt the binding of transcription factors which regulate gene expression). Input of rs11796, rs2071592, rs2075582 and rs3130059 into RegulomeDB reveals that they are likely to affect binding and are linked to expression of a gene target (i.e. there is enough evidence to suggest that the SNPs form part of expression quantitative trait loci and may disrupt/influence the binding of transcription factors, thus influencing gene expression).

3.2.1.2 Haplotype association analysis for HIV-SN susceptibility

An assessment of the previously identified haplotype was not possible as some of the SNPs in the haplotype could not be genotyped. Individual analysis of each of the SNPs in the haplotype that could be genotyped, however, revealed no significant association with HIV-SN susceptibility in any of the models.

Haplotype association analysis was therefore carried out using the sliding window approach, where two- and three-SNP haplotypes encompassing the chosen SNPs along the genes in the entire region were assessed. Numerous haplotypes passed the univariate threshold of $P_{EMP1}<0.05$ and were thus taken through to multivariate analysis (**Tables 3.4**). A number of these haplotypes also significantly associated with HIV-SN susceptibility after correcting for age and height, but none of them passed the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$ in all cases). Some were associated with increased risk of developing HV-SN (OR>1.0) and some with a decreased risk (OR<1.0).

All significantly associated haplotypes contain at least one of the SNPs significantly associated with HIV-SN susceptibility on their own and the alleles present determine the direction of the risk in each case. The C-allele (minor allele) of rs2075582 associates with decreased risk and the T-allele with increased risk. The A-allele (minor allele) of rs11796 associates with decreased risk and the T-allele with increased risk. The C-allele (major allele) of rs3130059 associates with an increased risk. The A-allele (minor allele) of rs2071592 associates with decreased risk and the T-allele with increased risk. Lastly, the T-allele (minor allele) of rs4947324 associates with decreased risk and the C-allele with increased risk.

Table 3.4. *TNFA* region HIV-SN susceptibility haplotype association results obtained using the sliding window approach

HAPLOTYPES										HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF HIV-SN
rs2734586	rs2075582	rs3130056	rs11796	rs2734583	rs2239709	rs11757236	rs3130059	rs2844509	rs9267487		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
C	C									0.10	0.040	0.58	0.031*	0.495	0.54	↓
C	C	T								0.06	0.011	0.45	0.021*	0.415	0.42	↓
	C	T								0.06	0.012	0.45	0.021*	0.438	0.42	↓
	C	T	A							0.05	0.026	0.47	0.041*	0.641	0.44	↓
	T	T	T							0.64	0.011	1.48	0.033*	0.619	1.43	↑
		T	A							0.31	0.028	0.70	0.089	0.895	0.74	↓
		T	T							0.64	0.028	1.43	0.069	0.809	1.37	↑
		T	A	A						0.31	0.026	0.69	0.101	0.915	0.75	↓
			A	A						0.36	0.025	0.69	0.062	0.783	0.72	↓
			A	A	T					0.05	0.037	0.49	0.041*	0.614	0.45	↓
				A	T					0.06	0.044	0.51	0.031*	0.562	0.44	↓
				A	T	C				0.05	0.040	0.49	0.057	0.765	0.47	↓
							C	A		0.64	0.012	1.53	0.036*	0.577	1.45	↑
							C	A	T	0.64	0.021	1.44	0.063	0.763	1.39	↑
GENE	***MCCD1***	***MCCD1***	***BAT1***	***BAT1***	***BAT1***	***BAT1***	***BAT1***	***BAT1***	***BAT1***	***ATP6V1G2 /BAT1***						

The region is split up for ease of representation. Each table is a continuation of the table before it. SNPs in green are those that were associated with HIV-SN susceptibility on their own. Alleles in red are those which form part of significantly associated haplotypes after correction for age and height. The gene to which each SNP belongs is indicated at the bottom of each table

Asterisks (*) indicate those SNPs that are significant following correction for covariates.

P_{EMP1} – pointwise empirical p-value

P_{EMP2} – Family-wise correction for multiple comparisons

OR – odds ratio

HAPLOTYPES										HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF HIV-SN
rs2251824	rs2239705	rs3219185	rs2071592	rs6929796	rs2230365	rs28445017	rs4947324	rs2516390	rs915654		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
	G	G	A							0.32	0.022	0.67	0.049*	0.658	0.70	↓
	G	G	T							0.58	0.004	1.56	0.020*	0.346	1.51	↑
		G	A							0.35	0.031	0.70	0.087	0.871	0.73	↓
		G	T							0.57	0.009	1.53	0.028*	0.488	1.47	↑
		G	A	G						0.35	0.013	0.66	0.053	0.710	0.71	↓
		G	T	G						0.39	0.001	1.80	0.001*	0.057	1.69	↑
			A	G						0.36	0.013	0.66	0.045*	0.620	0.69	↓
			T	G						0.39	0.001	1.77	0.004*	0.092	1.65	↑
			A	G	C					0.33	0.009	0.64	0.033*	0.514	0.68	↓
			T	G	C					0.39	0.001	1.78	0.001*	0.070	1.67	↑
					C	G	T			0.14	0.038	0.61	0.143	0.951	0.69	↓
						G	T			0.14	0.034	0.61	0.135	0.942	0.68	↓
						G	C	T		0.75	0.043	1.47	0.057	0.784	1.45	↑
							C	T		0.78	0.012	1.71	0.010*	0.278	1.68	↑
							T	T	T	0.03	0.006	0.14	0.030*	0.648	0.17	↓
							C	T	A	0.39	0.019	1.54	0.031*	0.513	1.49	↑
GENE	*BAT1 /ATP6V1G2*	*ATP6V1G2*	*NFKBIL1*	*NFKBIL1*	*NFKBIL1*	*NFKBIL1*	*NFKBIL1*	*NFKBIL1*	*LTA*	*LTA*						

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

Table 3.4. continued...

	HAPLOTYPES						HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF HIV-SN
	rs915654	rs2516312	rs2844482	rs2239704	rs2229094	rs1041981		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
	A	A					0.54	0.045	1.41	0.049*	0.698	1.43	↑
		G	C	A			0.03	0.046	0.41	0.012*	0.369	0.31	↓
			C	C	T		0.63	0.005	1.61	0.011*	0.143	1.61	↑
				C	C		0.29	0.025	0.67	0.062	0.802	0.71	↓
				C	T		0.63	0.006	1.61	0.012*	0.167	1.59	↑
				C	T	A	0.62	0.015	1.47	0.033*	0.551	1.44	↑
					C	C	0.27	0.046	0.71	0.218	0.997	0.78	↓
					T	A	0.62	0.028	1.45	0.058	0.739	1.40	↑
GENE	***LTA***	***LTA***	***LTA***	***LTA***	***LTA***	***LTA***							

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

Table 3.4. continued...

3.2.1.3 Single SNP association analysis for pain susceptibility

No SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant independent association between the presence or absence of pain and the allele or genotype present; having at least one minor allele versus not having any minor allele; and having two minor alleles versus having at least one major allele at any of the loci in the *TNFA* region.

3.2.1.4 Haplotype association analysis for pain susceptibility

Haplotype association analysis in the *TNFA* region followed the sliding window approach, where two- and three-SNP haplotypes encompassing the chosen SNPs along the genes in the entire region were assessed. Only one haplotype [rs2256974 (A) - rs1052248 (T) - rs3179003 (A)] passed the univariate threshold of $P_{EMP1}<0.05$ and was thus taken through to multivariate analysis. The haplotype also significantly associated with pain susceptibility after correcting for age, gender and CD4 T-cell count ($P_{EMP1}=0.013$, OR=0.02) and it passed the family-wise correction for multiple comparisons ($P_{EMP2}=0.049$). The haplotype associates with a decreased risk of developing pain (OR<0.1). The haplotype contains rs1052248, a SNP selected due to its previous association with HIV-SN susceptibility (Chew et al., 2010).

3.2.1.5 Single SNP association analysis for pain intensity

One SNP (rs28445017) passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the pain intensity analysis. The SNP also significantly associated with pain intensity after correcting for age, gender and CD4 T-cell count ($P_{EMP1}=0.046$, Beta=2.08) and it passed the family-wise

correction for multiple comparisons (P_{EMP2}=0.046). Carriage of the minor allele of the SNPs is associated with increased pain intensity relative to the major allele (positive beta-value). There is thus a significant association between the SNP alone and pain intensity.

An assessment of the BeadStudio SNP graph scatter plot showed distinct clusters. We can therefore rule out a spurious association

- **Post-analysis assessment of significant findings – pain intensity**

The population allele frequencies for rs28445017 in the five populations are shown in **Figure 3.4**.

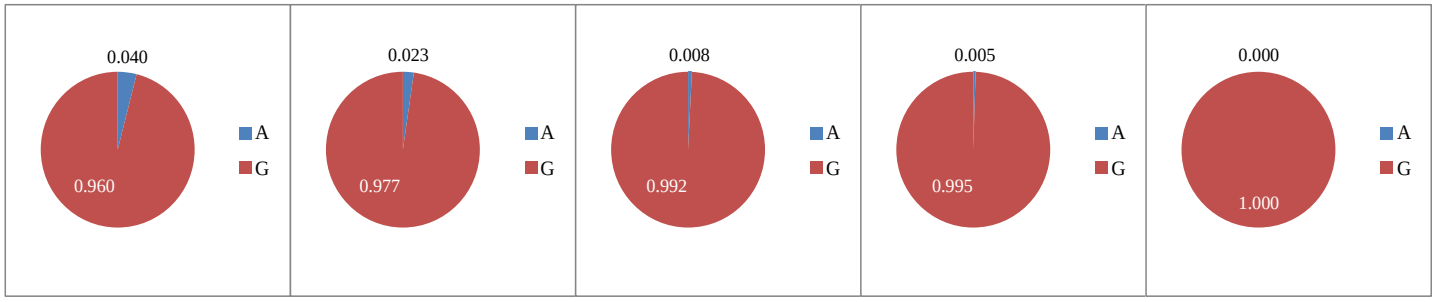

Figure 3.4. Allele frequency comparison of rs28445017 among five populations

rs28445017 is tagged along with four other SNPs in the YRI population (rs9282875, rs3093556, rs9469024 and rs3093666). The SNP is monomorphic in the CEU population. Input of rs28445017 into RegulomeDB reveals that it has minimal binding evidence.

3.2.1.6 Haplotype association analysis for pain intensity

The same haplotype association analysis approach used in the pain susceptibility analysis was used for the pain intensity association analysis. Several haplotypes passed the univariate threshold of $P_{EMP1}<0.05$ and were thus taken through to multivariate analysis (**Tables 3.5, 3.6 and 3.7**). All but one of the haplotypes also significantly associated with pain intensity after correcting for age, gender and CD4 T-cell count, although none passed the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$ in all cases). The haplotypes, including that which only passed the univariate threshold, seemed to be in "blocks" across the genes in the region investigated.

The first "block" (**Table 3.5**), falling over the 3' end of *NFKBIL1*and the intergenic region between *NFKBIL1* and *LTA*, is of particular interest. All haplotypes contain rs28445017, found in the 3' untranslated region (UTR) of *NFKBIL1*, which associated with pain intensity on its own. The allele present influenced whether the haplotype associated with increased or decreased pain intensity, with the A-allele (minor allele) leading to increased pain intensity (positive beta-value) and the G-allele leading to decreased pain intensity (negative beta-value). Controlling for rs28445017 resulted in all haplotypes containing the SNP to no longer be associated with pain intensity after correcting for age, gender and CD4 T-cell count (data not shown). This suggests that rs28445017 is the "causative SNP" or in strong LD with the "causative SNP" and influences, on its own, the intensity of pain experienced.

The haplotype in the second "block" (**Table 3.6**) contains two SNPs falling in the promoter region of *TNF*, between *LTA* and *TNF*. The haplotype did not associate with pain intensity after correcting for age, gender and CD4 T-cell count.

The haplotype associated with increased pain intensity in the third "block" (**Table 3.7**) contains two SNPs found in *LST1*. One of these SNPs, rs1052248, was selected due to its previous association with HIV-SN susceptibility and was also found to be part of a haplotype significantly associated with pain susceptibility.

Table 3.5. *TNFA* region pain intensity haplotype association results obtained using the sliding window approach – "Block" 1

HAPLOTYPES					HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
rs6929796	rs2230365	rs28445017	rs4947324	rs2516390		P_{EMP1}	BETA	P_{EMP1}	P_{EMP2}	BETA	
	C	A			0.04	0.036	2.07	0.043*	0.375	2.10	↑
	C	G			0.94	0.016	-1.99	0.009*	0.113	-2.03	↓
		A	C		0.03	0.033	2.27	0.042*	0.358	2.26	↑
G	C	A			0.04	0.035	2.07	0.041*	0.372	2.09	↑
	C	A	C		0.03	0.030	2.30	0.033*	0.325	2.31	↑
		A	C	T	0.03	0.032	2.28	0.034*	0.327	2.03	↑
GENE	***NFKBIL1***	***NFKBIL1***	***NFKBIL1***	***NFKBIL1***	***LTA***						

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

Table 3.6. *TNFA* region pain intensity haplotype association results obtained using the sliding window approach – "Block" 2

HAPLOTYPE		HAPLOTYPE FREQUENCY	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
rs1800630	rs1800629		P_{EMP1}	BETA	P_{EMP1}	P_{EMP2}	BETA	
C	G	0.72	0.044	-0.83	0.052	0.388	-0.86	↓
GENE	***LTA/TNF***	***TNF***						

P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

Table 3.7. *TNFA* region pain intensity haplotype association results obtained using the sliding window approach – "Block" 3

HAPLOTYPE		HAPLOTYPE FREQUENCY	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
rs2256974	rs1052248		P_{EMP1}	BETA	P_{EMP1}	P_{EMP2}	BETA	
C	A	0.27	0.034	0.91	0.036*	0.325	0.92	↑
GENE	***LST1***	***LST1***						

The asterisk (*) indicates that the SNP is significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

3.2.2 *IL12B*

tagSNP selection produced 18 SNPs for genotyping and analysis. Genotyping of four of the SNPs (rs3212227, rs3213092, rs3213110 and rs3213113) failed and were excluded from analysis. No SNPs had the minor allele frequency falling below the threshold of 0.01. The SNPs included in the analysis are shown in **Figure 3.5** with further details presented in **Table 3.8**.

For HIV-SN and pain susceptibility analyses, all SNPs were in HWE and therefore no SNPs were excluded from analysis. For pain intensity analysis, one SNP (rs2421047) failed HWE and was excluded from analysis.

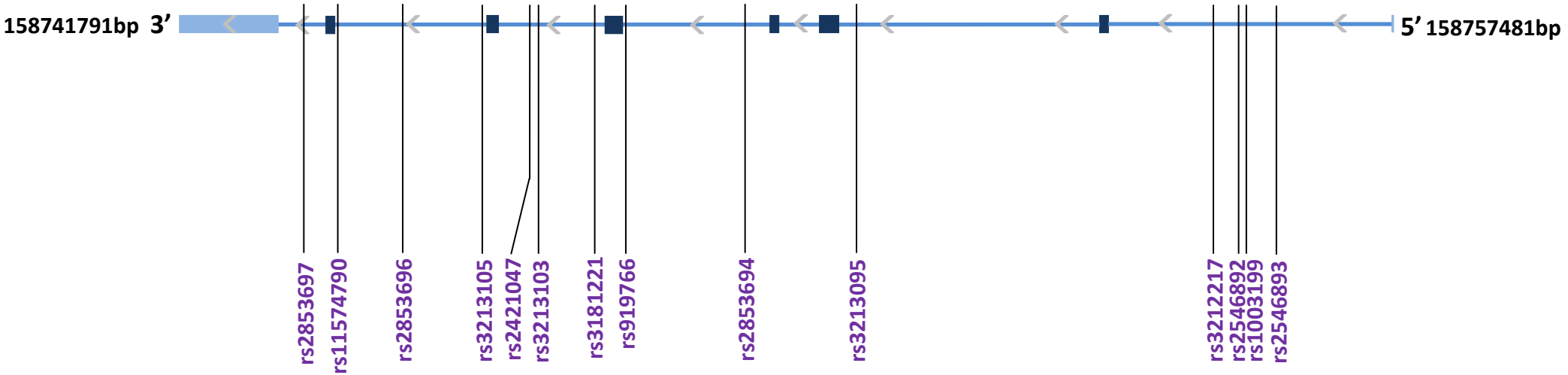

Figure 3.5. *IL12B* gene diagram with the SNPs chosen for analysis annotated on the diagram. *[SNPs in purple indicate the tagSNPs. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 3.8. Details and summary statistics of the *IL12B* tagSNPs included in analysis

SNP ID	Position (bp) [a]	Allele 1 [b]	Allele 2 [b]	Minor Allele (sample)	SN MAF (sample) [c]	Pain/PI MAF (sample) [d]	HWE (UNAFF - SN) [e]	HWE (UNAFF - PAIN) [f]	HWE (ALL) [g]
rs1003199	158755566	C	T	T	0.23	0.25	0.16	1.00	0.83
rs11574790	158743846	G	A	A	0.27	0.27	1.00	1.00	1.00
rs2421047	158746307	G	A	A	0.32	0.30	0.06	0.12	<0.01
rs2546892	158755475	G	A	A	0.04	0.04	0.24	1.00	1.00
rs2546893	158755960	G	A	A	0.24	0.26	0.24	0.51	0.83
rs2853694	158749088	G	T	G	0.24	0.24	0.64	1.00	0.67
rs2853696	158744660	T	C	T	0.03	0.02	0.02	1.00	1.00
rs2853697	158743403	T	G	G	0.04	0.04	0.17	1.00	1.00
rs3181221	158747158	A	G	G	0.04	0.04	1.00	1.00	1.00
rs3212217	158755130	C	G	C	0.31	0.28	0.03	0.11	0.55
rs3213095	158750543	T	A	T	0.07	0.07	1.00	1.00	1.00
rs3213103	158746436	G	A	A	0.10	0.11	0.60	1.00	1.00
rs3213105	158745699	C	T	T	0.07	0.07	1.00	1.00	1.00
rs919766	158747564	A	C	C	0.28	0.28	0.84	1.00	0.43

[a] Position on chromosome 5
[b] BioMart assigned alleles
[c] Minor allele frequency of the SNP in the entire sample used for HIV-SN susceptibility association analysis (case-control analysis – HIV-SN+ vs. HIV-SN-)
[d] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis
[e] Used to determine exclusion of SNPs due to deviation from HWE in the HIV-SN susceptibility (case-control) analysis. Individuals included in calculation are all individuals without HIV-SN (n=151)
[f] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)
[g] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

3.2.2.1 Single SNP and haplotype association analysis for HIV-SN susceptibility

No SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant independent association between HIV-SN susceptibility and the allele or genotype present; having at least one minor allele versus not having any minor allele; and having two minor alleles versus having at least one major allele at any of the loci in *IL12B*. No haplotypes passed the univariate analysis threshold of $P_{EMP1}<0.05$.

3.2.2.2 Single SNP association analysis for pain susceptibility

Table 3.9. *IL12B* pain susceptibility SNP association results

MODEL	SNP	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
Allelic	**rs3213095**	0.050	0.32	0.021*	0.021	0.27	↓
Genotypic	**rs3213095**	0.042	-	1.000	1.000	-	-
Dominant	**rs3213095**	0.049	-	0.025*	0.025	0.27	↓
Recessive	**rs2421047**	0.031	-	0.028*	0.028	0.27	↓

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

Allelic model:

One SNP (rs3213095) passed the univariate threshold of $P_{EMP1}<0.05$ in the allelic model and was thus taken through to multivariate analysis. The SNP still associated with pain susceptibility after correcting for age, gender and CD4 T-cell count and passed the family-wise correction for multiple comparisons ($P_{EMP2}<0.05$). Carriage of the minor allele of rs3213095 (T) is associated with a decreased risk of developing pain (OR<1.0). There is thus a significant association between pain susceptibility and the allele present at the particular locus.

Genotypic model:

One SNP (rs3213095) passed the univariate threshold of $P_{EMP1}<0.05$ in the allelic model and was thus taken through to multivariate analysis. The SNP no longer associated with pain susceptibility after correcting for age, gender and CD4 T-cell count. There is thus no significant independent association between pain susceptibility and the genotype present at the particular locus.

Dominant model:

One SNP (rs3213095) passed the univariate threshold of $P_{EMP1}<0.05$ in the dominant model. The SNP still associated with pain susceptibility after correcting for age, gender and CD4 T-cell count and passed the family-wise correction for multiple comparisons ($P_{EMP2}<0.05$). Carriage of the minor allele of rs3213095 (T) is again associated with a decreased risk of developing pain (OR<1.0). There is thus a significant association between pain susceptibility and having at least one minor allele versus not having any minor allele.

Recessive model:

One SNP (rs2421047) passed the univariate threshold of $P_{EMP1}<0.05$ in the recessive model. The SNP still associated with pain susceptibility after correcting for age, gender and CD4 T-cell count and passed the family-wise correction for multiple comparisons ($P_{EMP2}<0.05$). Carriage of the minor allele of rs2421047 (A) is also associated with a decreased risk of developing pain (OR<1.0). There is thus a significant association between pain susceptibility and having two minor alleles versus having at least one major allele at the particular locus.

An assessment of the BeadStudio SNP graph scatter plots for both rs3213095 and rs2421047 showed distinct clusters for both SNPs. We can therefore rule out spurious associations.

- **Post-analysis assessment of significant findings – pain susceptibility**

The population allele frequencies for the two positively associated SNPs in the five populations are shown in **Figure 3.6**.

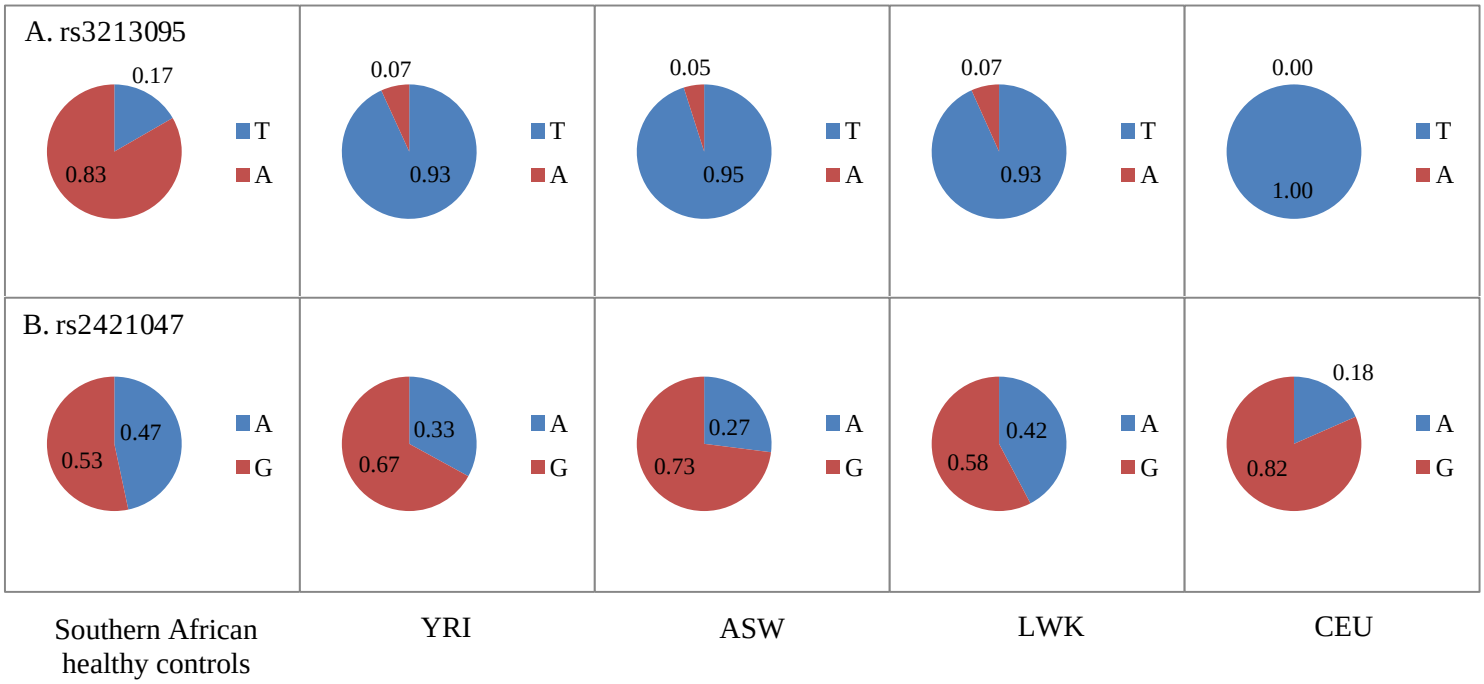

Figure 3.6. Allele frequency comparison of the two significantly associated SNPs (A-B) among five populations.

rs3213095 was tagged along with one other SNP in the YRI population and was monomorphic in the CEU population. rs2421047 was not tagged along with any other SNPs in the YRI population, but was tagged along with eight other SNPs in the CEU population. The SNPs tagged along with each of the significantly associated SNPs are presented in **Table 3.10**.

Table 3.10. SNPs tagged along with the SNPs significantly associated with pain susceptibility

	SNP	
Population	**rs3213095**	**rs2421047**
YRI	rs3213089	rs2421047
	rs3213095	
CEU	MONOMORPHIC	rs3212218
		rs2288831
		rs3212227
		rs3212219
		rs3212217
		rs3213097
		rs3213094
		rs2421047
		rs3212220

Input of rs3213095 into RegulomeDB revealed that it has minimal binding evidence. No data is available on RegulomeDB for rs2421047.

3.2.2.3 Haplotype association analysis for pain susceptibility

Haplotype association analysis in *IL12B* followed the sliding window approach, where two- and three-SNP haplotypes of the chosen SNPs were assessed. Several haplotypes passed the univariate threshold of $P_{EMP1}<0.05$ and was thus taken through to multivariate analysis (**Table 3.11**). All but one of the haplotypes also significantly associated with pain susceptibility after correcting for age, gender and CD4 T-cell count, although none of the haplotypes passed the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$ in all cases).

Table 3.11. *IL12B* pain susceptibility haplotype association results obtained using the sliding window approach

HAPLOTYPES									HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
rs3213105	rs2421047	rs3213103	rs3181221	rs919766	rs2853694	rs3213095	rs3212217	rs2546892		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
	A	G							0.30	0.040	0.52	0.056	0.412	0.49	↓
					T	T			0.07	0.014	0.27	0.031*	0.338	0.28	↓
						T	G		0.07	0.036	0.28	0.035*	0.432	0.28	↓
C	A	G							0.29	0.035	0.50	0.037*	0.385	0.49	↓
	A	G	A						0.30	0.044	0.51	0.049*	0.401	0.49	↓
				A	T	T			0.07	0.013	0.27	0.031*	0.330	0.27	↓
					T	T	G		0.07	0.014	0.27	0.031*	0.338	0.28	↓
						T	G	G	0.07	0.036	0.27	0.032*	0.339	0.27	↓

The striped columns represent SNPs that are present on either side of those shown, but not represented in the table
Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

3.2.2.4 Single SNP association analysis for pain intensity

No SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ and thus no SNPs were taken through to multivariate analysis. There is thus no significant independent association between *IL12B* and pain intensity.

3.2.2.5 Haplotype association analysis for pain intensity

No haplotypes passed the univariate analysis threshold of $P_{EMP1}<0.05$.

3.3 Discussion

3.3.1 *TNFA* region

The results for the *TNFA* region are inconsistent with those which were previously reported in the literature. rs1799964 was not significantly associated with HIV-SN susceptibility after correction for age and height in this study in a Southern African population. The minor allele of this SNP was, however, found to be associated with an increased risk of HIV-SN development in Caucasian (Cherry et al., 2008) and Indonesian (Affandi et al., 2008) populations. There was also no significant association between HIV-SN susceptibility and any of the SNPs from the previously associated haplotype (Chew et al., 2010). There is a significant difference in the LD and haplotype structure among populations (Tishkoff and Verrelli, 2003) and one would thus expect that different SNPs may be associated with HIV-SN susceptibility in different populations. One might expect rather low levels of LD among the SNPs in African populations (Campbell and Tishkoff, 2008). rs1799964 and the other

previously associated SNPs may be in LD with the causative SNP which influences HIV-SN susceptibility in the Caucasian and Indonesian populations, but not in the Southern African population.

Despite a lack of association between previously associated SNPs and HIV-SN susceptibility, a few tagSNPs, none of which have been identified in previous studies as being associated with HIV-SN susceptibility, were associated. Six SNPs are of particular interest as they were found to be associated with HIV-SN risk in either the allelic model, dominant model or both models after correction for age and height. In each case, the major allele was associated with an increased risk of developing HIV-SN. The six SNPs will be discussed in further detail below.

3.3.1.1 Allele frequency comparisons and possible functional role of associated SNPs

In all cases, one would expect the allele frequencies of the Southern African population to be most similar to the other three African populations due to the proposed history of human migration and the subsequent differences in genetic structure and diversity seen between African and non-African populations, as described in Chapter 1.

A comparison of the allele frequencies in **rs1041981** among different populations reveals some clear differences. The C-allele is the minor allele in the current sample with a MAF of 0.41 in healthy controls. This is comparable to the LWK population where the allele frequencies are the same. Although the minor allele in the YRI population is also C, the MAF (0.49) is not too different from the major allele frequency (0.51). The same is the case in the ASW population (A – 0.46, C – 0.54). Relatively small differences seen between the

frequencies of the minor and major alleles of the other African populations could suggest that the allele may not be associated with HIV-SN risk as the two alleles are in such similar proportions. However, one would have to investigate the proportions in affected and unaffected groups alone to make a solid conclusion as one of the alleles may be elevated in the affected group compared to the unaffected group. The minor (A – 0.36) and major (C – 0.64) alleles in the European (CEU) population are opposite to that which is seen in the current sample. If the A-allele (major allele in this Southern African sample) is the allele responsible for increased HIV-SN risk, this could suggest that there is an elevated risk or predisposition in Southern Africans to develop HIV-SN compared to the European population (where the A-allele is the minor allele). HIV-SN is very common in African individuals, so this may indeed be the case. rs1041981 is located in a coding region of *LTA* and is a synonymous mutation meaning that the function of the protein encoded by the DNA may or may not be altered due to a different amino acid being coded for. Input of rs1041981 into RegulomeDB reveals that it has minimal binding evidence.

A comparison of the allele frequencies in **rs11796** among different populations also reveals some clear differences and a very similar pattern to that which is seen with rs1041981. The allele frequencies in the current sample (A – 0.41, T – 0.59) are again most comparable to the LWK population (A – 0.42, T – 0.58). Similarly, the major and minor allele frequencies in the YRI (A – 0.48, T – 0.52) and ASW (A – 0.54, T – 0.46) populations are not too different from each other. The minor and major alleles in the CEU population are again opposite to that which is seen in the current sample (A – 0.64, T – 0.36). If the T-allele (major allele in this Southern African sample) is the allele responsible for increased HIV-SN risk, this could suggest that there is an elevated risk or predisposition in Southern Africans to develop HIV-SN compared to the European population (where the T-allele is the minor allele). rs11796 is

located in an intronic region of *BAT1*. Input of rs11796 into RegulomeDB reveals the possible functional role of this SNP is that it is likely to affect binding and is linked to expression of a gene target.

A comparison of the allele frequencies in **rs2071592** among different populations again reveals some differences. All populations, except the LWK population, have the A-allele as their minor allele. The differences between the minor and major allele frequencies in the YRI (A – 0.49, T – 0.51), ASW (A – 0.45, T – 0.55) and LWK (A – 0.56, T – 0.44) populations are again quite small. Again, if the T-allele (major allele in this Southern African sample) is the allele responsible for increased HIV-SN risk, this could suggest that there is an elevated risk or predisposition in Southern Africans to develop HIV-SN compared to the other African populations (where the T-allele is at a lower frequency). rs2071592 is located in an intronic region of *NFKBIL1*. Input of rs2071592 into RegulomeDB reveals the possible functional role of this SNP is that it too is likely to affect binding and is linked to expression of a gene target.

A comparison of the allele frequencies in **rs2075582** among the five populations reveals reasonably comparable frequencies. All have C as the minor allele and all MAFs are quite small (Southern African – 0.13, YRI – 0.17, ASW – 0.17, LWK – 0.13). The only slightly different population is the CEU population with a slightly higher MAF (0.25). Small MAFs can be a cause for concern as they can lead to false positives arising (Tabangin et al., 2009). Again, if the T-allele (major allele in this Southern African sample) is the allele responsible for increased HIV-SN risk, this could suggest that there is an elevated risk or predisposition in Southern Africans as well as the other African populations to develop HIV-SN compared to the European population (where the T-allele is at a slightly lower frequency). rs2075582 is

located in a coding region of the *MCCD1*, but is synonymous so doesn't affect the amino acid sequence of the gene product. Input of rs2075582 into RegulomeDB reveals the possible functional role of this SNP is that it is also likely to affect binding and is linked to expression of a gene target.

A comparison of the allele frequencies in **rs3130059** among different populations again reveals some clear differences and a very similar pattern to that which is seen with rs1041981 and rs11796. The allele frequencies in the current sample (G – 0.40, C – 0.60) are again most comparable to the LWK population (G – 0.42, C – 0.58). Similarly, the major and minor allele frequencies in the YRI (G – 0.48, C – 0.52) and ASW (G – 0.54, C – 0.46) populations are not too different from each other. The minor and major alleles in the CEU population (G – 0.64, C – 0.36) are again opposite to that which is seen in the current sample. If the C-allele (major allele in this Southern African sample) is the allele responsible for increased HIV-SN risk, this could suggest that there is an elevated risk or predisposition in Southern Africans to develop HIV-SN compared to the European population (where the C-allele is the minor allele). **rs3130059** is located in an intronic region of *BAT1*. Input of rs3130059 into RegulomeDB reveals the possible functional role of this SNP is that it too is likely to affect binding and is linked to expression of a gene target.

It is interesting to note that the findings for rs11796 and rs3130059 are very similar with regards to the associations found and the MAFs in the five populations mentioned above. They are in fact in the same gene and may be in LD with each other and collectively affect the expression of *BAT1*. Tagger does list the two SNPs together in the CEU population, and this may be the same in the current sample. *BAT1* does seem to be a gene of interest due to the previously reported association, although weak, of a minor allele in intron 10 of *BAT1*

with increased HIV-SN risk in a Caucasian cohort (Cherry et al., 2008). It should perhaps be studied on its own to assess any potential associations it has to HIV-SN susceptibility.

A comparison of the allele frequencies in **rs4947324** among the five populations reveals, as with rs2075582, reasonably comparable frequencies. All have T as the minor allele and all MAFs are quite small (Southern African – 0.17, YRI – 0.09, ASW – 0.13, LWK – 0.11). In this case, the MAF in the CEU population is also small (0.10). Again, small MAFs can be a cause for concern as they can lead to false positives arising (Tabangin et al., 2009). rs4947324 is intergenic and lies 3' of *NFKBIL1*. Input of rs4947324 into RegulomeDB reveals that it has minimal binding evidence.

Based on the outputs from RegulomeDB, it is possible from a functional point of view that rs11796, rs2071592, rs2075582 and rs3130059 could directly influence HIV-SN susceptibility.

3.3.1.2 Assessment of tagged SNPs

The SNPs found to be associated with HIV-SN susceptibility could be the actual causative SNPs determining the risk or they could be in LD with the causative SNPs. As there is no dataset for South(ern) Africans currently available, we cannot accurately and directly assess which SNPs are in LD with the associated SNPs in the current Southern African sample. Use was thus made of the YRI dataset, which is the best-matched dataset available, as well as the CEU dataset for an additional interesting comparison between African and European individuals. The tagger function in Haploview (using settings of MAF<0.01 and r^2=1) was used to assess which SNPs are tagged by the significantly associated SNPs in the YRI and

CEU populations in the hope of getting a crude idea of which SNPs are in LD with the SNPs found to be associated with HIV-SN susceptibility.

An assessment of **rs1041981** reveals that, in the YRI population, it is tagged along with two other SNPs – rs909253 and rs2071591. In the CEU population, rs11796 is tagged along with one other SNP - rs909253. As rs909253 SNP is common to both the YRI and CEU populations, it would be interesting to directly assess whether it is functionally associated with HIV-SN susceptibility. An assessment of **rs11796** reveals that, in the YRI population, it does not tag any other SNPs, suggesting that it is not in LD with any other SNPs in the YRI population under the settings specified. In the CEU population, rs11796 is tagged along with several SNPs including rs3130059. Thus if an association were to be found between rs11796 and HIV-SN susceptibility in Europeans, any one of the several SNPs could be pinned as the causative SNP. An assessment of **rs2071592** reveals that, in the YRI population as well as in the CEU population, it is not tagged along with any other SNPs. An assessment of **rs2075582** reveals that it is tagged along with seven other SNPs (rs2523507, rs2075580, rs3130055, rs1055388, rs2239528, rs933208 and rs2523504) in both the YRI and CEU population and the SNPs are the same for both populations. One of these seven SNPs, if not rs2075582, might have a functional role. An assessment of **rs3130059** reveals that, in the YRI population, it tags one other SNP, rs2071594. In the CEU population, however, it is tagged along with several SNPs including rs11796. An assessment of **rs4947324** reveals that, in the YRI and CEU populations, rs4947324 does not tag any other SNPs.

In the cases where the SNPs do not tag any other SNP(s) in the YRI population, and if the assumption is tentatively made that the current Southern African sample has a similar genetic structure to the YRI population, this could suggest that the SNPs may themselves be

functionally associated with the HIV-SN risk. Alternatively, they may be in LD with rare SNPs yet to be identified and documented and which need to be identified through sequencing or which are lost in the selection of tagSNPs due to the settings used. In the cases where the SNPs do tag other SNPs, HIV-SN risk could possibly be functionally inferred by one of the other SNPs and they would have to be directly assessed. It is possible, however, that the LD structure in black Southern Africans is entirely different to that which is seen in other African populations, including the YRI population from which the tagSNPs were selected. This could mean that either tagSNP selection may have missed important SNPs specific to the Southern African population or that the SNPs found to be associated with HIV-SN susceptibility may be in LD with other SNPs that may already be documented.

Haplotype association confirms some of the results seen in the individual SNP analysis. At least one of the six SNPs discussed above is contained within each of the associated haplotypes and the alleles present influence the direction of the risk in each case. As with the individual SNP analysis, the major allele is associated with an increased risk of developing HIV-SN in each case. Interestingly, the region containing the significantly associated haplotypes seems to "cut off" abruptly at the end of the *LTA* gene. No haplotypes falling in the region containing *TNF*, the lymphotoxin-beta gene (*LTB), LST1* and the natural cytotoxicity triggering receptor 3 gene *(NCR3)* are significantly associated with HIV-SN susceptibility. The region associated with HIV-SN risk in an African population can therefore perhaps be defined to just encompass the genes further upstream of these genes relative to the forward strand, namely *MCCD1*, *BAT1*, the ATPase, H+ transporting, lysosomal 13kDa, V1 subunit G2 gene *(ATP6V1G2), NFKBIL1* and *LTA*.

Further analysis into the *TNFA* region revealed little association with pain susceptibility and only a few findings with pain intensity. TNF-α has been found to be a key mediator in the pathogenesis of neuropathic pain (Leung and Cahill, 2010) and a greater or clearer association would, therefore, not have been surprising. No individual SNPs associated with pain susceptibility, but quite a significant finding was found between a 3-SNP haplotype and a decreased risk of pain. The haplotype could incorporate the actual causative SNP(s) or the SNPs in the haplotypes may be in LD with the causative SNP(s). The tagging process checks for this.

Pain intensity analysis revealed a SNP, rs28445017, whose minor allele (A) was associated with increased pain intensity. This SNP was also found in most haplotypes associated with pain intensity. A comparison of the allele frequencies among different populations revealed that the SNP is monomorphic in the CEU population and has very low MAFs in the three African populations (YRI – 0.023, ASW – 0.008, LWK – 0.005). The MAF in this population is also quite low (0.04). Again, small MAFs can be a cause for concern as they can lead to false positive associations arising (Tabangin et al., 2009). An assessment of rs28445017 in Haploview reveals that it is tagged along with four other SNPs (rs9282875, rs3093556, rs9469024 and rs3093666) in the YRI population. One of these four SNPs, if not rs28445017, might have a functional role. The SNP is confirmed to be monomorphic in the CEU population. rs28445017 is intergenic and lies 3' of *NFKBIL1*. Input of rs28445017 into RegulomeDB reveals that it has minimal binding evidence. All of these findings provide weak support for the role of rs28445017 in pain intensity. The SNP should probably be investigated in further detail before any definite conclusions about its role in pain intensity can be made. For example, one can assess the effect the SNP, as well as those seen to be tagged along with rs28445017, has on the expression of *NFKBIL1* and the

resultant function of the gene product. One could also conduct a more in depth LD analysis to identify a possible causative SNP.

The current study provides strong evidence in support of previous studies for the role of the so-called "*TNFA* region" in HIV-SN susceptibility, as well as some evidence for a potential role in pain susceptibility and pain intensity. Apart from the SNPs identified, other SNPs that weren't looked at in this study may be associated with HIV-SN susceptibility, pain susceptibility or pain intensity. The tagging efficiency, although quite high when SNPs were initially selected (91%), was not 100% and some SNPs were excluded following the genotyping process or due to failure of HWE or low MAF. It is therefore possible that some important associations may have been missed. The exact functional role of the identified SNPs and SNPs in LD with these needs to be investigated further to determine what may cause altered gene expression in the genes in this region and ultimately lead to changes in HIV-SN susceptibility or pain experienced. Differences in the findings between HIV-SN susceptibility and pain susceptibility and intensity could point to different mechanisms at play in neuropathy and pain development.

Important to note is the complexity of the region in which the genes lie and the subsequent complications this may cause in identifying the exact causative gene(s) and SNP(s). The *TNFA* region in this study lies within the central MHC, a 4 Mb region spanning part of the short arm of chromosome 6 (de Bakker and Raychaudhuri, 2012). The MHC region is a very complex region and has the largest number of genetic associations for numerous diseases (de Bakker and Raychaudhuri, 2012). The complexity of this region arises due to the diversity of the genetic sequence, the large number of genes in the region and the extensive LD (de Bakker and Raychaudhuri, 2012). The LD may, however, not be as extensive in African

populations, although this does not completely simplify this region in Africans. Due to this genetic diversity and LD, it is difficult to identify the exact causative SNP associated with a particular disease and several SNPs may in fact have the same statistical association (Ioannidis et al., 2009). Looking specifically at the *TNF* region within the MHC, it has been reported that 17 haplotypes defined the TNF region in a European population (Allcock et al., 2004) and 22 haplotypes defined the region in an African population (Ackerman et al., 2003).

3.3.2 *IL12B*

Analysis carried out in *IL12B* showed no association with HIV-SN susceptibility, as has been reported in a previous study in a Caucasian cohort (Cherry et al., 2008). There was also no association found between *IL12B* and pain intensity. The only positive association found was between *IL12B* and pain susceptibility.

Two SNPs in particular came up as being significantly associated after correcting for age, gender and CD4 T-cell count – rs3213095 in both the allelic and dominant models and rs2421047 in the recessive model. The minor allele of each of the SNPs was associated with a decreased risk of developing pain. In all cases, the results were robust as all passed the family-wise correction for multiple comparisons, but need to be interpreted with caution because of the small sample of patients without pain (n =15).

3.3.2.1 Allele frequency comparisons and possible functional role of associated SNPs

A comparison of the allele frequencies in **rs3213095** among the five populations reveals a complete switch between the current sample and the other three African populations.

Whereas the minor allele in the current sample is the T-allele (with a MAF of 0.17), the alternative allele (A) is the minor allele in the other African populations. The MAFs are all quite small (YRI – 0.07, ASW – 0.05, LWK – 0.07) and as mentioned previously, small MAFs can be a cause for concern as they can lead to false positives arising (Tabangin et al., 2009). The SNP in the CEU population is monomorphic and likely won't have any association with HIV-SN susceptibility, pain susceptibility or pain sensitivity. If the A-allele (major allele in this Southern African sample) is the allele responsible for increased pain susceptibility, this could suggest that there is an elevated risk or predisposition in Southern Africans to develop pain compared to the other African populations (where the A-allele is the minor allele). rs3213095 is located in an intronic region of *IL12B*. *IL12B* encodes a subunit of IL-12 which is a cytokine that acts on T and natural killer cells (http://www.ncbi.nlm.nih.gov/gene). Input of the SNP into RegulomeDB reveals that it has minimal binding evidence. Based on this weak evidence, functional studies are required to determine any functional role that the SNPs may play. It is possible, however that, based on their location in the intronic regions of IL12B, that they have no functional role (although this is not always the case with introns), suggesting that SNPs in LD with these two SNPs are more likely the causative SNP(s).

A comparison of the allele frequencies in **rs2421047** among different populations reveals some clear differences. The A-allele is the minor allele in the current sample with a MAF of 0.47 in healthy controls. This is most comparable to the LWK population (MAF – 0.42). If the G-allele (major allele in this Southern African sample) is the allele responsible for increased pain susceptibility, this could suggest that there is an elevated risk or predisposition in the other African populations and more specifically the European population to develop

pain compared to the Southern African population. rs2421047 is also located in an intronic region of *IL12B*. No data is available on RegulomeDB for this SNP.

3.3.2.2 Assessment of tagged SNPs

An assessment of **rs3213095** reveals that, in the YRI population, it is tagged along with one other SNP (rs3213089). Pain risk could be functionally inferred by rs3213095 itself or the SNP with which it is in LD. It would have to be directly assessed to determine this. As the SNP is monomorphic in the CEU population, it does not appear in Haploview.

An assessment of **rs2421047** reveals that, in the YRI population, it does not tag any other SNPs, suggesting that it is not in LD with any other SNPs in the YRI population under the settings specified. Again, if the tentative assumption is made that the current Southern African sample has a similar genetic structure to the YRI population, this could suggest that the SNP may itself be functionally associated with the pain risk. Alternatively, it may be in LD with rare SNPs yet to be identified and documented and which need to be identified through sequencing or which are lost in the selection of tagSNPs due to the settings used. In the CEU population, rs2421047 is tagged along with several SNPs including rs3212217 which was investigated in this study, but had no significant association with pain susceptibility. If an association were to be found between rs2421047 and pain susceptibility in Europeans, any one of the several SNPs could be pinned as the causative SNP.

Interestingly, all significantly associated haplotypes contained either one of the SNPs (rs2421047 or rs3213095) which was significantly associated with pain susceptibility in one or more of the models on their own. Controlling for these SNPs individually caused all

haplotypes containing either of the SNPs to no longer be associated with pain susceptibility after correcting for age, gender and CD4 T-cell count (data not shown). This suggests that rs2421047 and rs3213095 could be "causative SNPs" and influence, on their own, the susceptibility to developing pain or may be in strong LD with the causative SNP(s).

The initial tagging efficiency for *IL12B* was 85%. Taking this into account and the fact that four SNPs were excluded due to failed genotyping, some important associations may have been missed. Further studies also need to be carried out on the identified SNPs to determine their functional role or the functional role of SNPs that are in LD with these two SNPs. Functional studies could include the assessment of the effect the SNPs have on the expression of *IL12B* and the possible altered capacity the gene product may have as a subunit of IL-12. This can be investigated by assessing its action on T and natural killer cells.

3.4 Conclusions

The *TNFA* region showed significant associations with HIV-SN susceptibility, although the associated SNPs were different to those previously identified in other populations. Some associations were also found between the TNFA region and pain susceptibility and pain intensity. Due to the complexity of the MHC in which the TNFA region lies, the exact causative SNP(s) are difficult to identify and further studies should be carried out. On the other hand, IL12B showed no association with HIV-SN susceptibility, unlike what has previously been reported. There was also no association with pain intensity. Significant associations were found between *IL12B* and p*ain s*usceptibility with the significant SNPs being associated alone and in haplotypes. There is little evidence supporting the functional role of these SNPs and further studies should therefore be conducted.

CHAPTER 4

The association of *KCNS1*, but not *GCH1*, with pain susceptibility and pain intensity

A portion of the contents of this chapter has been peer-reviewed and accepted for publication in an ISI-accredited journal (Appendix F):

HENDRY LM, LOMBARD Z, WADLEY AL, KAMERMAN PR

KCNS1, but not GCH1, is associated with pain intensity in a black Southern African population with HIV-associated sensory neuropathy: a genetic association study.

Journal of Acquired Immune Deficiency Syndromes

4.1 Background

As discussed in Chapter 1, *GCH1* and *KCNS1* have both been associated with pain intensity in previous studies in non-African populations. A recent study by Wadley et al. (2012), using the same Southern African samples as used for this current study, reported on the first investigation into *GCH1* and pain in a black Southern African population. They looked at six of the SNPs from the 15-SNP "pain-protective" haplotype, but found no association with pain intensity or pain susceptibility (Wadley et al., 2012).

The current study involves a more detailed investigation into *GCH1* and neuropathic pain using population-specific tagSNPs as well as the first investigation into *KCNS1* and neuropathic pain in a black African population. Analysis in both genes involved an investigation of whether the previously associated haplotype/SNPs as well as population specific tagSNPs were associated with pain intensity and pain susceptibility in the black Southern African population. The methodology used is described in detail in **Chapter 2**.

4.2 Results

4.2.1 *GCH1*

Twelve of the fifteen "pain-protective" haplotype SNPs were genotyped and included in the analysis. The three SNPs that were not included (rs2183081, rs7147286 and rs7492600) failed ADT evaluation when developing the SNP list. None of these three SNPs were those reported by Lotsch et al. (2007) to define the "pain-protective" haplotype. tagSNP selection produced a further 20 SNPs for genotyping and analysis. Genotyping of one of these SNPs

(rs1753589) failed and was thus not included in any analysis. One SNP was excluded from analysis as the minor allele frequency fell below the threshold of 0.01. The SNPs included in the analysis (and those excluded due to MAF<0.01) are shown in **Figure 4.1** with further details presented in **Table 4.1**.

For pain susceptibility analysis, all SNPs were in HWE and therefore no SNPs were excluded from analysis. For pain intensity analysis, one SNP (rs10483639) failed HWE and was excluded from analysis.

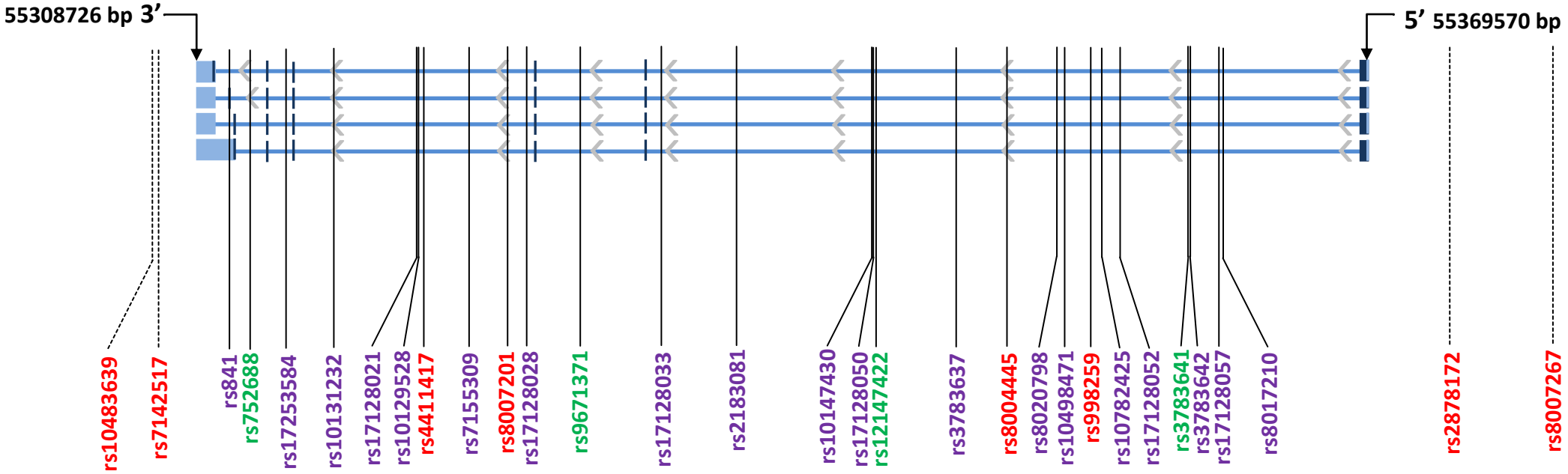

Figure 4.1. *GCH1* gene diagram with the SNPs included in analysis annotated on the diagram. *[SNPs in red indicate the previously associated SNPs; SNPs in purple indicate the tagSNPs; and SNPs in green indicate SNPs that were both previously associated and resulted from tagSNP selection. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 4.1. Details and summary statistics of the *GCH1* SNPs included in analysis

SNP ID	T/P [a]	Position (bp) [b]	Allele 1 [c]	Allele 2 [c]	Minor allele (sample)	MAF (sample) [d]	HWE (UNAFF) [e]	HWE (ALL) [f]
rs10483639	P	55306457	G	C	C	0.22	1.00	<0.01
rs7142517	P	55306804	A	C	A	0.02	1.00	1.00
rs4411417	P	55320563	T	C	C	0.09	1.00	0.62
rs8007201	P	55324848	A	G	A	0.25	1.00	1.00
rs8004445	P	55350666	G	T	G	0.47	0.60	0.07
rs998259 [g]	P	55355031	C	T	T	<0.01	1.00	1.00
rs2878172	P	55373670	A	G	A	0.26	1.00	0.01
rs8007267	P	55378991	C	T	C	0.34	1.00	0.22
rs841	T	55310492	G	A	A	0.09	1.00	0.12
rs17253584	T	55313331	T	C	C	0.12	1.00	1.00
rs10131232	T	55315908	G	A	G	0.22	1.00	0.82
rs17128021	T	55320197	G	A	A	0.42	1.00	0.33
rs10129528	T	55320270	C	T	T	0.34	1.00	0.21
rs7155309	T	55322851	T	C	C	0.09	1.00	1.00
rs17128028	T	55325823	C	T	T	0.24	0.23	0.25
rs17128033	T	55332799	C	T	T	0.17	1.00	0.13
rs2183081	T	55336751	A	G	A	0.24	1.00	0.66
rs10147430	T	55343699	G	A	A	0.05	1.00	0.34
rs17128050	T	55343879	T	C	C	0.08	1.00	1.00
rs3783637	T	55348118	C	T	T	0.15	1.00	0.75
rs8020798	T	55353368	C	T	T	0.25	1.00	1.00
rs10498471	T	55353717	G	A	A	0.47	1.00	0.87
rs10782425	T	55355661	A	G	A	0.03	1.00	1.00
rs17128052	T	55356525	G	C	C	0.13	1.00	1.00
rs3783642	T	55360203	T	C	T	0.26	1.00	0.68

rs17128057	T	55361671	C	T	T	0.04	1.00	1.00
rs8017210	T	55361836	G	A	A	0.33	1.00	0.71
rs752688	T and P	55311569	C	T	T	0.10	1.00	0.05
rs9671371	T and P	55328635	C	T	T	0.32	1.00	0.71
rs12147422	T and P	55344015	T	C	T	0.47	0.60	0.11
rs3783641	T and P	55360139	T	A	A	0.30	0.62	0.45

[a] T=tagSNP; P=Previously associated SNP

[b] Position on chromosome 14

[c] BioMart assigned alleles

[d] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis

[e] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)

[f] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

[g] The SNP was excluded from analysis as the minor allele frequency fell below the threshold of 0.01

Only significant results are shown in this chapter. The full results set appears in **Appendix G**.

4.2.1.1 Single SNP association analysis for pain susceptibility

Table 4.2. *GCH1* pain susceptibility SNP association results

MODEL	SNP	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
Allelic	**rs7155309**	0.034	0.40	0.351	0.599	0.57	↓
	rs8007267	0.037	2.85	0.082	0.131	0.32	↓
Genotypic	**rs752688**	0.024	-	0.416	0.416	-	-
Dominant	**rs10483639**	0.050	-	0.058	0.111	-	-
	rs7155309	0.021	-	0.145	0.370	-	-
	rs752688	0.021	-	0.063	0.133	-	-

P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

Allelic model:

Two SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the allelic model and were thus taken through to multivariate analysis. These SNPs, however, showed no significant association with pain susceptibility after correcting for age, gender and CD4 T-cell count. There is thus no significant independent association between the allele present at the various loci and the presence or absence of pain.

Genotypic model:

One SNP passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the genotypic model and was thus taken through to multivariate analysis. The SNP, which was previously identified as being part of the 15-SNP "pain-protective" haplotype, was not significantly associated with pain susceptibility after correcting for age, gender and CD4 T-cell count. There is thus no significant independent association between the genotype present at the various loci and the presence or absence of pain.

Dominant model:

Three SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the dominant model (although rs10483639 was borderline) and were thus taken through to multivariate analysis. None of the SNPs were significantly associated with pain susceptibility after correcting for age, gender and CD4 T-cell count. There is thus no significant independent association between the presence of pain and having at least one minor allele versus not having any minor allele at these two SNPs.

Recessive model:

No SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$. There is thus no significant independent association between the presence or absence of pain and having two minor alleles versus having at least one major allele.

4.2.1.2 Haplotype association analysis for pain susceptibility

Wadley et al. (2012) carried out an analysis on the three-SNP haplotype which was thought to define the "pain-protective" haplotype (Lotsch et al., 2007). The haplotype associated with pain susceptibility on univariate analysis ($P_{EMP1}=0.02$; OR=0.31), but no longer associated with pain susceptibility after correcting for age, gender and CD4 T-cell count ($P_{EMP1}=0.05$; OR=0.35). A more detailed haplotype association analysis was carried out in the current study using the sliding window approach, where two- and three-SNP haplotypes encompassing the 31 chosen SNPs along the gene were assessed. Two two-SNP and two three-SNP haplotypes (**Table 4.3**) passed the univariate threshold of $P_{EMP1}<0.05$ but did not associate with pain susceptibility after correcting for age, gender and CD4 T-cell count.

Table 4.3. *GCH1* pain susceptibility haplotype association results obtained using the sliding window approach

HAPLOTYPES										HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
rs17253584	rs10131232	rs17128021	rs4411417	rs7155309	rs2183081	rs10147430	rs17128050	rs2878172	rs8007267		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
T	A	G								0.25	0.013	0.44	0.065	0.451	0.49	↓
			C	C						0.08	0.034	0.34	0.162	0.934	0.50	↓
					G	G	T			0.65	0.019	0.35	0.066	0.454	0.39	↓
								G	T	0.61	0.035	0.38	0.060	0.437	0.41	↓

The striped columns represent SNPs that are present on either side of those shown, but not represented in the table
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

4.2.1.3 Single SNP association analysis for pain intensity

No SNPs passed the univariate threshold of $P_{EMP1}<0.05$. There is thus no significant independent association between the SNPs alone and pain intensity.

4.2.1.4 Haplotype association analysis for pain intensity

Wadley et al. (2012) also carried out an analysis on the association between the three-SNP haplotype which was thought to define the "pain-protective" haplotype (Lotsch et al., 2007) and pain intensity. There was, however, no significant association between the haplotype and pain intensity on univariate analysis ($P_{EMP1}=0.23$; $\beta=-0.91$).

Haplotype analysis using the same sliding window approach as in the pain susceptibility analysis produced one two-SNP haplotype that passed the univariate threshold of $P_{EMP1}<0.05$. It was no longer associated with pain intensity after correcting for age, gender and CD4 T-cell count (**Table 4.4**).

Table 4.4. *GCH1* pain intensity haplotype association results obtained using the sliding window approach

HAPLOTYPES		HAPLOTYPE	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
rs10782425	rs17128052	FREQUENCY	P_{EMP1}	BETA	P_{EMP1}	P_{EMP2}	BETA	
G	G	0.85	0.049	0.99	0.101	0.223	0.84	↑

P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

4.2.2 *KCNS1*

rs734784 was selected due to its previous association with pain intensity. tagSNP selection produced a further three SNPs for genotyping and analysis. The SNPs included in the analysis are shown in **Figure 4.2** with further details presented in **Table 4.5**.

For pain susceptibility and pain intensity analyses, all SNPs were in HWE and therefore no SNPs were excluded from analysis.

4.2.2.1 Single SNP association analysis for pain susceptibility

Allelic, genotypic, dominant and recessive models:

No SNPs passed the univariate threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant independent association between pain susceptibility and the alleles or genotype present; having at least one minor allele versus not having any minor allele; or having two minor alleles versus having at least one major allele.

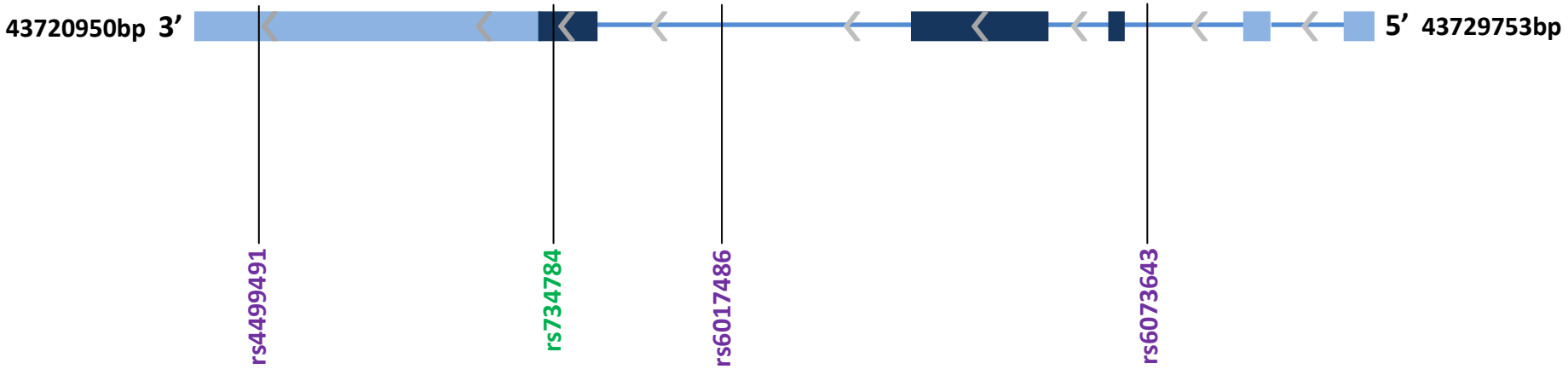

Figure 4.2. *KCNS1* gene diagram with the SNPs included in analysis annotated on the diagram. *[SNPs in purple indicate the tagSNPs; and the SNP in green indicates the SNP that was both previously associated and resulted from tagSNP selection. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 4.5. Details and summary statistics of the *KCNS1* SNPs included in analysis

SNP ID	T/P [a]	Position (bp) [b]	Allele 1 [c]	Allele 2 [c]	Minor allele (sample)	MAF (sample) [d]	HWE (UNAFF) [e]	HWE (ALL) [f]
rs734784	T and P	43723627	T	C	T	0.46	0.62	0.02
rs4499491	T	43721419	C	A	A	0.30	0.29	0.44
rs6017486	T	43724879	G	A	A	0.32	1.00	1.00
rs6073643	T	43728070	T	C	C	0.32	1.00	0.46

[a] T=tagSNP; P=Previously associated SNP

[b] Position on chromosome 20

[c] BioMart assigned alleles

[d] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis

[e] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)

[f] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

4.2.2.2 Haplotype association analysis for pain susceptibility

Two-, three- and four-SNP haplotypes in *KCNS1* were constructed using the four SNPs. Analysis produced two haplotypes that passed the univariate threshold of $P_{EMP1}<0.05$ (**Table 4.6**). Only one of these haplotypes associated with pain susceptibility after correcting for age, gender and CD4 T-cell count. The haplotype contains both rs4499491 (A-allele) and rs734784 (T-allele), the SNP previously identified to be associated with increased pain intensity. The haplotype associated with a decreased risk of developing pain (OR=0.27). The haplotype did not pass the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$).

Table 4.6. *KCNS1* pain susceptibility haplotype association results

HAPLOTYPES			HAPLOTYPE	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
rs4499491	rs734784	rs6073643	FREQUENCIES	P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
A	**T**	**T**	0.21	0.049	0.34	0.057	0.271	0.33	↓
A	**T**		0.19	0.026	0.27	0.024*	0.162	0.27	↓

The asterisk (*) indicates that the SNP is significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

4.2.2.3 Single SNP association analysis for pain intensity

No SNPs passed the univariate threshold of $P_{EMP1}<0.05$. There is thus no significant independent association between pain intensity and the alleles present.

4.2.2.4 Haplotype association analysis for pain intensity

As with pain susceptibility analysis, haplotypes were constructed from the four SNPs. Analysis produced four haplotypes that passed the univariate threshold of $P_{EMP1}<0.05$ (although one was borderline) (**Table 4.7**). All still associated with pain intensity after

correcting for age, gender and CD4 T-cell count. Interestingly, none of these four haplotypes contained the previously associated SNP (rs734784). Two of the haplotypes associated with decreased pain (negative beta-values) and two associated with increased pain (positive beta-values). The rs6017486 SNP seemed to influence whether the pain increased or decreased depending on which allele was present – the A-allele (minor allele) associated with decreased pain and the G-allele with increased pain. This SNP was present in all associated haplotypes and could therefore be considered a key SNP, although it showed no association with pain intensity on its own (P_{EMP1}=0.27). None of the haplotypes passed the family-wise correction for multiple comparisons (P_{EMP2}>0.05 in all cases).

Table 4.7. *KCNS1* pain intensity haplotype association results

HAPLOTYPES			HAPLOTYPE	UNIVARIATE		MULTIVARIATE			PAIN
rs4499491	**rs6017486**	**rs6073643**	**FREQUENCIES**	**P_{EMP1}**	**BETA**	**P_{EMP1}**	**P_{EMP2}**	**BETA**	**INTENSITY**
C	**G**	**T**	0.33	0.050	0.78	0.024*	0.145	0.89	↑
C	**G**		0.40	0.028	0.84	0.012*	0.106	0.91	↑
A	**A**		0.01	0.015	-5.08	0.007*	0.106	-5.45	↓
	A	**T**	0.06	0.039	-1.61	0.017*	0.115	-1.93	↓

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

4.3 Discussion

4.3.1 *GCH1*

Analysis carried out in *GCH1* revealed no significant association between SNPs or haplotypes and pain susceptibility or pain intensity after correcting for age, gender and CD4 T-cell count. Despite this lack of association, *GCH1* has been shown to be a strong candidate as a "pain gene" in previous studies, and perhaps cannot be ruled out completely until further investigations (involving, for example, increasing the sample size or sequencing the gene to

identify novel variants) have been carried out. The difficulty in defining the exact role that *GCH1* plays, however, arises due to the fact that previous studies have been inconsistent in their conclusions.

The role of *GCH1* in pain intensity was first outlined in a study in a Caucasian cohort which revealed an association between a particular haplotype and pain intensity in patients with a neuropathic pain syndrome and in healthy individuals with experimentally induced pain (Tegeder et al., 2006). These findings were verified in a later study by Tegeder et al. (2008). Further positive associations were reported in studies on pain induced by capsaicin (Campbell et al., 2009), pain related to surgery for spinal injury (Kim et al., 2010) and cancer-related pain (Lotsch et al., 2010). The findings in the current study, however, are more consistent with the studies by Kim and Dionne (2007) and Lazarev et al. (2008). Kim and Dionne (2007) reported that there was no association between *GCH1* polymorphisms and sensitivity to experimentally induced pain and an inflammatory pain state in European Americans, Hispanic Americans, Asian Americans or African Americans. Lazarev et al. (2008) reported a lack of association between the "pain-protective" haplotype and pain associated with chronic pancreatitis. Similarly, in an earlier investigation of *GCH1* in the same population investigated in this study, no association was found between the haplotype and pain associated with HIV-SN in black Southern Africans (Wadley et al., 2012).

Some of the findings by other investigators, both positive and negative, should, however, be treated with caution mainly due to the design of the studies. Although reported to have been carried out in a Caucasian cohort, Kim and Dionne (2007) wondered whether the study by Tegeder et al. (2006) may in fact have been conducted in a population of mixed ethnicity. This was based on the fact that previous cohorts of Tegeder et al. had cited previous

publications with mixed population groups. The study by Kim and Dionne (2007) however was also conducted in a population of mixed ethnicity, although the cohort was subdivided into separate ethnic groups for association analysis. Campbell et al. (2009) also used a mixed population, but corrected for ethnicity and also found no ethnic differences in pain ratings. Most studies have been conducted in Caucasian and mixed populations. However, it is important to carry out studies in separate and distinct ethnic groups due to the differences in LD and haplotype structure among populations (Tishkoff and Verrelli, 2003) and the resultant expected differences in associated SNPs. Studies carried out in African populations, particularly, require a larger density of SNPs to obtain the same amount of haplotype coverage (Tishkoff and Verrelli, 2003, Campbell and Tishkoff, 2008). The current findings could point to the fact that *GCH1* has no association with pain in African individuals and that the previously associated "pain-protective" haplotype could be unique to the Caucasian population in which it was identified.

All of the studies have also used relatively small samples, and the sample sizes were further reduced in some studies by the subdivision of the samples into separate ethnic groups for association analysis. Genetic association studies require significantly large samples for the results to be reliable. Small samples may result in the studies being underpowered and may produce false-positive or false-negative results (Kim and Dionne, 2007). The current study, itself, has a relatively small sample size (n=158 for pain analyses) and should therefore be reproduced in a larger sample.

Another possible reason for the conflicting results could be due to the fact that the association between *GCH1* and pain may be "model-specific" (Kim et al., 2010). It has also been suggested that the differences in the findings may be due to the differences in experimental

stimuli used (Edwards et al., 2005 cited in Lazarev et al., 2008). The studies that have been conducted have looked at several different forms of pain and some have used different experimental stimuli. Thus, the lack of association in the study by Wadley et al. (2012) and in the more detailed current study could indicate that *GCH1* has no association with HIV-related pain and, more specifically, pain associated with HIV-SN.

At a biological level, the "pain-protective" haplotype is believed to decrease *GCH1* upregulation and decrease BH_4 production (Tegeder et al., 2008). The exact mechanism of how *GCH1* may (or may not) lead to changes in pain intensity, however, is not known and the exact SNPs which play a functional role in regulating or altering *GCH1* transcription remain to be identified (Doehring et al., 2008). Tegeder et al. (2006) suggested that the location of regulation of *GCH1* transcription probably lies 5' of exon one and in the 20kb region of intron 1. Tegeder et al. (2008) further suggested that the entire "pain-protective" haplotype or one or more of the SNPs in the haplotype or even other SNPs outside of the haplotype may be involved. The tagging efficiency achieved for *GCH1* when selecting tagSNPs was only about 71%. Taking this into account and the fact that some SNPs were excluded following the genotyping process or due to failure of HWE or low MAF, it is possible that the exact "causative SNP" or SNPs in LD with this SNP may not have been investigated in the current study and we therefore may have missed an important association. It is also possible that the previously identified SNPs in the haplotype could perhaps tag other SNPs in *GCH1* (not included in the SNP list to be investigated) which may be associated with pain intensity or pain susceptibility in the African population.

4.3.2 *KCNS1*

The results also show conflicting results to the previously published work on *KCNS1*. The previously identified SNP, rs734784 (Costigan et al., 2010), showed no association with pain susceptibility or pain intensity on its own and was only present in one haplotype significantly associated with pain susceptibility on univariate and multivariate analysis. Analysis carried out in *KCNS1* using tagSNPs did, however, reveal a significant association between several haplotypes and pain susceptibility and pain intensity after correcting for age, gender and CD4 T-cell count. This could indicate that the significantly associated haplotypes could incorporate the causative SNP(s) or that SNPs in the haplotypes may be in LD with the causative SNP(s). The key SNP in the pain intensity haplotypes is rs6017486. Although it showed no individual association with pain intensity, it is present in all of the haplotypes and appears to play a key role in the effect on the intensity of the pain experienced depending on the allele present. It could be in LD with another SNP, not tested in this study, which could be causative and be significantly associated with pain intensity if tested on its own. Again, the tagging efficiency achieved for *KCNS1* when selecting tagSNPs was only about 57%, indicating that an important association, other than that found, could have been missed.

4.4 Conclusions

GCH1 showed no association with pain susceptibility or pain intensity. Although no association was found, one cannot rule out the role of *GCH1*. A further investigation in an entirely African population, using a much larger sample and ensuring 100% tagging and genotyping efficiency should be carried out. On the other hand, *KCNS1* did associate with pain susceptibility and pain intensity, thus supporting the role of *KCNS1* in neuropathic pain

as reported in non-African populations (Costigan et al., 2010). The association was not attributed to the same previously associated SNP, but rather was seen with several haplotypes, which could incorporate the causative SNP. This result indicates that further investigation of this gene is needed, perhaps through sequencing to identify novel variants or through investigation of other SNPs present in the gene. Any novel variants identified would represent SNPs associated with pain that are perhaps unique to this Southern African population and that have not previously been investigated for their association. One could again conclude from this that there are differences in associated SNPs among different population groups.

CHAPTER 5

Preliminary investigation into the associations between *IL1B*, *IL6*, *CCL2* and *CCR2* and pain susceptibility and pain intensity

5.1 Background

This chapter is the second in the current study to investigate the role of cytokines in pain susceptibility and pain intensity. The genes in this chapter, however, are minor to those investigated in chapter three and don't involve any investigation into HIV-SN susceptibility. This chapter also describes, in very minor detail, a particular chemokine and its receptor's role in pain susceptibility and pain intensity.

As discussed in Chapter 1, the products of *IL1B, IL6, CCL2* and *CCR2* have all been implicated in neuropathic pain. The current study involves a very small and preliminary investigation into *IL1B, IL6, CCL2* and *CCR2*. The genes were chosen purely on the basis of the involvement of the gene products in neuropathic pain and not on whether SNPs in the genes have been found to be previously associated with pain. Analysis in all genes involved an investigation of whether some population specific tagSNPs were associated with pain intensity and pain susceptibility in the black Southern African population.

5.2 Results

5.2.1 *IL1B*

tagSNP selection produced four SNPs for genotyping and analysis. No SNPs were excluded due to genotyping failure. The SNPs included in the analysis are shown in **Figure 5.1** with further details presented in **Table 5.1**. For pain susceptibility and pain intensity analyses, all SNPs were in HWE and therefore no SNPs were excluded from analysis.

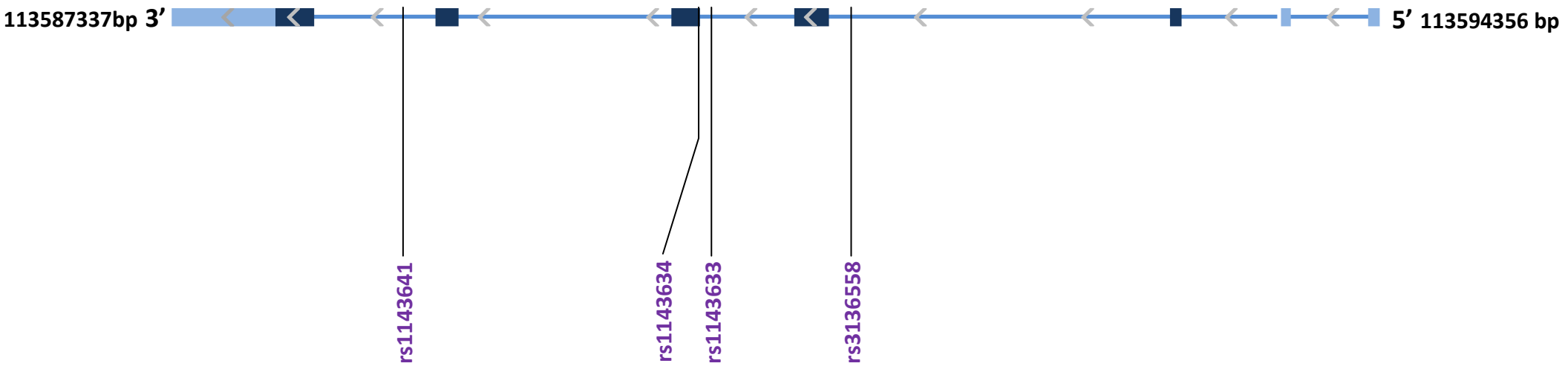

Figure 5.1. *IL1B* gene diagram with the SNPs included in analysis annotated on the diagram. *[SNPs in purple indicate the tagSNPs. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 5.1. Details and summary statistics of the *IL1B* tagSNPs included in analysis

SNP ID	Position (bp) [a]	Allele 1 [b]	Allele 2 [b]	Minor Allele (sample)	MAF (sample) [c]	HWE (UNAFF) [d]	HWE (ALL) [e]
rs1143633	113590467	C	T	T	0.22	0.06	0.49
rs1143634	113590390	G	A	A	0.12	1.00	1.00
rs1143641	113588666	G	A	A	0.11	1.00	1.00
rs3136558	113591275	A	G	G	0.08	1.00	1.00

[a] Position on chromosome 2

[b] BioMart assigned alleles

[c] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis

[d] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)

[e] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

Only significant results are shown in this chapter. The full results set appears in **Appendix H**.

5.2.1.1 Single SNP association analysis for pain susceptibility

No SNPs passed the univariate analysis threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant independent association between pain susceptibility and the allele or genotype present; having at least one minor allele versus not having any minor allele; and having two minor alleles versus having at least one major allele at any of the loci in *IL1B*.

5.2.1.2 Haplotype association analysis for pain susceptibility

Two-, three- and four-SNP haplotypes in *IL1B* were constructed using the four SNPs. No haplotypes passed the univariate analysis threshold of $P_{EMP1}<0.05$.

5.2.1.3 Single SNP association analysis for pain intensity

No SNPs passed the univariate threshold of $P_{EMP1}<0.05$. There is thus no significant independent association between the SNPs alone and pain intensity.

5.2.1.4 Haplotype association analysis for pain intensity

As with pain susceptibility analysis, haplotypes were constructed from the four SNPs. Two haplotypes passed the univariate threshold of $P_{EMP1}<0.05$ and were taken through to multivariate analysis (**Table 5.2**). The haplotypes still associated with pain intensity after

correcting for age, gender and CD4 T-cell count, but did not pass the family-wise correction for multiple comparisons (P_{EMP2}>0.05 in both cases). Both haplotypes were associated with increased pain intensity (positive beta-values).

Table 5.2. *IL1B* pain intensity haplotype association results

HAPLOTYPES			HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
rs1143634	rs1143633	rs3136558		P_{EMP1}	BETA	P_{EMP1}	P_{EMP2}	BETA	
G	C	A	0.58	0.032	0.76	0.010*	0.059	0.89	↑
	C	A	0.70	0.033	0.80	0.021*	0.082	0.89	↑

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

5.2.2 *IL6*

tagSNP selection produced ten SNPs for genotyping and analysis. One SNP (rs2069840) failed genotyping and was thus excluded from analysis. The SNPs included in the analysis are shown in **Figure 5.2** with further details presented in **Table 5.3**.

For pain susceptibility and pain intensity analyses, one SNP (rs2069842) failed HWE and was excluded from analysis.

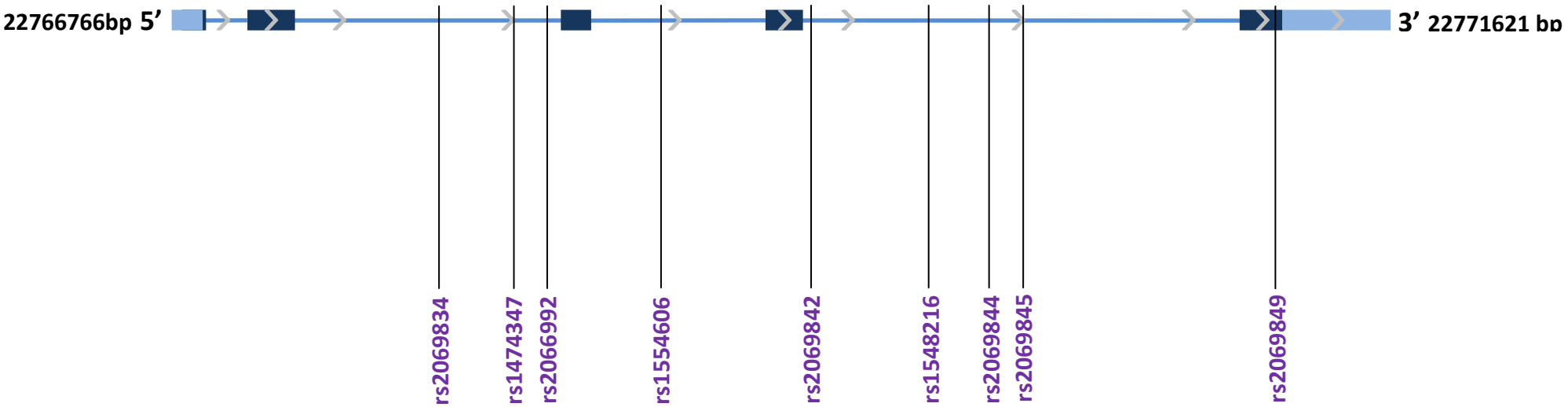

Figure 5.2. *IL6* gene diagram with the SNPs included in analysis annotated on the diagram. *[SNPs in purple indicate the tagSNPs. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 5.3. Details and summary statistics of the *IL6* tagSNPs included in analysis

SNP ID	Position (bp) [a]	Allele 1 [b]	Allele 2 [b]	Minor Allele (sample)	MAF (sample) [c]	HWE (UNAFF) [d]	HWE (ALL) [e]
rs1474347	22768124	C	A	C	0.07	1.00	0.58
rs1548216	22769773	G	C	C	0.17	0.46	0.77
rs1554606	22768707	T	G	T	0.24	1.00	1.00
rs2066992	22768249	G	T	T	0.08	1.00	0.22
rs2069834	22767828	C	T	T	0.04	1.00	1.00
rs2069842	22769310	G	A	A	0.14	<0.01	<0.01
rs2069844	22770010	C	A	A	0.19	1.00	0.80
rs2069845	22770149	G	A	G	0.25	0.30	0.52
rs2069849	22771156	C	T	T	0.16	0.35	1.00

[a] Position on chromosome 7

[b] BioMart assigned alleles

[c] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis

[d] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)

[e] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

5.2.2.1 Single SNP association analysis for pain susceptibility

No SNPs passed the univariate threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant independent association between pain susceptibility and the alleles or genotype present; having at least one minor allele versus not having any minor allele; or having two minor alleles versus having at least one major allele.

5.2.2.2 Haplotype association analysis for pain susceptibility

Haplotype association analysis in *IL6* followed the sliding window approach, where two- and three-SNP haplotypes of the chosen SNPs were assessed. Analysis produced two haplotypes that passed the univariate threshold of $P_{EMP1}<0.05$ (**Table 5.4**). Both of these haplotypes still associated with pain susceptibility after correcting for age, gender and CD4 T-cell count, but did not pass the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$ in both cases). Both associated with a decreased risk of developing pain (OR<1.0).

Table 5.4. *IL6* pain susceptibility haplotype association results obtained using the sliding window approach

HAPLOTYPES			HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
rs2069844	rs2069845	rs2069849		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
C	**G**		0.05	0.048	0.33	0.029*	0.140	0.29	↓
C	**G**	**C**	0.04	0.044	0.33	0.028*	0.132	0.28	↓

The striped column represents SNPs that are present to the left of those shown, but not represented in the table
Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

5.2.2.3 Single SNP association analysis for pain intensity

One SNP (rs2066992; **Table 5.5**) passed the univariate threshold of $P_{EMP1}<0.05$ and was thus taken through to multivariate analysis. The SNP was no longer associated with pain intensity after correcting for age, gender and CD4 T-cell count. There is thus no significant independent association between pain intensity and the alleles present.

Table 5.5. *IL6* pain intensity SNP association results

	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
SNP	**P_{EMP1}**	**BETA**	**P_{EMP1}**	**P_{EMP2}**	**BETA**	
rs2066992	0.024	-1.47	0.060	0.060	-1.27	↓

P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

5.2.2.4 Haplotype association analysis for pain intensity

As with pain susceptibility analysis, haplotype association analysis followed the sliding window approach. Analysis produced eight haplotypes that passed the univariate threshold of $P_{EMP1}<0.05$ (**Table 5.6**). Only four still associated with pain intensity after correcting for age, gender and CD4 T-cell count and none of the haplotypes passed the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$ in all cases). Three of the haplotypes associated with decreased pain intensity (negative beta-values) and one associated with increased pain intensity (positive beta-value).

Table 5.6. *IL6* pain intensity haplotype association results obtained using the sliding window approach

HAPLOTYPES									HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			PAIN INTENSITY
rs2069834	rs1474347	rs2066992	rs1554606	rs2069842	rs1548216	rs2069844	rs2069845	rs2069849		P_{EMP1}	BETA	P_{EMP1}	P_{EMP2}	BETA	
C	C								0.03	0.019	-2.26	0.018*	0.173	-2.32	↓
	A	T							0.07	0.025	-1.48	0.069	0.489	-1.24	↓
		T	G						0.08	0.017	-1.47	0.060	0.432	-1.25	↓
C	A	T							0.07	0.028	-1.45	0.078	0.526	-1.21	↓
C	C	G							0.03	0.025	-2.27	0.022*	0.213	-2.34	↓
C	A	G							0.85	0.037	1.10	0.042*	0.341	1.05	↑
	A	T	G						0.07	0.022	-1.46	0.072	0.481	-1.24	↓
					G	C	G		0.02	0.048	-2.14	0.035*	0.295	-2.26	↓

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons

5.2.3 *CCL2* and *CCR2*

tagSNP selection in *CCL2* and *CCR2* produced two SNPs and four SNPs for genotyping and analysis, respectively. No SNPs were excluded due to genotyping failure. The SNPs included in the analysis are shown in **Figures 5.3 and 5.4** with further details presented in **Tables 5.7 and 5.8**.

For pain susceptibility and pain intensity analyses, all SNPs were in HWE and therefore no SNPs were excluded from analysis.

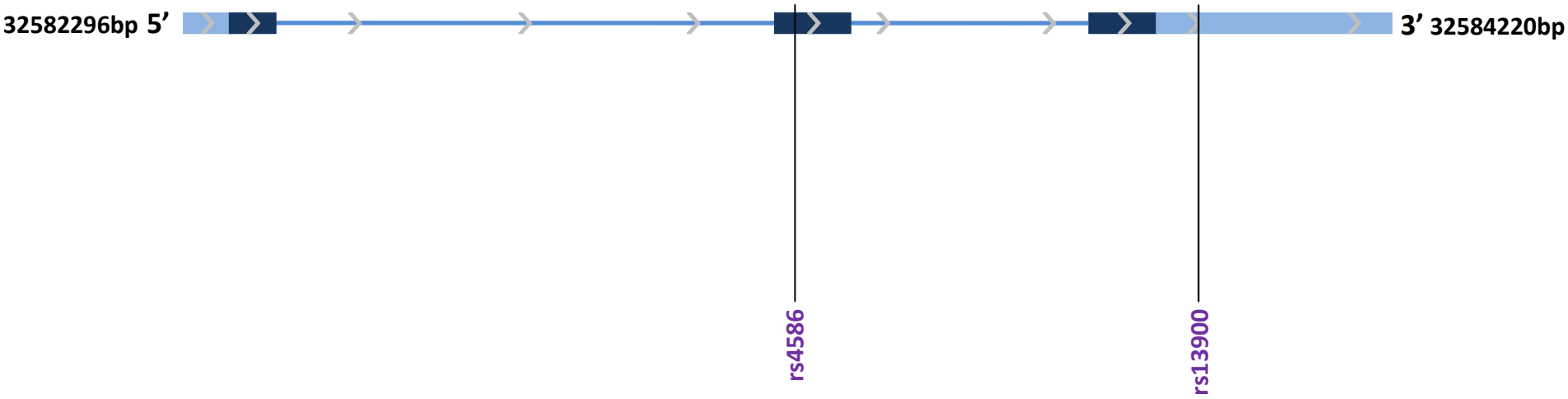

Figure 5.3. *CCL2* gene diagram with the SNPs included in analysis annotated on the diagram. *[SNPs in purple indicate the tagSNPs. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

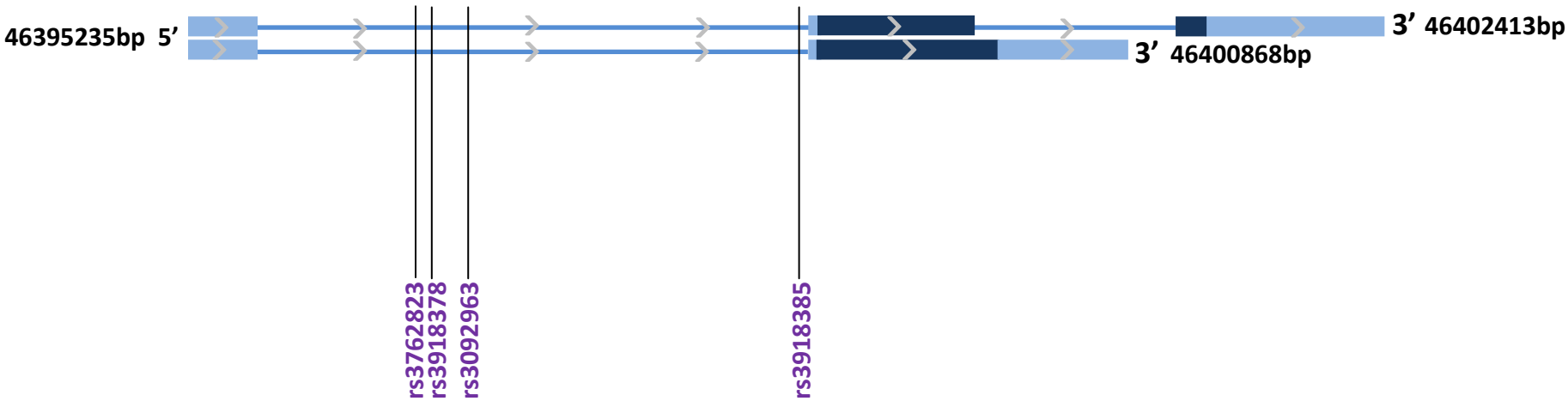

Figure 5.4. *CCR2* gene diagram with the SNPs included in analysis annotated on the diagram. *[SNPs in purple indicate the tagSNPs. The introns are represented by the thin blue lines; the coding regions of the exons are represented by the dark blue blocks; and the rest of the exons are represented by the light blue blocks.]* (Information obtained from The NCBI Gene Resource (build 37.1) (http://www.ncbi.nlm.nih.gov/gene))

Table 5.7. Details and summary statistics of the *CCL2* tagSNPs included in analysis

SNP ID	Position (bp) [a]	Allele 1 [b]	Allele 2 [b]	Minor Allele (sample)	MAF (sample) [c]	HWE (UNAFF) [d]	HWE (ALL) [e]
rs13900	32583911	C	T	T	0.32	1.00	1.00
rs4586	32583269	T	C	T	0.17	1.00	0.36

Table 5.8. Details and summary statistics of the *CCR2* tagSNPs included in analysis

SNP ID	Position (bp) [a]	Allele 1 [b]	Allele 2 [b]	Minor Allele (sample)	MAF (sample) [c]	HWE (UNAFF) [d]	HWE (ALL) [e]
rs3092963	46396938	A	G	G	0.33	0.60	0.72
rs3762823	46396616	G	A	A	0.09	1.00	1.00
rs3918378	46396706	G	T	T	0.15	0.03	0.10
rs3918385	46398911	A	G	G	0.10	1.00	1.00

[a] Position on chromosomes 17 (CCL2) and 3 (CCR2)

[b] BioMart assigned alleles

[c] Minor allele frequency of the SNP in the portion of the sample used for pain susceptibility (case-control analysis – pain+ vs. pain-) and intensity (cohort quantitative trait analysis – only HIV-SN+ with pain) association analysis

[d] Used to determine exclusion of SNPs due to deviation from HWE in the pain susceptibility (case-control) analysis. Individuals included in calculation are all individuals with HIV-SN and without pain (n=15)

[e] Used to determine exclusion of SNP due to deviation from HWE in the pain intensity (cohort quantitative trait) analysis. Individuals included in calculation are all individuals included in the pain analyses (n=158)

5.2.3.1 Single SNP association analysis for pain susceptibility

- *CCL2*

Neither of the SNPs in *CCL2* passed the univariate threshold of $P_{EMP1}<0.05$ in the allelic, genotypic, dominant or recessive models. There is thus no significant independent association between pain susceptibility and the alleles or genotype present; having at least one minor allele versus not having any minor allele; or having two minor alleles versus having at least one major allele at either SNP in *CCL2*.

- *CCR2*

Table 5.9. *CCR2* pain susceptibility SNP association results

MODEL	SNP	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
Genotypic	**rs3918378**	0.047	-	0.428	0.145	-	-
Dominant	**rs3918378**	0.037	-	0.035*	0.035	0.32	↓

The asterisk (*) indicates that the SNP is significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

Genotypic model:

One SNP passed the univariate threshold of $P_{EMP1}<0.05$ in the genotypic model and was thus taken through to multivariate analysis. It was no longer associated with pain susceptibility after correcting for age, gender and CD4 T-cell count.

Dominant model:

One SNP passed the univariate threshold of $P_{EMP1}<0.05$ in the dominant model and was thus taken through to multivariate analysis. It still associated with pain susceptibility after correcting for age, gender and CD4 T-cell count and it also passed the family-wise correction for multiple comparisons ($P_{EMP2}<0.05$). Carriage of the minor allele of this SNP (T) was associated with a decreased risk of developing pain (OR<1.0).

Allelic and recessive models:

None of the SNPs in *CCR2* passed the univariate threshold of $P_{EMP1}<0.05$ in the allelic or recessive models. There is thus no significant independent association between pain susceptibility and the alleles present or having two minor alleles versus having at least one major allele at any of the SNPs in *CCR2*.

An assessment of the BeadStudio SNP graph scatter plot for rs3918378 showed that, although clear clusters can be seen, the heterozygous cluster is in quite close proximity to one of the homozygous clusters and the results for this SNP should perhaps be treated with caution (**Appendix I**).

Post-analysis assessment of significant findings – pain susceptibility

The population allele frequencies for rs3918378 in the five populations are shown in **Figure 5.5**.

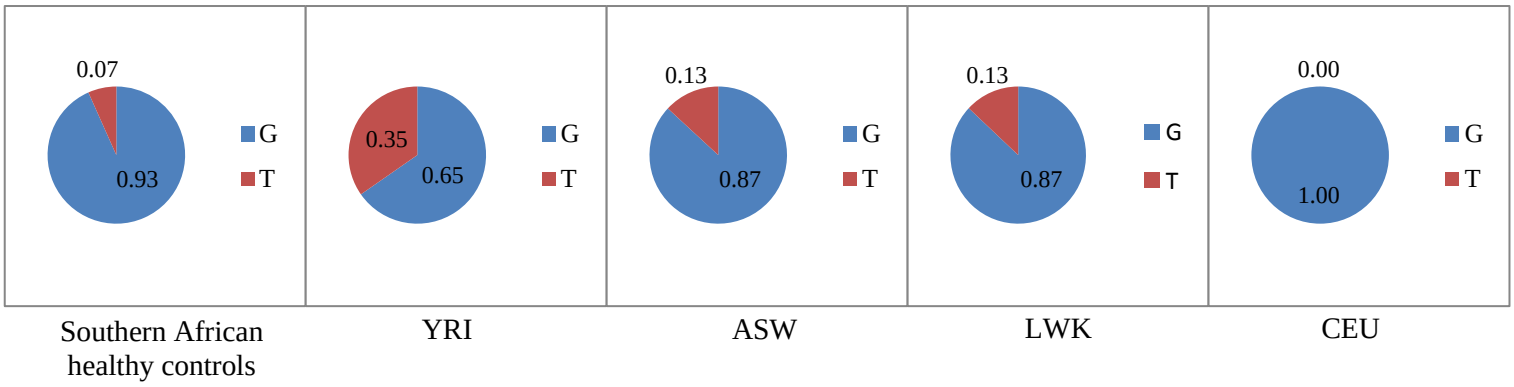

Figure 5.5. Allele frequency comparison of rs3918378 among five populations

The SNP is not tagged along with any other SNPs in the YRI population and is monomorphic in the CEU population. Input of rs3918378 into RegulomeDB revealed that it is likely to affect binding of transcription factors, thus influencing gene expression.

5.2.3.2 Haplotype association analysis for pain susceptibility

- *CCL2*

The two SNPs combined did not pass the univariate threshold of $P_{EMP1}<0.05$.

- *CCR2*

Two-, three- and four-SNP haplotypes in *CCR2* were constructed using the four SNPs. Numerous haplotypes passed the univariate threshold of $P_{EMP1}<0.05$, but only two still associated with pain susceptibility after correcting for age, gender and CD4 T-cell count, although neither haplotype passed the family-wise correction for multiple comparisons ($P_{EMP2}>0.05$ in both cases) (**Table 5.10**).

Table 5.10. *CCR2* pain susceptibility haplotype association results

HAPLOTYPES				HAPLOTYPE FREQUENCIES	UNIVARIATE		MULTIVARIATE			RISK OF PAIN
rs3762823	rs3918378	rs3092963	rs3918385		P_{EMP1}	OR	P_{EMP1}	P_{EMP2}	OR	
G	G	G	G	0.10	0.031	0.36	0.059	0.454	0.41	↓
G	G	G		0.24	0.026	0.42	0.010*	0.073	0.32	↓
G		G	G	0.10	0.031	0.36	0.059	0.454	0.41	↓
	G	G	G	0.10	0.032	0.36	0.059	0.454	0.32	↓
G	G			0.77	0.031	0.24	0.997	1.00	<0.01	↓
G		G		0.24	0.041	0.42	0.010*	0.075	0.32	↓
G			G	0.10	0.031	0.36	0.059	0.454	0.41	↓
	G		G	0.10	0.038	0.37	0.060	0.436	0.40	↓
		G	G	0.10	0.042	0.37	0.062	0.458	0.41	↓

Asterisks (*) indicate those SNPs that are significant following correction for covariates.
P_{EMP1} – pointwise empirical p-value
P_{EMP2} – Family-wise correction for multiple comparisons
OR – odds ratio

5.2.3.3 Single SNP association analysis for pain intensity

No SNPs passed the univariate threshold of $P_{EMP1}<0.05$ in *CCL2* or *CCR2* and therefore no SNPs were taken through to multivariate analysis. There is thus no significant independent association between pain intensity and the alleles present at any of the SNPs in *CCL2* or *CCR2*.

5.2.3.4 Haplotype association analysis for pain intensity

No haplotypes passed the univariate threshold of $P_{EMP1}<0.05$ in *CCL2* or *CCR2*.

5.3 Discussion

Analysis carried out in *IL1B, IL6, CCL2* and *CCR2* showed very limited associations with pain susceptibility and pain intensity.

5.3.1 *IL1B*

Neither SNPs nor haplotypes in *IL1B* were associated with pain susceptibility. No individual SNPs associated with pain intensity either, although two haplotypes, both which contain rs1143633 and rs3136558, associated with increased pain intensity. As with *KCNS1*, this may suggest that the haplotypes incorporate the causative SNP(s) and a more detailed investigation needs to be carried out on this gene. Although the initial tagging efficiency is 100%, it is possible that there are additional, undiscovered SNPs in *IL1B* that are perhaps unique to the Southern African population. Sequencing of the gene may reveal novel SNPs that may have some association with pain susceptibility and pain intensity. A more detailed review of the literature revealed that rs1143634 has previously been associated with lower back pain, and more specifically the number of days that an individual experiences pain, in a Finnish cohort (Solovieva et al., 2004). There was, however, no individual association between this SNP and pain susceptibility or pain intensity, although it was contained in one of the haplotypes associated with pain intensity. It is likely though that the association between this SNP and pain is population-specific or model-specific and shows no association with pain associated with HIV-SN in a Southern African population.

5.3.2 *IL6*

No individual SNPs in IL6 associated with pain susceptibility or pain intensity. Several haplotypes, however, did associate with pain susceptibility and pain intensity, suggesting, once again, that the haplotypes could incorporate the causative SNP(s). The initial tagging efficiency for *IL6* was 68%. It is highly possible that an important association may have been missed and further studies with better coverage of the gene need to be carried out.

5.3.3 *CCL2* and *CCR2*

CCL2 showed no association with pain susceptibility or pain intensity. This does, however, not mean that there is no association. Only two SNPs came up in tagSNP selection and were genotyped and included in analysis. Although there was 100% tagging efficiency, this is only based on those SNPs that have been identified and genotyped. The two SNPs may have been insufficient to detect any effect that *CCL2* has on pain susceptibility and pain intensity. There may be other SNPs in *CCL2* that require identification through sequencing.

CCR2, whose gene product is functionally very closely linked to the gene product of *CCL2*, also showed no association with pain intensity, but showed a more positive association with pain susceptibility. The minor allele (T) of one SNP in particular, rs3918378, was associated with decreased pain susceptibility in the dominant model. This association has not previously been reported.

A comparison of the allele frequencies in rs3918378 among different populations reveals some clear differences (**Figure 5.5**). The T-allele is the minor allele in the current sample with a MAF of 0.07 in healthy controls. This frequency may be lower than expected due to the small number of healthy controls in the pain samples. The frequency is most comparable to the ASW and LWK (both 0.13) populations. The MAF in the YRI population (0.35) is much higher than in all other African populations investigated. If the G-allele (major allele in this Southern African sample) is the allele responsible for increased pain susceptibility, this could suggest that there is an elevated risk or predisposition in Southern Africans to develop pain compared to the other African populations. The SNP is monomorphic in the CEU population. An assessment in

Haploview reveals that, in the YRI population, rs3918378 does not tag any other SNPs, suggesting that it is not in LD with any other SNPs in the YRI population under the settings specified. Again, if the assumption is made that the current Southern African sample has the same genetic structure to the YRI population, this could suggest that the SNP may itself be functionally associated with the pain risk. Alternatively, it may be in LD with rare SNPs yet to be identified and documented and which need to be identified through sequencing or which are lost in the selection of tagSNPs due to the settings used. rs3918378 is located in an intronic region of *CCR2*, a gene encoding a receptor for monocyte chemoattractant protein-1 (or CCL2), a chemokine which controls monocyte chemotaxis (www.ncbi.nlm.nih.gov/gene). Input of the SNP into RegulomeDB revealed that it is likely to affect binding. It is possible therefore that this SNP may play a direct functional role in determining pain risk, although further, functional studies should be conducted to confirm this.

rs3918378 was also found to be present in one of the two haplotypes associated with pain susceptibility after correction for age, gender and CD4 T-cell count. Correction for this SNP leads to the haplotype being no longer significantly associated with pain susceptibility, suggesting that rs3918378 is either the causative SNP or in LD with the causative SNP(s).

The initial tagging efficiency for *CCR2* was 100%. As with *CCL2* only a small number of SNPs were studied and there may be other SNPs in the gene which require identification through sequencing.

5.4 Conclusions

The only individual SNP association seen was between a SNP in *CCR2* and pain susceptibility. Haplotypes of *IL6* and *CCR2* also associated with pain susceptibility and haplotypes of *IL1B* and *IL6* associated with pain intensity. No definite conclusions can be made, however, until complete coverage of the genes is obtained, in the cases where the tagging efficiency is not 100%, or until sequencing is carried out to determine possible novel variants, in cases where the number of SNPs studied for the particular gene(s) was quite low. Functionally, gene products of *IL1B*, *IL6*, *CCL2* and CCR2 have been found to play a role in neuropathic pain, as previously outlined in Chapter 1. It would therefore not be surprising if polymorphisms in these genes are associated with altered pain susceptibility and pain intensity. More detailed genetic investigations need to be carried out in all of the genes to determine their genetic role in neuropathic pain.

CHAPTER 6

GENERAL CONCLUSIONS

Positive associations were found between SNPs and haplotypes in the *TNFA* region and HIV-SN susceptibility. In addition significant associations were found between the *TNFA* region, *IL12B*, *KCNS1*, *IL6* and *CCR2* and pain susceptibility. Pain intensity analysis revealed significant associations between the *TNFA* region, *KCNS1*, *IL1B* and *IL6* and pain intensity. No associations were found between *GCH1* and *CCL2* and HIV-SN susceptibility, pain susceptibility or pain intensity. The findings support some previous studies where associations were found between *TNFA* and surrounding genes and HIV-SN risk and *KCNS1* and pain intensity as well as the role of *IL1B*, *IL6* and *CCR2* in neuropathic pain. However, the SNPs and haplotypes responsible for the associations are different to those previously reported. Additionally, in some instances, as in the case of *GCH1*, the previously reported positive association could not be replicated at all, even with the use of population-specific tagSNPs.

The reasons for the conflicting results could be due to one or more of the following reasons. Firstly, the associations may be population-specific. The genes and polymorphisms previously reported in non-African populations may have no association with HIV-SN susceptibility or pain susceptibility and intensity in African individuals. This is likely due to the substructure observed in the genome of different populations, with African individuals being genetically a lot more diverse than non-African individuals. The level of LD in African populations is significantly lower than in non-African populations resulting in more specific and a larger density of SNPs required to obtain the same amount of haplotype coverage (Campbell and Tishkoff, 2008). The difference in LD structure and the fact that different SNPs may be in LD with the causal SNP in the different populations, suggests that different SNPs may be associated in different populations and markers identified in other populations as being associated with disease susceptibility or intensity may in fact have no association in

an African population. Secondly, the associations may be model-specific. Many of the previously reported associations for pain sensitivity have not been in HIV-positive individuals with painful HIV-SN and therefore genes and polymorphisms reported to be associated with other forms of pain may not be associated with painful HIV-SN if the dominant regulatory pathways that mediate the pain hypersensitivity differ between pain syndromes.

6.1 Limitations

There were several limitations to the study. Firstly, the sample size is relatively small (n=340 for HIV-SN analysis and n=158 for pain analyses), which is a disadvantage, especially in genetic association studies. Small samples may not have enough power to detect very small effects that may be present when looking at the association of a particular gene and polymorphisms within the gene with the disease/condition/trait in question. This may lead to important associations being overlooked or, when associations are found (as indicated by significant p-values), it is not certain whether these are true findings or merely random significant p-values (Pickrell et al., 2011). The number of individuals without pain in the sample is very small (n=15). This group acts as the control group and may not be of adequate size to act as a representative control group to the case group. For example, the allele frequency in the control group may be unrepresentative of the overall population, leading to spurious associations. Secondly, the method of sampling used (convenience sampling) may have introduced some bias into the sample as the sample was not chosen at random and conclusions drawn may only apply to the sample of individuals chosen. The choice of sampling may also have lead to the sample being non-representative of the population (in this case HIV-positive black Southern African individuals). Any conclusions made may not be a

true reflection of what exists in the actual population and should be treated with caution. Thirdly, genotyping failure and tagging inefficiency lead to a reduction in the sample size and gene coverage, respectively. Loss of data due to failed genotyping reduced the already small sample size. Any tagging efficiency that is not 100% could have resulted in significant associations being missed. Some SNPs also had to be excluded due to genotyping failure. Lastly, tagSNP selection was carried out using the YRI population. It is possible that the genetic structure of individuals in Southern Africa is different to that of individuals in the YRI population. The tagging may, therefore, not have been accurate and, again, important SNPs may have been missed. This is seen by the fact that genes previously thought to play a role in the particular phenotype are now not seen to be associated when analysing the selected SNPs. There is perhaps an association, but with SNPs specific to the Southern African population that would not have been selected from the YRI population.

6.2 Future work

Future work in this area of research could include conducting the study in a much bigger sample to adequately power the study. Additionally, when a Southern African dataset becomes available, tagSNPs could be selected using this dataset. A more complete coverage of the genes investigated should also be achieved and, when genotyping failure occurs, individuals should, if possible, be re-genotyped to prevent a reduction in sample size or a loss of SNPs from the study. Some SNPs were originally excluded from the SNPs to be genotyped based on the fact that they could not be genotyped by the genotyping method chosen. Alternative genotyping methods could be employed to genotype these SNPs and thus overcome the unnecessary exclusion of SNPs which may have been significant. This was not possible in the current study due to time and cost constraints. The SNPs responsible for the

altered susceptibility or sensitivity may not be present in the list of recorded SNPs. Sequencing entire genomes could be conducted to identify novel variants, particularly in the genes of interest. There are, however, limitations to this, namely the high cost involved in sequencing whole genomes and the extremely large sample size that would be required. Lastly, although significant associations have been found, the actual functional role that these variations in the gene play is largely unknown. Genetic association studies need to be coupled with functional studies to determine the role that these identified polymorphisms play in altering the gene product or regulation and binding associated with the gene which ultimately causes the variations in disease susceptibility and intensity.

The current study has allowed for an investigation into the role of genetics in the susceptibility for HIV-SN and the variability of pain susceptibility and intensity in HIV-positive individuals of black Southern African ancestry. The research has revealed that, although some previously reported genes associated with HIV-SN susceptibility, pain susceptibility or pain intensity in non-African populations are also associated in the Southern African population, the SNPs and haplotypes responsible for these associations are different. This highlights the importance of conducting genetic association studies in independent populations and ethnic groups.

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UCSC Genome Browser (Assembly: Feb. 2009 (GRCh37/hg19)) – Available from: http://genome.ucsc.edu/cgi-bin/hgGateway (First accessed 24 January 2012)

Appendix A – Ethical approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Liesl Hendry

CLEARANCE CERTIFICATE	M110754
PROJECT	Genetics of Pain Sensitivity Associated with and Susceptibility to the Development of Anti-Retroviral Toxic Neuropathy in Human Immunodeficiency Virus (HIV) Positive Individuals of Black Ancestry
INVESTIGATORS	Ms Liesl Hendry.
DEPARTMENT	School of Pathology/Div of Human Genetics
DATE CONSIDERED	Ad hoc
M1107540DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 15/07/2011 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Zane Lombard

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 B - Equipment, kit and reagent details

Equipment	Manufacturer	Country
2720 Thermal Cycler	Applied Biosystems	USA
3130*xl* Genetic Analyzer	Applied Biosystems	USA
7900HT Fast Real-Time PCR System	Applied Biosystems	USA
Allegra® X-15R Centrifuge	Beckman Coulter	USA
BeadXpress® Reader	Illumina	USA
Centrifuge 5810	Eppendorf	Germany
Freedom EVO®	Tecan	Switzerland
G:BOX Gel Documentation System	Vacutec, Syngene	UK
Heat Sealer	Eppendorf	Germany
Hybex® Microsample Incubator	SciGene	USA
Infinite® 200 PRO NanoQuant	Tecan	Switzerland
Milli-Q Biocel System	Merck Millipore	Germany
Millivac™ Maxi Vacuum Pump	Millipore	USA
MixMate®	Eppendorf	Germany
Quantum® EX Ultrapure Organex Cartridge	Merck Millipore	Germany
VorTemp™ 56 Shaking Incubator	Labnet	USA

Reagent/Kit	Manufacturer	Country
10 X VeraCode Read Buffer	Illumina	USA
10% Ficoll	Promega	USA
2-Propanol	Sigma	USA
96-plex BeadXpress Kit	Illumina	USA
Agarose Molecular Grade (DNase/RNase-free)	Bioline	UK
AmpliTaq Gold®	Applied Biosystems (Roche)	USA
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems	USA
DyeEx™ 2.0 Spin Kit	QIAGEN	Germany
EDTA Disodium Salt	Merck	Germany
Ethanol Absolute	Merck	Germany
Ethidium Bromide	Sigma-Aldrich	USA
GeneAmp® 10X PCR Gold Buffer	Applied Biosystems (Roche)	USA
GeneAmp® dNTPs	Applied Biosystems (Roche)	USA
GeneRuler™ 50bp DNA ladder	Fermentas	Germany
$MgCl_2$	Applied Biosystems (Roche)	USA
Nucleospin® Extract II Kit	MACHEREY-NAGEL GmbH & Co. KG	Germany
Potassium Hydroxide Pellets	Sigma-Aldrich	USA
Quantifiler™ Human DNA Quantification Kit	Applied Biosystems	UK
TaqMan® SNP genotyping assay	Applied Biosystems	USA
Tris Base	Roche	USA
VeraCode Test and Calibration Kit	Illumina	USA

Appendix C – Sequencing protocol

PCR CLEAN UP

1. PCR amplify samples.

2. Check if PCR product is present AND if only one band is present on agarose gel.

NUCLEOSPIN
From PCR product **(If there are no spurious bands)**
⇒ Adjust PCR product's volume to 50 µl with TE buffer (pH = 7.5).
⇒ Mix 2 volumes of NT buffer with 1 volume PCR product:
∴100 µl NT + 50 µl PCR product
⇒ Place column in collection tube and load sample onto column.
⇒ Centrifuge: 1 min @ 14 000 rpm
⇒ Discard flow-through and place column back in collection tube.

⇒ Pipette **600 µl NT3 buffer** onto column.
⇒ Centrifuge: 1 min @ 14 000 rpm
⇒ Discard flow-through and place column back in collection tube.

⇒ Centrifuge: 2 min @ 14 000 rpm
⇒ Incubate samples for 2-5 min @ 70ºC (to evaporate ETOH).
⇒ Discard flow-through and place column into a clean Eppendorf tube.

⇒ Pipette **15-50 µl ddH_2O** (volume equal to starting volume)
⇒ Incubate samples for 1 min @ RT (to increase yield).
⇒ Centrifuge: 1 min @ 14 000 rpm.
⇒ Discard column.

CYCLE SEQUENCING

Set up the cycle sequence rxn.
a. One tube for forward and one tube for reverse for each PCR product.
b. Remember product is light sensitive

⇒ Cycle sequencing PCR mix ($1/8^{th}$ reaction):

2 µl	cleaned PCR product
1 µl	Bigdye v3.1 (in -20°C freezer with Taq)
1.5 µl	5xBigdye buffer (in 4°C coke fridge)
1 µl	Primer (forward OR reverse – not both!)
4.5 µl	ddH_2O
10µl	

NB: If sequencing reaction fails either dilute PCR product 1/10 or add 1u PCR product and 2 µl **Big Dye.**

⇒ Cycle sequencing PCR program:

30 sec	@	96ºC	} 25 cycles
15 sec	@	50ºC	
4 min	@	60ºC	
Hold	@	24ºC	

CYCLE SEQUENCING PRODUCT CLEAN UP AND PREPARATION

QIAGEN DyeEX

(NB: Switch the cooling unit of the vacuum system on ½ hour before use)

- Loosen column cap by ½ turn vortex to resuspend resin then snap of the bottom of column tube.
- Place column in a collection tube.
- Centrifuge: 3 min @ 3000 rpm
- Discard eluent and place column in a clean collection tube.
- Transfer column to a clean tube
- Carefully apply the sequencing reaction to the gel bed in the centre without piercing the column.
- Centrifuge: 3 min @ 3000 rpm
- Remove the column from the tube - the eluant contains the purified DNA
- Dry (concentrate) samples under vacuum without heat, for approximately 1 hour with sample lids open.
- Remove dried samples and store in the dark at 4ºC(Remember product is light sensitive)
- Add 10µl HiDye (aliquots in the -20ºC chest freezer) to each sample no more than an hour before sequencing, making sure the dried product is re-dissolved in the dye.
- Denature samples for 2 min at 94ºC and keep on ice until loaded on the capillary sequencer

CAPILLARY SEQUENCER

SET UP A RUN

1. Expand "Foundation data collection window on bottom of screen
2. Select "Plate manager" and Click NEW
    a. Give plate a name without any spaces
    b. Leave description blank
    c. Application – Sequence analysis
    d. Plate type – 96-well
    e. Owner – sero
    f. Operator – Own name
3. Click OK
4. Sequencing analysis plate editor window will open
5. Enter your sample ID's in the first column of the window
6. Fill in the blanks so you have complete runs which are 2 columns/runs
7. Fill in the other columns:
    a. Priority – 100

b. Results group – "sequencing'
c. Instrument protocols – Z_Seq POP7-36 Ultra (for shorter sequences 400-500 bp) or Rapid (for longer sequences 700-900bp)
d. Analysis protocol – POP7_BDTv3 kb

8. Click OK. Go to Plate view and hit "Find All" then look for your plate ID
9. Select your plate then right click on the appropriate plate on the display of the two plates in the sequencer to link your data to your plate

LOADING SAMPLE

1. After denaturing and chilling plate, place special sequencing cover on plate with black mark on lid aligned with the notch on the plate.
2. Snap the plate into the sequencing adaptor with the notches on plate and black base aligned. Place the white adaptor lid on so the A on the lid aligns with notch on base.
3. If plate is clipped on properly then the round spaces should all be black when you look through the assembly.
4. Make sure the green light on the sequencer is not blinking indicating that noo runs are in progress
5. Press "Tray" button and wait until the tray moves forward.
6. Open the door
7. Place the plate assembly into position with notch towards the back of machine ensuring tha tit lies flush with the buffer chambers in the front of the machine.
8. Check the buffer levels in the chambers which should be on or above the black lines.
9. Open the door on the right side of the sequencer and check the probe in the feeder bottle for bubbles. If there is a bubble remove the bottle gently and replace – bubble should disappear. Close both doors of the sequencer

STARTING RUN

1. On plate view, find and select your plate then right click on the yellow box indicating the filled plate positions in the sequencer. The plate should turn green
2. Click the green button on the top panel fo the window to start the run
3. On instrument status on the menu on the right it'll show what the machine is doing
4. Leave the computer on **INSTRUMENT SETTING** for the duration of the run.

VIEWING SEQUENCES

1. On desktop, click "shortcut to data"
2. Open the folder you saved the run under - "sequencing" to view the raw data
3. To analyse the samples, Open sequence analysis on the desktop
4. **User**: 3130x1 seq **Password** – Sequencer
5. Under File select "Add samples"
6. Under "sequencing" open your run, select all, ADD selected samples and Press OK. NB: in the results under the column SPACING the value should be 12-14 if sequence runs worked well
7. Click PLAY button to analyze the samples (base call)
8. In SAMPLE MANAGER view you can select SHOW to view individual sequences or select " P" box to print individual sequences. Push PLAY to print the selected sequences.
9. After checking all sequences close window and Save "Yes to all"
10. Open shortcut to data and select your folder. All three sequence files will be shown and you can select and move to a flash drive.

Appendix D – Full results set: Chapter 3

TNFA region – Single SNP analysis

UNIVARIATE - HIV-SN									
SNP	ALLELIC MODEL			GENOTYPIC MODEL		DOMINANT MODEL		RECESSIVE MODEL	
	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}
rs2734586	0.821	1.000	0.96	0.793	1.000	0.899	1.000	0.466	1.000
rs2075582	0.028	0.574	0.58	**0.032**	0.550	0.091	0.831	**0.002**	0.259
rs3130056	0.997	1.000	0.99	0.506	1.000	0.705	1.000	0.502	1.000
rs11796	0.027	0.497	0.70	0.053	0.725	**0.020**	0.319	0.288	0.994
rs2734583	0.320	0.999	1.26	0.204	0.993	0.169	0.956	0.331	1.000
rs2239709	0.087	0.942	0.59	0.106	0.942	0.096	0.907	1.000	1.000
rs11757236	0.746	1.000	1.39	0.760	1.000	0.758	1.000	1.000	1.000
rs2079170	-	-	-	-	-	-	-	-	-
rs3130059	0.018	0.366	0.67	**0.019**	0.376	**0.008**	0.107	0.411	1.000
rs2844509	0.167	0.974	0.72	0.210	0.995	0.089	0.861	0.901	1.000
rs9267487	0.560	1.000	0.85	0.495	1.000	0.472	1.000	0.890	1.000
rs2251824	0.191	0.993	0.74	0.446	1.000	0.229	0.993	0.665	1.000
rs2239705	0.522	1.000	0.78	0.346	1.000	0.380	1.000	0.682	1.000
rs3219185	0.379	1.000	0.77	0.360	1.000	0.594	1.000	0.190	0.961
rs2071592	0.008	0.179	0.64	**0.008**	0.187	**0.004**	0.049	0.237	0.991
rs6929796	0.309	0.999	0.83	0.558	1.000	0.293	1.000	0.746	1.000
rs2230365	0.695	1.000	0.86	0.683	1.000	0.688	1.000	1.000	1.000
rs28445017	0.257	0.999	1.92	0.221	1.000	0.250	1.000	1.000	1.000
rs4947324	0.047	0.743	0.65	**0.004**	0.124	**0.014**	0.307	0.223	0.985
rs2516390	0.106	0.946	0.66	0.084	0.842	0.065	0.807	0.946	1.000
rs915654	0.228	0.996	0.82	0.112	0.931	0.856	1.000	**0.044**	0.385
rs2516312	0.124	0.960	0.52	0.103	0.965	0.131	0.928	1.000	1.000
rs2844482	0.438	1.000	0.84	0.543	1.000	0.594	1.000	0.314	0.998
rs2239704	0.151	0.968	0.68	0.081	0.909	0.102	0.868	0.731	1.000
rs2229094	0.056	0.692	0.71	0.134	0.955	0.070	0.761	0.252	0.986
rs1041981	0.033	0.595	0.71	**0.040**	0.620	**0.017**	0.215	0.494	1.000
rs3093544	-	-	-	-	-	-	-	-	-
rs1799964	0.462	1.000	0.86	0.743	1.000	0.490	1.000	0.672	1.000
rs1800630	0.972	1.000	1.00	0.375	1.000	0.800	1.000	0.184	0.993
rs1800629	0.493	1.000	1.15	0.808	1.000	0.495	1.000	1.000	1.000
rs2228088	0.320	0.999	0.78	0.284	1.000	0.506	1.000	**0.024**	0.893
rs3093662	0.227	0.987	0.71	0.138	0.965	0.133	0.939	0.573	1.000
rs3093665	0.246	0.989	0.61	0.113	0.949	0.143	0.975	0.622	1.000
rs3093671	0.415	1.000	0.82	0.733	1.000	0.443	1.000	0.761	1.000
rs7769073	0.667	1.000	0.91	0.888	1.000	0.746	1.000	0.558	1.000
rs2256974	0.370	0.999	1.15	0.660	1.000	0.430	1.000	0.622	1.000
rs1052248	0.983	1.000	1.01	0.890	1.000	0.900	1.000	0.738	1.000
rs3179003	0.979	1.000	1.01	0.999	1.000	0.975	1.000	0.978	1.000

SNP	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
	ALLELIC MODEL			GENOTYPIC MODEL		DOMINANT MODEL		RECESSIVE MODEL		ALLELIC MODEL		
	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	BETA
rs2734586	0.761	1.000	1.41	1.000	1.000	0.772	1.000	1.000	1.000	0.362	1.000	0.51
rs2075582	0.719	1.000	0.86	0.755	1.000	0.742	1.000	1.000	1.000	0.947	1.000	-0.05
rs3130056	0.227	0.968	0.47	0.135	0.890	0.639	1.000	0.081	0.534	0.989	1.000	-0.02
rs11796	0.974	1.000	1.09	0.166	0.941	0.432	1.000	0.383	0.973	0.804	1.000	0.10
rs2734583	0.289	0.993	2.68	0.532	1.000	0.226	0.972	1.000	1.000	0.246	0.993	0.61
rs2239709	0.900	1.000	1.54	1.000	1.000	0.875	1.000	1.000	1.000	0.427	1.000	-0.71
rs11757236	0.130	0.896	0.25	0.133	0.905	0.132	0.867	1.000	1.000	0.105	0.892	-2.01
rs2079170	0.770	1.000	NA	0.998	1.000	1.000	1.000	1.000	1.000	-	-	-
rs3130059	0.536	1.000	1.44	0.229	0.983	0.290	0.990	0.637	0.998	0.397	1.000	0.34
rs2844509	1.000	1.000	1.04	0.780	1.000	1.000	1.000	1.000	1.000	0.953	1.000	0.03
rs9267487	0.164	0.912	0.44	0.208	0.962	0.111	0.816	0.914	1.000	0.440	1.000	-0.53
rs2251824	0.551	1.000	1.81	0.746	1.000	0.744	1.000	0.999	1.000	0.585	1.000	0.33
rs2239705	0.947	1.000	NA	0.942	1.000	0.947	1.000	0.917	1.000	0.910	1.000	-0.14
rs3219185	0.585	1.000	2.18	0.581	1.000	0.578	1.000	1.000	1.000	0.685	1.000	-0.31
rs2071592	0.542	1.000	1.45	0.182	0.955	0.190	0.933	0.630	0.997	0.377	1.000	0.35
rs6929796	0.657	1.000	1.37	0.820	1.000	0.583	1.000	0.878	1.000	0.288	1.000	-0.43
rs2230365	0.898	1.000	NA	0.585	1.000	0.612	1.000	1.000	1.000	0.142	0.967	1.59
rs28445017	0.595	1.000	NA	0.589	1.000	0.600	1.000	1.000	1.000	**0.036**	0.598	2.05
rs4947324	0.334	1.000	0.63	0.340	1.000	0.170	0.995	1.000	1.000	0.654	1.000	-0.27
rs2516390	1.000	1.000	1.19	0.964	1.000	0.942	1.000	0.865	1.000	0.839	1.000	0.14
rs915654	0.332	1.000	1.45	0.724	1.000	0.541	1.000	0.683	0.999	0.365	1.000	-0.36
rs2516312	0.586	1.000	NA	0.627	1.000	0.650	1.000	1.000	1.000	0.827	1.000	0.23
rs2844482	0.390	1.000	2.25	0.597	1.000	0.366	0.999	0.995	1.000	0.339	1.000	0.51
rs2239704	0.918	1.000	1.09	0.911	1.000	0.884	1.000	0.741	1.000	0.994	1.000	0.01
rs2229094	0.998	1.000	1.04	0.621	1.000	0.999	1.000	0.523	0.990	0.478	1.000	0.33
rs1041981	0.880	1.000	0.99	0.165	0.952	0.471	1.000	0.113	0.842	0.634	1.000	0.19
rs3093544	0.936	1.000	NA	0.934	1.000	0.935	1.000	0.905	1.000	-	-	-
rs1799964	0.998	1.000	1.16	0.440	1.000	0.734	1.000	0.467	0.984	0.328	1.000	0.46
rs1800630	0.756	1.000	1.70	0.762	1.000	0.746	1.000	1.000	1.000	0.184	0.972	0.85
rs1800629	0.654	1.000	1.51	0.328	1.000	0.350	0.999	0.540	0.993	0.220	0.991	0.57
rs2228088	0.950	1.000	1.36	1.000	1.000	0.997	1.000	1.000	1.000	0.123	0.909	-1.07
rs3093662	0.386	1.000	0.55	0.305	0.999	0.373	0.999	0.999	1.000	0.839	1.000	0.15
rs3093665	0.981	1.000	NA	0.999	1.000	0.998	1.000	0.999	1.000	0.467	1.000	0.88
rs3093671	1.000	1.000	1.30	0.952	1.000	0.997	1.000	0.854	1.000	0.908	1.000	0.08
rs7769073	1.000	1.000	1.35	0.973	1.000	0.969	1.000	0.916	1.000	0.531	1.000	0.43
rs2256974	0.255	0.998	0.65	0.527	1.000	0.713	1.000	0.304	0.941	0.115	0.891	-0.63
rs1052248	1.000	1.000	1.14	0.999	1.000	0.839	1.000	0.998	1.000	0.154	0.972	0.54
rs3179003	0.435	1.000	0.61	0.360	1.000	0.211	0.995	0.754	1.000	0.753	1.000	0.22

TNFA region – Haplotype analysis

HAPLOTYPE				UNIVARIATE – HIV-SN			UNIVARIATE - PAIN			UNIVARIATE – PAIN INTENSITY		
				OR	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	BETA	P_{EMP1}	P_{EMP2}
rs2734586	rs2075582	rs3130056	222	0.92	0.784	1.000	0.70	0.764	1.000	0.35	0.687	1.000
rs2734586	rs2075582	rs3130056	224	0.45	**0.011**	0.582	1.17	0.561	1.000	-0.56	0.586	1.000
rs2734586	rs2075582	rs3130056	344	0.99	0.973	1.000	2.30	0.283	1.000	0.67	0.231	1.000
rs2734586	rs2075582	rs3130056	244	1.28	0.182	1.000	0.87	0.698	1.000	-0.31	0.491	1.000
rs2075582	rs3130056	rs11796	221	0.81	0.578	1.000	0.71	0.730	1.000	0.35	0.665	1.000
rs2075582	rs3130056	rs11796	241	0.47	**0.026**	0.812	1.16	0.782	1.000	-0.56	0.580	1.000
rs2075582	rs3130056	rs11796	441	0.81	0.227	1.000	1.49	0.417	1.000	0.26	0.552	1.000
rs2075582	rs3130056	rs11796	444	1.48	**0.011**	0.503	0.92	0.967	1.000	-0.11	0.772	1.000
rs3130056	rs11796	rs2734583	443	1.20	0.407	1.000	2.76	0.117	0.992	0.61	0.230	1.000
rs3130056	rs11796	rs2734583	211	0.99	0.962	1.000	0.49	0.214	1.000	-0.03	0.968	1.000
rs3130056	rs11796	rs2734583	411	0.69	**0.026**	0.720	1.45	0.516	1.000	0.12	0.784	1.000
rs3130056	rs11796	rs2734583	441	1.24	0.174	1.000	0.63	0.185	0.999	-0.40	0.279	1.000
rs11796	rs2734583	rs2239709	114	0.49	**0.037**	0.862	1.55	0.543	1.000	-0.53	0.571	1.000
rs11796	rs2734583	rs2239709	432	1.22	0.377	1.000	2.76	0.117	0.992	0.61	0.230	1.000
rs11796	rs2734583	rs2239709	112	0.81	0.188	1.000	1.03	0.953	1.000	0.17	0.690	1.000
rs11796	rs2734583	rs2239709	412	1.23	0.189	1.000	0.61	0.205	0.999	-0.32	0.384	1.000
rs2734583	rs2239709	rs11757236	124	1.94	0.439	1.000	0.35	0.133	1.000	-1.29	0.358	1.000
rs2734583	rs2239709	rs11757236	142	0.49	**0.040**	0.908	17.70	0.221	1.000	-0.23	0.815	1.000
rs2734583	rs2239709	rs11757236	322	1.21	0.373	1.000	2.75	0.187	0.996	0.62	0.230	1.000
rs2734583	rs2239709	rs11757236	122	0.99	0.957	1.000	0.54	0.340	1.000	-0.16	0.719	1.000
rs2239709	rs11757236	rs2079170	222	-	-	-	5.98E+191	0.960	1.000	-	-	-
rs2239709	rs11757236	rs2079170	243	-	-	-	0.36	0.090	1.000	-	-	-
rs2239709	rs11757236	rs2079170	423	-	-	-	18.50	0.179	1.000	-	-	-
rs2239709	rs11757236	rs2079170	223	-	-	-	0.75	0.675	1.000	-	-	-
rs11757236	rs2079170	rs3130059	223	-	-	-	3.39E+81	0.961	1.000	-	-	-
rs11757236	rs2079170	rs3130059	233	-	-	-	1.28	0.569	1.000	-	-	-
rs11757236	rs2079170	rs3130059	432	-	-	-	0.23	0.082	0.955	-	-	-
rs11757236	rs2079170	rs3130059	232	-	-	-	0.89	0.711	1.000	-	-	-
rs2079170	rs3130059	rs2844509	233	-	-	-	1.37E+08	0.993	1.000	-	-	-
rs2079170	rs3130059	rs2844509	333	-	-	-	0.68	0.485	1.000	-	-	-
rs2079170	rs3130059	rs2844509	331	-	-	-	1.75	0.250	1.000	-	-	-
rs2079170	rs3130059	rs2844509	321	-	-	-	0.68	0.365	1.000	-	-	-
rs3130059	rs2844509	rs9267487	332	0.77	0.398	1.000	0.55	0.191	1.000	-0.24	0.749	1.000
rs3130059	rs2844509	rs9267487	334	0.67	0.198	1.000	2.35E+41	0.991	1.000	0.70	0.471	1.000
rs3130059	rs2844509	rs9267487	314	0.75	0.122	0.994	1.77	0.252	1.000	0.41	0.379	1.000
rs3130059	rs2844509	rs9267487	214	1.44	**0.021**	0.664	0.78	0.571	1.000	-0.21	0.582	1.000
rs2844509	rs9267487	rs2251824	141	0.76	0.253	1.000	1.71	0.389	1.000	0.30	0.589	1.000
rs2844509	rs9267487	rs2251824	323	0.76	0.379	1.000	0.55	0.233	1.000	-0.24	0.761	1.000
rs2844509	rs9267487	rs2251824	123	-	-	-	0.20	0.059	0.997	-2.25	0.215	1.000
rs2844509	rs9267487	rs2251824	343	0.71	0.269	1.000	2.18E+73	0.974	1.000	0.29	0.761	1.000
rs2844509	rs9267487	rs2251824	143	1.38	0.052	0.913	0.91	0.866	1.000	-0.04	0.917	1.000

rs9267487	rs2251824	rs2239705	431	0.83	0.657	1.000	4.28E+117	0.705	1.000	0.02	0.985	1.000
rs9267487	rs2251824	rs2239705	413	0.76	0.261	1.000	1.79	0.405	1.000	0.35	0.534	1.000
rs9267487	rs2251824	rs2239705	233	0.88	0.659	1.000	0.43	0.095	0.981	-0.49	0.504	1.000
rs9267487	rs2251824	rs2239705	433	1.30	0.157	0.997	1.01	0.981	1.000	0.01	0.979	1.000
rs2251824	rs2239705	rs3219185	332	0.95	0.851	1.000	2.35	0.470	1.000	-0.30	0.671	1.000
rs2251824	rs2239705	rs3219185	313	0.95	0.918	1.000	1.90E+09	0.893	1.000	-0.11	0.928	1.000
rs2251824	rs2239705	rs3219185	133	0.80	0.359	1.000	1.81	0.346	1.000	0.37	0.507	1.000
rs2251824	rs2239705	rs3219185	333	1.29	0.186	1.000	0.40	0.159	0.984	-0.10	0.815	1.000
rs2239705	rs3219185	rs2071592	321	0.12	0.118	0.997	-	-	-	-	-	-
rs2239705	rs3219185	rs2071592	131	0.95	0.935	1.000	1.15E+08	0.766	1.000	-0.14	0.917	1.000
rs2239705	rs3219185	rs2071592	331	0.67	**0.022**	0.579	1.30	0.608	1.000	0.41	0.312	1.000
rs2239705	rs3219185	rs2071592	324	1.00	0.990	1.000	2.39	0.452	1.000	-0.32	0.656	1.000
rs2239705	rs3219185	rs2071592	334	1.56	**0.004**	0.255	0.60	0.248	0.999	-0.26	0.481	1.000
rs3219185	rs2071592	rs6929796	241	0.85	0.644	1.000	2.15	0.420	1.000	-0.17	0.824	1.000
rs3219185	rs2071592	rs6929796	341	0.86	0.412	1.000	1.13	0.809	1.000	-0.46	0.299	1.000
rs3219185	rs2071592	rs6929796	213	0.39	0.228	1.000	-	-	-	-	-	-
rs3219185	rs2071592	rs6929796	313	0.66	**0.013**	0.449	1.39	0.510	1.000	0.40	0.330	1.000
rs3219185	rs2071592	rs6929796	343	1.80	**0.001**	0.017	0.59	0.207	0.996	0.02	0.962	1.000
rs2071592	rs6929796	rs2230365	134	0.88	0.810	1.000	1.82E+08	0.645	1.000	1.59	0.159	0.999
rs2071592	rs6929796	rs2230365	412	0.85	0.368	1.000	1.35	0.597	1.000	-0.45	0.277	1.000
rs2071592	rs6929796	rs2230365	132	0.64	**0.009**	0.315	1.21	0.649	1.000	0.20	0.625	1.000
rs2071592	rs6929796	rs2230365	432	1.78	**0.001**	0.021	0.59	0.207	0.996	0.01	0.984	1.000
rs6929796	rs2230365	rs28445017	321	1.96	0.190	1.000	1.85E+08	0.860	1.000	2.07	**0.035**	0.829
rs6929796	rs2230365	rs28445017	343	0.90	0.834	1.000	8.07E+133	0.990	1.000	1.59	0.157	0.999
rs6929796	rs2230365	rs28445017	123	0.85	0.364	1.000	1.36	0.481	1.000	-0.43	0.284	1.000
rs6929796	rs2230365	rs28445017	323	1.11	0.552	1.000	0.51	0.178	0.987	-0.13	0.757	1.000
rs2230365	rs28445017	rs4947324	234	0.61	**0.038**	0.779	0.62	0.305	1.000	-0.29	0.653	1.000
rs2230365	rs28445017	rs4947324	212	2.50	0.111	0.994	2.72E+12	0.869	1.000	2.30	**0.030**	0.741
rs2230365	rs28445017	rs4947324	432	0.86	0.683	1.000	6.14E+157	0.793	1.000	1.60	0.157	0.999
rs2230365	rs28445017	rs4947324	232	1.32	0.161	1.000	0.84	0.779	1.000	-0.63	0.192	1.000
rs28445017	rs4947324	rs2516390	322	0.67	0.170	0.999	1.28	0.639	1.000	0.20	0.797	1.000
rs28445017	rs4947324	rs2516390	344	0.64	0.064	0.932	0.66	0.423	1.000	-0.25	0.705	1.000
rs28445017	rs4947324	rs2516390	124	1.99	0.192	1.000	8.14E+11	0.844	1.000	2.28	**0.032**	0.744
rs28445017	rs4947324	rs2516390	324	1.47	**0.043**	0.801	0.95	0.878	1.000	-0.35	0.453	1.000
rs4947324	rs2516390	rs915654	444	0.14	**0.006**	0.582	-	-	-	-	-	-
rs4947324	rs2516390	rs915654	244	0.95	0.793	1.000	1.46	0.422	1.000	-0.39	0.319	1.000
rs4947324	rs2516390	rs915654	221	0.70	0.238	1.000	1.31	0.627	1.000	0.24	0.760	1.000
rs4947324	rs2516390	rs915654	441	0.74	0.286	1.000	0.61	0.403	1.000	-0.34	0.625	1.000
rs4947324	rs2516390	rs915654	241	1.54	**0.019**	0.421	0.80	0.560	1.000	0.40	0.284	1.000
rs2516390	rs915654	rs2516312	213	0.42	0.055	0.911	3.06E+10	0.587	1.000	-0.54	0.656	1.000
rs2516390	rs915654	rs2516312	441	0.86	0.379	1.000	1.45	0.433	1.000	-0.43	0.272	1.000
rs2516390	rs915654	rs2516312	211	1.00	0.999	1.000	0.80	0.834	1.000	0.34	0.652	1.000
rs2516390	rs915654	rs2516312	411	1.36	0.075	0.932	0.64	0.301	1.000	0.29	0.434	1.000
rs915654	rs2516312	rs2844482	414	0.82	0.420	1.000	2.27	0.330	1.000	0.45	0.429	1.000
rs915654	rs2516312	rs2844482	132	0.49	0.097	0.991	1.05E+11	0.903	1.000	-0.02	0.984	1.000
rs915654	rs2516312	rs2844482	412	0.93	0.672	1.000	1.13	0.807	1.000	-0.64	0.135	0.994

rs915654	rs2516312	rs2844482	112	1.33	0.084	0.973	0.54	0.156	0.985	0.30	0.438	1.000
rs2516312	rs2844482	rs2239704	321	0.41	**0.046**	0.875	7.93E+18	0.608	1.000	-0.68	0.582	1.000
rs2516312	rs2844482	rs2239704	121	0.90	0.727	1.000	0.71	0.524	1.000	0.14	0.859	1.000
rs2516312	rs2844482	rs2239704	142	0.81	0.389	1.000	2.24	0.288	1.000	0.40	0.478	1.000
rs2516312	rs2844482	rs2239704	122	1.34	0.116	0.994	0.56	0.241	1.000	-0.35	0.457	1.000
rs2844482	rs2239704	rs2229094	422	0.87	0.569	1.000	2.21	0.230	1.000	0.68	0.222	1.000
rs2844482	rs2239704	rs2229094	222	0.64	0.053	0.861	0.69	0.506	1.000	-0.05	0.935	1.000
rs2844482	rs2239704	rs2229094	214	0.66	0.201	1.000	1.71	0.539	1.000	0.22	0.773	1.000
rs2844482	rs2239704	rs2229094	224	1.61	**0.005**	0.200	0.83	0.687	1.000	-0.27	0.505	1.000
rs2239704	rs2229094	rs1041981	222	0.74	0.069	0.971	1.00	0.982	1.000	0.22	0.634	1.000
rs2239704	rs2229094	rs1041981	142	0.71	0.233	1.000	1.07	0.675	1.000	-0.03	0.972	1.000
rs2239704	rs2229094	rs1041981	221	0.51	0.329	1.000	18.50	0.324	1.000	0.81	0.685	1.000
rs2239704	rs2229094	rs1041981	241	1.47	**0.015**	0.502	0.89	0.826	1.000	-0.28	0.465	1.000
rs2229094	rs1041981	rs3093544	423	-	-	-	-	-	-	-	-	-
rs2229094	rs1041981	rs3093544	213	-	-	-	-	-	-	-	-	-
rs2229094	rs1041981	rs3093544	221	-	-	-	0.95	0.897	1.000	-	-	-
rs2229094	rs1041981	rs3093544	421	-	-	-	1.09	0.893	1.000	-	-	-
rs2229094	rs1041981	rs3093544	211	-	-	-	inf	0.470	1.000	-	-	-
rs2229094	rs1041981	rs3093544	411	-	-	-	0.89	0.834	1.000	-	-	-
rs1041981	rs3093544	rs1799964	212	-	-	-	1.11	0.867	1.000	-	-	-
rs1041981	rs3093544	rs1799964	234	-	-	-	7.30E+07	0.770	1.000	-	-	-
rs1041981	rs3093544	rs1799964	134	-	-	-	-	-	-	-	-	-
rs1041981	rs3093544	rs1799964	214	-	-	-	0.83	0.743	1.000	-	-	-
rs1041981	rs3093544	rs1799964	114	-	-	-	0.93	0.853	1.000	-	-	-
rs3093544	rs1799964	rs1800630	121	-	-	-	1.74	0.380	1.000	-	-	-
rs3093544	rs1799964	rs1800630	122	-	-	-	0.81	0.568	1.000	-	-	-
rs3093544	rs1799964	rs1800630	342	-	-	-	7.96E+141	0.451	1.000	-	-	-
rs3093544	rs1799964	rs1800630	142	-	-	-	0.78	0.586	1.000	-	-	-
rs1799964	rs1800630	rs1800629	421	1.14	0.513	1.000	1.47	0.535	1.000	0.56	0.236	1.000
rs1799964	rs1800630	rs1800629	213	0.97	0.921	1.000	1.74	0.380	1.000	0.85	0.171	0.999
rs1799964	rs1800630	rs1800629	223	0.79	0.356	1.000	0.81	0.620	1.000	-0.11	0.871	1.000
rs1799964	rs1800630	rs1800629	423	1.02	0.899	1.000	0.69	0.390	1.000	-0.71	0.059	0.949
rs1800630	rs1800629	rs2228088	234	0.77	0.360	1.000	1.41	0.619	1.000	-1.09	0.109	0.993
rs1800630	rs1800629	rs2228088	213	1.13	0.550	1.000	1.45	0.511	1.000	0.57	0.221	1.000
rs1800630	rs1800629	rs2228088	133	1.02	0.950	1.000	1.74	0.380	1.000	0.85	0.171	0.999
rs1800630	rs1800629	rs2228088	233	1.01	0.956	1.000	0.55	0.179	0.995	-0.43	0.281	1.000
rs1800629	rs2228088	rs3093662	333	0.69	0.175	1.000	0.53	0.184	1.000	0.15	0.863	1.000
rs1800629	rs2228088	rs3093662	341	0.77	0.351	1.000	1.37	0.635	1.000	-1.14	0.096	0.988
rs1800629	rs2228088	rs3093662	131	1.14	0.520	1.000	1.48	0.480	1.000	0.59	0.210	1.000
rs1800629	rs2228088	rs3093662	331	1.15	0.385	1.000	0.86	0.734	1.000	-0.12	0.753	1.000
rs2228088	rs3093662	rs3093665	332	0.59	0.243	1.000	4.74E+12	0.940	1.000	0.94	0.614	1.000
rs2228088	rs3093662	rs3093665	331	0.85	0.638	1.000	0.40	0.092	0.987	-0.11	0.906	1.000
rs2228088	rs3093662	rs3093665	411	0.79	0.419	1.000	1.32	0.833	1.000	-1.21	0.088	0.979
rs2228088	rs3093662	rs3093665	311	1.39	0.124	0.988	1.13	0.884	1.000	0.49	0.330	1.000
rs3093662	rs3093665	rs3093671	111	0.79	0.375	1.000	1.23	0.772	1.000	0.30	0.649	1.000
rs3093662	rs3093665	rs3093671	323	0.59	0.243	1.000	6.43E+25	0.384	1.000	1.26	0.533	1.000

rs3093662	rs3093665	rs3093671	313	0.83	0.571	1.000	0.47	0.182	0.999	-0.08	0.923	1.000
rs3093662	rs3093665	rs3093671	113	1.39	0.107	0.986	1.15	0.720	1.000	-0.30	0.519	1.000
rs3093665	rs3093671	rs7769073	111	0.83	0.491	1.000	1.12	0.844	1.000	0.52	0.440	1.000
rs3093665	rs3093671	rs7769073	114	0.32	0.155	0.998	1.76E+08	0.708	1.000	-0.17	0.931	1.000
rs3093665	rs3093671	rs7769073	234	0.60	0.213	1.000	2.69E+294	0.724	1.000	1.34	0.295	1.000
rs3093665	rs3093671	rs7769073	134	1.35	0.181	1.000	0.54	0.427	1.000	-0.36	0.538	1.000
rs3093671	rs7769073	rs2256974	311	-	-	-	1.76E+08	0.708	1.000	-0.17	0.931	1.000
rs3093671	rs7769073	rs2256974	341	1.15	0.427	1.000	0.62	0.208	0.999	-0.64	0.104	0.991
rs3093671	rs7769073	rs2256974	112	0.83	0.486	1.000	1.12	0.844	1.000	0.52	0.440	1.000
rs3093671	rs7769073	rs2256974	342	0.94	0.707	1.000	1.41	0.428	1.000	0.63	0.121	0.994
rs7769073	rs2256974	rs1052248	411	0.63	0.608	1.000	-	-	-	-	-	-
rs7769073	rs2256974	rs1052248	121	0.92	0.775	1.000	1.14	0.819	1.000	0.72	0.298	1.000
rs7769073	rs2256974	rs1052248	421	1.09	0.671	1.000	0.99	0.984	1.000	0.43	0.302	1.000
rs7769073	rs2256974	rs1052248	114	0.79	0.829	1.000	1.06E+04	0.288	1.000	-1.71	0.401	1.000
rs7769073	rs2256974	rs1052248	414	1.21	0.261	1.000	0.63	0.244	0.999	-0.41	0.269	1.000
rs7769073	rs2256974	rs1052248	424	0.79	0.216	1.000	1.67	0.325	1.000	-0.02	0.965	1.000
rs2256974	rs1052248	rs3179003	211	0.87	0.611	1.000	1.09	0.845	1.000	1.02	0.164	1.000
rs2256974	rs1052248	rs3179003	141	5.71	0.187	1.000	0.08	**0.011**	0.505	-2.33	0.147	0.999
rs2256974	rs1052248	rs3179003	112	0.67	0.673	1.000	-	-	-	-	-	-
rs2256974	rs1052248	rs3179003	212	1.14	0.517	1.000	1.09	0.867	1.000	0.46	0.320	1.000
rs2256974	rs1052248	rs3179003	142	1.15	0.407	1.000	0.75	0.488	1.000	-0.45	0.225	1.000
rs2256974	rs1052248	rs3179003	242	0.77	0.164	0.998	1.91	0.257	0.999	0.07	0.874	1.000
rs2734586	rs2075582		22	0.58	**0.040**	0.762	0.88	0.931	1.000	-0.03	0.962	1.000
rs2734586	rs2075582		34	1.02	0.961	1.000	1.47	0.522	1.000	0.51	0.356	1.000
rs2734586	rs2075582		24	1.32	0.156	0.996	0.85	0.674	1.000	-0.33	0.478	1.000
rs2075582	rs3130056		22	0.82	0.583	1.000	0.71	0.556	1.000	0.34	0.691	1.000
rs2075582	rs3130056		24	0.45	**0.012**	0.623	1.18	0.375	1.000	-0.58	0.578	1.000
rs2075582	rs3130056		44	1.61	0.063	0.898	1.62	0.308	1.000	0.26	0.681	1.000
rs3130056	rs11796		21	0.89	0.751	1.000	0.50	0.184	1.000	-0.07	0.929	1.000
rs3130056	rs11796		41	0.70	**0.028**	0.752	1.43	0.490	1.000	0.14	0.732	1.000
rs3130056	rs11796		44	1.43	**0.028**	0.703	0.93	0.873	1.000	-0.11	0.782	1.000
rs11796	rs2734583		43	1.26	0.308	1.000	2.81	0.105	0.989	0.56	0.270	1.000
rs11796	rs2734583		11	0.69	**0.025**	0.623	1.09	0.846	1.000	0.10	0.802	1.000
rs11796	rs2734583		41	1.25	0.146	0.998	0.62	0.190	0.999	-0.37	0.308	1.000
rs2734583	rs2239709		14	0.51	**0.044**	0.908	1.55	0.497	1.000	-0.74	0.408	1.000
rs2734583	rs2239709		32	1.21	0.393	1.000	2.77	0.128	0.991	0.62	0.239	1.000
rs2734583	rs2239709		12	1.04	0.842	1.000	0.41	0.159	0.981	-0.29	0.519	1.000
rs2239709	rs11757236		24	1.97	0.411	1.000	0.37	0.138	1.000	-1.31	0.349	1.000
rs2239709	rs11757236		42	0.59	0.137	0.997	15.10	0.160	1.000	-0.21	0.837	1.000
rs2239709	rs11757236		22	1.37	0.291	1.000	1.09	0.730	1.000	0.91	0.212	1.000
rs11757236	rs2079170		22	0.89	0.734	1.000	7.86E+07	0.657	1.000	0.30	0.780	1.000
rs11757236	rs2079170		43	1.72	0.513	1.000	0.25	0.129	0.959	-1.99	0.100	0.994
rs11757236	rs2079170		23	0.99	0.981	1.000	1.32	0.417	1.000	0.56	0.492	1.000
rs2079170	rs3130059		23	1.03	0.901	1.000	2.83E+08	0.696	1.000	-0.09	0.952	1.000
rs2079170	rs3130059		33	0.68	**0.023**	0.637	1.24	0.582	1.000	0.38	0.368	1.000
rs2079170	rs3130059		32	1.48	**0.010**	0.525	0.71	0.410	1.000	-0.40	0.318	1.000

rs3130059	rs2844509	33	0.70	0.108	0.988	0.95	0.804	1.000	0.15	0.810	1.000
rs3130059	rs2844509	31	0.73	0.097	0.971	1.77	0.257	1.000	0.35	0.462	1.000
rs3130059	rs2844509	21	1.53	**0.012**	0.400	6.74E-01	0.356	1.000	-0.26	0.501	1.000
rs2844509	rs9267487	32	0.82	0.505	1.000	0.57	0.162	1.000	-0.22	0.767	1.000
rs2844509	rs9267487	12	-	-	-	0.20	0.224	0.998	-2.21	0.244	1.000
rs2844509	rs9267487	34	0.75	0.344	1.000	1.53E+08	0.725	1.000	0.35	0.694	1.000
rs2844509	rs9267487	14	1.25	0.309	1.000	1.23	0.664	1.000	0.18	0.730	1.000
rs9267487	rs2251824	41	0.81	0.408	1.000	1.82	0.437	1.000	0.33	0.557	1.000
rs9267487	rs2251824	23	0.88	0.637	1.000	0.43	0.113	0.982	-0.56	0.455	1.000
rs9267487	rs2251824	43	1.23	0.306	1.000	1.17	0.741	1.000	0.02	0.960	1.000
rs2251824	rs2239705	31	0.72	0.435	1.000	1.27E+10	0.755	1.000	-0.11	0.932	1.000
rs2251824	rs2239705	13	0.75	0.229	1.000	1.80	0.401	1.000	0.36	0.521	1.000
rs2251824	rs2239705	33	1.38	0.144	0.996	0.47	0.261	1.000	-0.27	0.601	1.000
rs2239705	rs3219185	32	1.06	0.866	1.000	2.27	0.358	1.000	-0.31	0.666	1.000
rs2239705	rs3219185	13	0.87	0.811	1.000	1.09E+08	0.689	1.000	0.09	0.939	1.000
rs2239705	rs3219185	33	1.09	0.758	1.000	0.34	0.198	1.000	0.20	0.763	1.000
rs3219185	rs2071592	21	0.11	0.053	0.967	-	-	-	-	-	-
rs3219185	rs2071592	31	0.70	**0.031**	0.723	1.50	0.357	1.000	0.35	0.393	1.000
rs3219185	rs2071592	24	0.92	0.827	1.000	2.29	0.313	1.000	-0.30	0.676	1.000
rs3219185	rs2071592	34	1.53	**0.009**	0.348	0.59	0.225	0.999	-0.25	0.525	1.000
rs2071592	rs6929796	41	0.81	0.230	1.000	1.31	0.602	1.000	-0.45	0.280	1.000
rs2071592	rs6929796	13	0.66	**0.013**	0.465	1.46	0.400	1.000	0.36	0.369	1.000
rs2071592	rs6929796	43	1.77	**0.001**	0.025	0.59	0.190	0.990	0.04	0.917	1.000
rs6929796	rs2230365	34	1.04	0.951	1.000	2.13E+08	0.835	1.000	1.58	0.165	0.999
rs6929796	rs2230365	12	0.83	0.281	1.000	1.34	0.542	1.000	-0.43	0.295	1.000
rs6929796	rs2230365	32	1.20	0.288	1.000	0.64	0.328	1.000	0.19	0.622	1.000
rs2230365	rs28445017	21	2.43	0.120	0.995	1.86E+08	0.911	1.000	2.07	**0.036**	0.834
rs2230365	rs28445017	43	0.85	0.668	1.000	1.84E+08	0.899	1.000	1.59	0.161	0.999
rs2230365	rs28445017	23	0.76	0.478	1.000	4.99E-09	0.917	1.000	-1.99	**0.016**	0.384
rs28445017	rs4947324	34	0.61	**0.034**	0.745	0.61	0.307	1.000	-0.29	0.653	1.000
rs28445017	rs4947324	12	2.51	0.112	0.995	6.97E+12	0.993	1.000	2.27	**0.033**	0.761
rs28445017	rs4947324	32	1.33	0.188	1.000	1.12	0.794	1.000	-0.35	0.522	1.000
rs4947324	rs2516390	22	0.66	0.164	0.998	2.67	0.181	1.000	0.41	0.575	1.000
rs4947324	rs2516390	44	0.63	0.055	0.913	0.64	0.329	1.000	-0.19	0.771	1.000
rs4947324	rs2516390	24	1.71	**0.012**	0.276	1.03	0.870	1.000	-0.06	0.922	1.000
rs2516390	rs915654	44	0.87	0.432	1.000	1.50	0.363	1.000	-0.35	0.388	1.000
rs2516390	rs915654	21	0.80	0.492	1.000	1.17	0.753	1.000	0.23	0.739	1.000
rs2516390	rs915654	41	1.27	0.160	0.997	0.66	0.352	1.000	0.25	0.490	1.000
rs915654	rs2516312	13	0.48	0.113	0.994	1.88E+08	0.715	1.000	0.57	0.599	1.000
rs915654	rs2516312	41	0.80	0.186	1.000	1.51	0.366	1.000	-0.36	0.373	1.000
rs915654	rs2516312	11	1.41	**0.045**	0.835	0.58	0.190	0.998	0.26	0.503	1.000
rs2516312	rs2844482	14	0.83	0.443	1.000	2.22	0.296	1.000	0.40	0.497	1.000
rs2516312	rs2844482	32	0.53	0.140	0.996	5.82E+17	0.795	1.000	-0.32	0.771	1.000
rs2516312	rs2844482	12	1.35	0.143	0.997	0.34	0.154	0.984	-0.38	0.441	1.000
rs2844482	rs2239704	21	0.66	0.137	0.996	1.10	0.745	1.000	0.01	0.995	1.000
rs2844482	rs2239704	42	0.78	0.286	1.000	2.15	0.242	1.000	0.49	0.383	1.000

rs2844482	rs2239704	22	1.43	0.071	0.917	0.60	0.359	1.000	-0.33	0.480	1.000
rs2239704	rs2229094	22	0.67	**0.025**	0.618	1.05	0.893	1.000	0.32	0.477	1.000
rs2239704	rs2229094	14	0.67	0.155	0.997	1.22	0.616	1.000	0.04	0.966	1.000
rs2239704	rs2229094	24	1.61	**0.006**	0.178	0.94	0.881	1.000	-0.25	0.551	1.000
rs2229094	rs1041981	22	0.71	**0.046**	0.921	0.89	0.774	1.000	0.20	0.652	1.000
rs2229094	rs1041981	42	0.83	0.554	1.000	1.17	0.899	1.000	0.06	0.938	1.000
rs2229094	rs1041981	21	0.61	0.355	1.000	1.66E+08	0.943	1.000	2.19	0.259	1.000
rs2229094	rs1041981	41	1.45	**0.028**	0.666	0.99	0.999	1.000	-0.27	0.495	1.000
rs1041981	rs3093544	23	0.89	0.753	1.000	6.91E+07	0.772	1.000	0.68	0.640	1.000
rs1041981	rs3093544	13	0.44	0.234	1.000	-	-	-	-	-	-
rs1041981	rs3093544	21	0.73	0.058	0.926	0.94	0.845	1.000	0.10	0.826	1.000
rs1041981	rs3093544	11	1.41	**0.030**	0.738	0.99	0.987	1.000	-0.21	0.562	1.000
rs3093544	rs1799964	12	0.89	0.525	1.000	1.16	0.769	1.000	0.43	0.343	1.000
rs3093544	rs1799964	34	0.77	0.409	1.000	1.33E+04	0.255	1.000	1.15	0.361	1.000
rs3093544	rs1799964	14	1.22	0.227	1.000	0.80	0.757	1.000	-0.56	0.202	1.000
rs1799964	rs1800630	21	0.99	0.994	1.000	1.70	0.424	1.000	0.94	0.130	0.995
rs1799964	rs1800630	22	0.81	0.423	1.000	0.81	0.560	1.000	-0.12	0.849	1.000
rs1799964	rs1800630	42	1.13	0.553	1.000	0.87	0.742	1.000	-0.46	0.313	1.000
rs1800630	rs1800629	21	1.13	0.559	1.000	1.47	0.483	1.000	0.57	0.228	1.000
rs1800630	rs1800629	13	0.98	0.940	1.000	1.60	0.464	1.000	0.82	0.182	1.000
rs1800630	rs1800629	23	0.92	0.679	1.000	0.60	0.321	1.000	-0.83	**0.044**	0.867
rs1800629	rs2228088	34	0.72	0.254	1.000	1.32	0.735	1.000	-0.99	0.149	0.999
rs1800629	rs2228088	13	1.12	0.560	1.000	1.46	0.542	1.000	0.49	0.310	1.000
rs1800629	rs2228088	33	1.06	0.756	1.000	0.65	0.441	1.000	-0.06	0.903	1.000
rs2228088	rs3093662	33	0.70	0.231	1.000	0.52	0.150	1.000	0.16	0.868	1.000
rs2228088	rs3093662	41	0.79	0.422	1.000	1.37	0.577	1.000	-1.22	0.086	0.978
rs2228088	rs3093662	31	1.38	0.137	0.994	1.20	0.680	1.000	0.61	0.245	1.000
rs3093662	rs3093665	32	0.49	0.136	0.997	-	-	-	-	-	-
rs3093662	rs3093665	31	0.82	0.558	1.000	0.48	0.160	0.999	-0.07	0.927	1.000
rs3093662	rs3093665	11	1.50	0.134	0.995	1.49	0.498	1.000	-0.35	0.632	1.000
rs3093665	rs3093671	11	0.71	0.228	1.000	1.18	0.665	1.000	0.25	0.715	1.000
rs3093665	rs3093671	23	0.65	0.262	1.000	1.14E+15	0.769	1.000	1.13	0.384	1.000
rs3093665	rs3093671	13	1.48	0.093	0.981	0.66	0.504	1.000	-0.43	0.478	1.000
rs3093671	rs7769073	11	0.90	0.699	1.000	1.12	0.733	1.000	0.49	0.462	1.000
rs3093671	rs7769073	14	0.46	0.257	1.000	1.77E+08	0.525	1.000	-0.20	0.905	1.000
rs3093671	rs7769073	34	1.13	0.663	1.000	0.64	0.521	1.000	-0.05	0.933	1.000
rs7769073	rs2256974	11	9.07	0.204	1.000	4.60E+37	0.809	1.000	-0.92	0.638	1.000
rs7769073	rs2256974	41	1.10	0.562	1.000	0.58	0.140	0.994	-0.67	0.088	0.983
rs7769073	rs2256974	12	0.83	0.524	1.000	2.25	0.356	1.000	1.23	0.115	0.994
rs7769073	rs2256974	42	0.93	0.624	1.000	1.38	0.452	1.000	0.41	0.313	1.000
rs2256974	rs1052248	11	0.64	0.642	1.000	-	-	-	-	-	-
rs2256974	rs1052248	21	1.08	0.693	1.000	1.27	0.637	1.000	0.91	**0.034**	0.775
rs2256974	rs1052248	14	1.19	0.313	1.000	0.53	0.106	0.972	-0.71	0.070	0.954
rs2256974	rs1052248	24	0.77	0.164	0.998	1.86	0.252	1.000	-0.01	0.970	1.000
rs1052248	rs3179003	11	0.84	0.527	1.000	1.03	0.857	1.000	0.82	0.287	1.000
rs1052248	rs3179003	41	2.57	0.260	1.000	0.23	0.068	0.951	-1.36	0.294	1.000

rs1052248	rs3179003	12	1.10	0.619	1.000	1.05	0.937	1.000	0.23	0.602	1.000
rs1052248	rs3179003	42	0.93	0.686	1.000	1.20	0.668	1.000	-0.26	0.510	1.000

IL12B – Single SNP analysis

UNIVARIATE - HIV-SN									
SNP	**ALLELIC MODEL**			**GENOTYPIC MODEL**		**DOMINANT MODEL**		**RECESSIVE MODEL**	
	P_{EMP1}	**P_{EMP2}**	**OR**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**
rs2853697	0.919	1.000	0.94	0.612	1.000	1.000	1.000	0.188	0.979
rs11574790	0.900	1.000	1.02	0.990	1.000	0.921	1.000	0.910	1.000
rs2853696	0.359	0.992	0.65	0.425	0.989	0.561	1.000	0.099	0.716
rs3213105	0.534	1.000	1.22	0.529	1.000	0.513	1.000	1.000	1.000
rs2421047	0.235	0.924	0.81	0.471	0.999	0.193	0.905	0.546	1.000
rs3213103	0.535	1.000	1.18	0.658	1.000	0.665	1.000	0.515	0.996
rs3181221	0.558	1.000	0.79	0.527	1.000	0.550	0.999	1.000	1.000
rs919766	0.873	1.000	0.98	0.984	1.000	0.935	1.000	0.823	1.000
rs2853694	0.409	0.996	1.16	0.697	1.000	0.397	0.995	0.705	1.000
rs3213095	0.726	1.000	0.89	0.735	1.000	0.603	1.000	1.000	1.000
rs3212217	0.122	0.646	0.76	0.181	0.910	0.360	0.982	0.085	0.382
rs2546892	0.632	1.000	0.85	0.687	1.000	0.758	1.000	0.162	0.969
rs1003199	0.839	1.000	1.05	0.675	1.000	0.551	0.999	0.629	1.000
rs2546893	0.464	1.000	1.15	0.631	1.000	0.326	0.982	0.874	1.000

UNIVARIATE - PAIN										UNIVARIATE - PAIN INTENSITY		
SNP	**ALLELIC MODEL**			**GENOTYPIC MODEL**		**DOMINANT MODEL**		**RECESSIVE MODEL**		**ALLELIC MODEL**		
	P_{EMP1}	**P_{EMP2}**	**OR**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**BETA**
rs2853697	1.000	1.000	1.05	1.000	1.000	1.000	1.000	1.000	1.000	0.656	1.000	0.49
rs11574790	0.682	1.000	1.27	0.835	1.000	0.583	0.998	1.000	1.000	0.854	1.000	0.07
rs2853696	0.252	0.990	0.59	0.253	0.999	0.253	0.993	1.000	1.000	0.990	1.000	0.03
rs3213105	0.514	1.000	2.22	0.545	1.000	0.558	1.000	1.000	1.000	0.284	0.960	0.83
rs2421047	0.069	0.324	0.45	0.075	0.543	0.319	0.939	**0.031**	0.112	-	-	-
rs3213103	0.748	1.000	1.78	0.754	1.000	0.739	1.000	0.919	1.000	0.226	0.935	-0.73
rs3181221	1.000	1.000	1.27	1.000	1.000	1.000	1.000	1.000	1.000	0.217	0.900	1.23
rs919766	1.000	1.000	1.10	0.919	1.000	0.790	1.000	1.000	1.000	0.845	1.000	0.08
rs2853694	0.352	0.975	1.68	0.666	1.000	0.613	0.998	0.998	1.000	0.924	1.000	0.04
rs3213095	**0.050**	0.264	0.32	**0.042**	0.307	**0.049**	0.251	1.000	1.000	0.770	1.000	-0.21
rs3212217	0.536	0.999	0.76	0.064	0.496	0.180	0.743	0.329	0.827	0.987	1.000	0.00
rs2546892	1.000	1.000	1.18	1.000	1.000	0.997	1.000	1.000	1.000	0.892	1.000	0.13
rs1003199	0.524	0.994	0.75	0.546	1.000	0.438	0.978	1.000	1.000	0.840	1.000	0.09
rs2546893	0.847	1.000	0.94	0.332	0.974	0.593	0.998	0.616	0.987	0.629	1.000	0.20

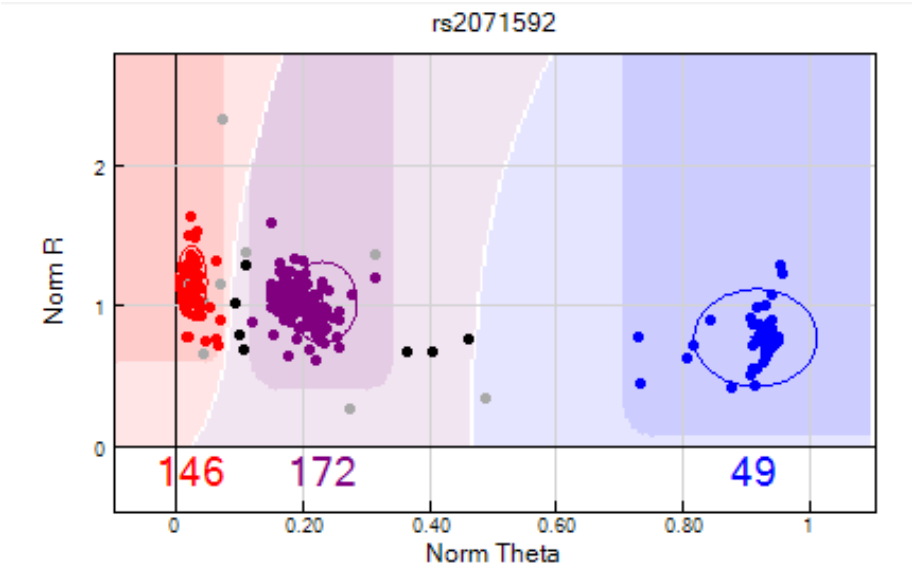
IL12B – Haplotype analysis

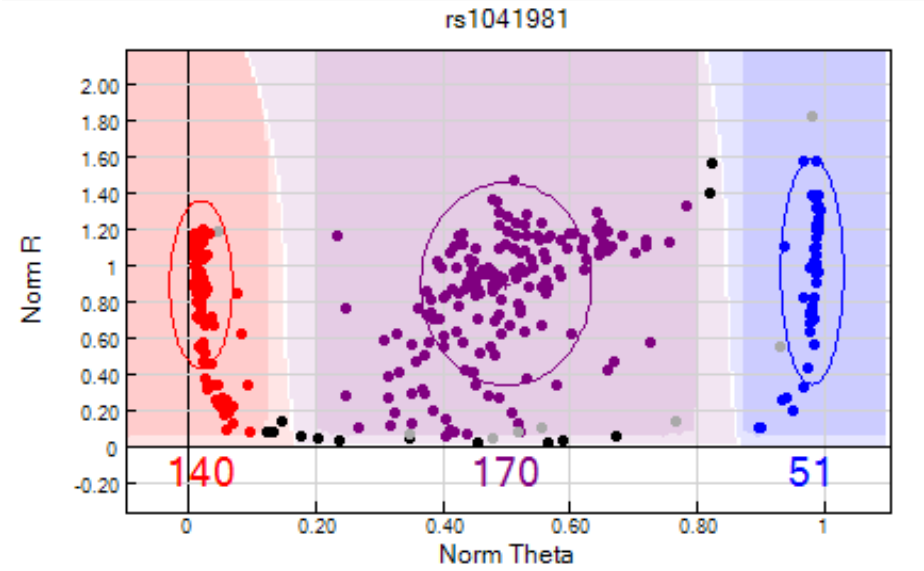
HAPLOTYPE				UNIVARIATE – HIV-SN			UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
				OR	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	BETA	P_{EMP1}	P_{EMP2}
rs2853697	rs11574790	rs2853696	434	0.87	0.807	1.000	0.58	0.329	1.000	-0.01	0.999	1.000
rs2853697	rs11574790	rs2853696	412	1.08	0.700	1.000	1.26	0.595	1.000	0.05	0.898	1.000
rs2853697	rs11574790	rs2853696	332	0.93	0.873	1.000	0.94	0.502	1.000	0.42	0.715	1.000
rs2853697	rs11574790	rs2853696	432	1.01	0.967	1.000	0.86	0.760	1.000	-0.12	0.765	1.000
rs11574790	rs2853696	rs3213105	124	1.71	0.217	0.989	2.04	0.526	1.000	1.32	0.084	0.891
rs11574790	rs2853696	rs3213105	324	0.93	0.929	1.000	2110.00	0.479	1.000	-1.30	0.504	1.000
rs11574790	rs2853696	rs3213105	342	0.99	0.976	1.000	0.58	0.351	1.000	0.04	0.983	1.000
rs11574790	rs2853696	rs3213105	122	0.98	0.925	1.000	1.02	0.986	1.000	-0.31	0.497	1.000
rs11574790	rs2853696	rs3213105	322	0.99	0.969	1.000	0.86	0.749	1.000	0.00	0.998	1.000
rs2853696	rs3213105	rs2421047	221	0.84	0.259	0.997	0.57	0.119	0.862	-	-	-
rs2853696	rs3213105	rs2421047	243	1.65	0.230	0.990	3.04	0.284	0.998	-	-	-
rs2853696	rs3213105	rs2421047	423	0.94	0.893	1.000	0.58	0.283	1.000	-	-	-
rs2853696	rs3213105	rs2421047	223	1.15	0.363	1.000	1.56	0.241	0.971	-	-	-
rs3213105	rs2421047	rs3213103	231	1.13	0.687	1.000	1.66	0.445	1.000	-	-	-
rs3213105	rs2421047	rs3213103	413	1.03	0.977	1.000	-	-	-	-	-	-
rs3213105	rs2421047	rs3213103	213	0.84	0.275	0.998	0.50	**0.035**	0.560	-	-	-
rs3213105	rs2421047	rs3213103	433	1.35	0.469	1.000	2.77	0.316	1.000	-	-	-
rs3213105	rs2421047	rs3213103	233	1.07	0.660	1.000	1.48	0.268	0.988	-	-	-
rs2421047	rs3213103	rs3181221	333	0.78	0.427	1.000	1.29	0.662	1.000	-	-	-
rs2421047	rs3213103	rs3181221	311	1.18	0.550	1.000	1.83	0.405	1.000	-	-	-
rs2421047	rs3213103	rs3181221	131	0.83	0.218	0.993	0.51	**0.044**	0.597	-	-	-
rs2421047	rs3213103	rs3181221	331	1.17	0.283	0.998	1.54	0.187	0.959	-	-	-
rs3213103	rs3181221	rs919766	332	0.75	0.417	1.000	1.28	0.571	1.000	1.22	0.201	0.974
rs3213103	rs3181221	rs919766	112	1.11	0.734	1.000	1.30	0.781	1.000	-0.98	0.223	0.984
rs3213103	rs3181221	rs919766	312	1.02	0.928	1.000	0.98	0.929	1.000	0.13	0.795	1.000
rs3213103	rs3181221	rs919766	111	1.33	0.608	1.000	25.70	0.328	1.000	-0.48	0.663	1.000
rs3213103	rs3181221	rs919766	311	0.98	0.912	1.000	0.76	0.560	1.000	0.01	0.982	1.000
rs3181221	rs919766	rs2853694	113	1.18	0.397	1.000	1.75	0.297	0.991	0.02	0.966	1.000
rs3181221	rs919766	rs2853694	324	0.78	0.445	1.000	1.28	0.571	1.000	1.22	0.201	0.974
rs3181221	rs919766	rs2853694	124	1.05	0.816	1.000	1.07	0.887	1.000	-0.19	0.685	1.000
rs3181221	rs919766	rs2853694	114	0.90	0.490	1.000	0.66	0.276	0.988	-0.07	0.856	1.000
rs919766	rs2853694	rs3213095	144	0.97	0.915	1.000	0.27	**0.013**	0.385	-0.21	0.763	1.000
rs919766	rs2853694	rs3213095	131	1.23	0.300	0.998	1.75	0.339	0.990	0.01	0.984	1.000
rs919766	rs2853694	rs3213095	241	1.01	0.947	1.000	1.13	0.748	1.000	0.08	0.866	1.000
rs919766	rs2853694	rs3213095	141	0.89	0.450	1.000	0.99	0.973	1.000	-0.01	0.975	1.000
rs2853694	rs3213095	rs3212217	412	0.79	0.149	0.954	0.78	0.426	1.000	0.00	0.986	1.000
rs2853694	rs3213095	rs3212217	443	1.03	0.947	1.000	0.27	**0.014**	0.398	-0.27	0.726	1.000
rs2853694	rs3213095	rs3212217	313	1.17	0.441	1.000	1.72	0.299	0.991	0.04	0.909	1.000
rs2853694	rs3213095	rs3212217	413	1.16	0.358	1.000	1.43	0.353	0.998	0.04	0.904	1.000
rs3213095	rs3212217	rs2546892	131	0.96	0.918	1.000	1.17	0.362	1.000	0.07	0.945	1.000
rs3213095	rs3212217	rs2546892	123	0.79	0.153	0.957	0.78	0.589	1.000	-0.01	0.973	1.000

rs3213095	rs3212217	rs2546892	433	1.09	0.803	1.000	0.27	**0.036**	0.398	-0.21	0.780	1.000
rs3213095	rs3212217	rs2546892	133	1.26	0.115	0.923	1.74	0.138	0.854	0.05	0.905	1.000
rs3212217	rs2546892	rs1003199	314	0.85	0.662	1.000	1.36	0.363	1.000	0.16	0.868	1.000
rs3212217	rs2546892	rs1003199	234	1.14	0.861	1.000	0.18	0.071	0.892	-1.81	0.269	0.997
rs3212217	rs2546892	rs1003199	334	1.14	0.523	1.000	0.83	0.677	1.000	0.22	0.627	1.000
rs3212217	rs2546892	rs1003199	232	0.78	0.139	0.936	0.88	0.785	1.000	0.05	0.910	1.000
rs3212217	rs2546892	rs1003199	332	1.18	0.237	0.998	1.37	0.435	0.998	-0.10	0.773	1.000
rs2546892	rs1003199	rs2546893	141	0.96	0.886	1.000	1.18	0.546	1.000	0.13	0.888	1.000
rs2546892	rs1003199	rs2546893	341	1.14	0.496	1.000	0.83	0.658	1.000	0.27	0.521	1.000
rs2546892	rs1003199	rs2546893	321	1.21	0.836	1.000	1.85E+139	0.886	1.000	0.44	0.757	1.000
rs2546892	rs1003199	rs2546893	323	0.95	0.753	1.000	1.22	0.641	1.000	-0.14	0.742	1.000
rs2853697	rs11574790		41	1.02	0.933	1.000	1.25	0.621	1.000	0.04	0.919	1.000
rs2853697	rs11574790		33	0.92	0.863	1.000	0.93	0.617	1.000	0.46	0.695	1.000
rs2853697	rs11574790		43	1.00	0.998	1.000	0.81	0.606	1.000	-0.12	0.765	1.000
rs11574790	rs2853696		34	1.22	0.708	1.000	0.60	0.244	1.000	0.08	0.962	1.000
rs11574790	rs2853696		12	1.12	0.570	1.000	1.20	0.653	1.000	-0.04	0.902	1.000
rs11574790	rs2853696		32	0.92	0.655	1.000	0.90	0.784	1.000	0.03	0.943	1.000
rs2853696	rs3213105		24	1.37	0.399	1.000	2.13	0.453	1.000	0.99	0.183	0.983
rs2853696	rs3213105		42	0.96	0.932	1.000	0.50	0.295	1.000	-0.08	0.954	1.000
rs2853696	rs3213105		22	0.88	0.679	1.000	0.77	0.712	1.000	-0.78	0.247	0.996
rs3213105	rs2421047		21	0.80	0.166	0.976	0.56	0.101	0.839	-	-	-
rs3213105	rs2421047		43	1.74	0.195	0.985	2.98	0.251	0.998	-	-	-
rs3213105	rs2421047		23	1.13	0.423	1.000	1.53	0.244	0.984	-	-	-
rs2421047	rs3213103		31	1.09	0.748	1.000	1.73	0.431	1.000	-	-	-
rs2421047	rs3213103		13	0.83	0.229	0.993	0.52	**0.040**	0.616	-	-	-
rs2421047	rs3213103		33	1.16	0.326	1.000	1.59	0.185	0.925	-	-	-
rs3213103	rs3181221		33	0.75	0.418	1.000	1.29	0.662	1.000	1.20	0.208	0.980
rs3213103	rs3181221		11	1.18	0.523	1.000	1.83	0.405	1.000	-0.73	0.220	0.986
rs3213103	rs3181221		31	0.97	0.908	1.000	0.57	0.466	0.998	0.19	0.744	1.000
rs3181221	rs919766		32	0.75	0.422	1.000	1.31	0.423	1.000	1.22	0.207	0.978
rs3181221	rs919766		12	1.04	0.829	1.000	1.06	0.929	1.000	-0.18	0.695	1.000
rs3181221	rs919766		11	1.02	0.892	1.000	0.90	0.799	1.000	-0.08	0.866	1.000
rs919766	rs2853694		13	1.22	0.308	1.000	1.78	0.270	0.985	0.04	0.924	1.000
rs919766	rs2853694		24	1.00	0.973	1.000	1.13	0.797	1.000	0.01	0.995	1.000
rs919766	rs2853694		14	0.88	0.417	1.000	0.65	0.297	0.982	-0.03	0.944	1.000
rs2853694	rs3213095		44	0.94	0.850	1.000	0.27	**0.014**	0.398	-0.27	0.726	1.000
rs2853694	rs3213095		31	1.20	0.364	1.000	1.72	0.299	0.991	0.04	0.909	1.000
rs2853694	rs3213095		41	0.89	0.518	1.000	1.11	0.772	1.000	0.04	0.909	1.000
rs3213095	rs3212217		12	0.79	0.165	0.959	0.82	0.640	1.000	0.01	0.985	1.000
rs3213095	rs3212217		43	1.00	0.999	1.000	0.28	**0.036**	0.523	-0.26	0.754	1.000
rs3213095	rs3212217		13	1.26	0.119	0.935	1.78	0.121	0.843	0.06	0.871	1.000
rs3212217	rs2546892		31	0.79	0.529	1.000	1.26	0.387	1.000	0.09	0.925	1.000
rs3212217	rs2546892		23	0.79	0.136	0.940	0.77	0.487	1.000	-0.05	0.876	1.000
rs3212217	rs2546892		33	1.30	0.096	0.847	1.24	0.607	1.000	0.04	0.933	1.000
rs2546892	rs1003199		14	0.85	0.690	1.000	1.19	0.575	1.000	0.10	0.908	1.000
rs2546892	rs1003199		34	1.15	0.453	1.000	0.72	0.474	1.000	0.07	0.859	1.000

rs2546892	rs1003199	32	0.92	0.647	1.000	1.33	0.482	1.000	-0.09	0.831	1.000
rs1003199	rs2546893	41	1.15	0.451	1.000	0.88	0.777	1.000	0.22	0.585	1.000
rs1003199	rs2546893	21	1.19	0.915	1.000	1.82E+08	0.413	1.000	0.43	0.770	1.000
rs1003199	rs2546893	23	0.91	0.554	1.000	1.18	0.735	1.000	-0.08	0.857	1.000

Appendix E – rs2071592 and rs1041981 BeadStudio SNP graph scatter plots





Appendix F – Publication arising from results in Chapter 4

KCNS1, but Not *GCH1*, Is Associated With Pain Intensity in a Black Southern African Population With HIV-Associated Sensory Neuropathy: A Genetic Association Study

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Abstract: *KCNS1* and *GCH1* were investigated for their association with pain intensity in black Southern Africans with HIV-associated sensory neuropathy. Previously associated single nucleotide polymorphisms (SNPs) were supplemented with population-specific tagSNPs. No SNPs in *KCNS1* were individually associated with pain intensity. However, several haplotypes of population-specific tagSNPs correlated with pain intensity on univariate analysis and after correcting for age, gender, and CD4 T-cell count. This suggests that the haplotypes incorporate the causative SNP(s). No SNPs or haplotypes in *GCH1* were associated with pain intensity. The study shows the importance of conducting association analyses in different ethnic groups, using population-based marker selection.

Key Words: *KCNS1*, *GCH1*, HIV, pain intensity, neuropathy, Africans

(*J Acquir Immune Defic Syndr* 2013;63:27–30)

INTRODUCTION

HIV-associated sensory neuropathy (HIV-SN) is a common neurological complication of HIV infection and its treatment, and it is frequently painful. The pain causes significant decreases in the health-related quality of life,[1] and no commercially available agents have proven efficacy for the treatment of the pain.[2,3] The intensity of the pain experienced can differ significantly between individuals,[4] and although psychosocial factors probably contribute to this difference,[5] recent evidence from other types of peripheral neuropathy indicates that genetic variation also may be a contributing factor.[6]

Received for publication November 16, 2012; accepted January 2, 2013.
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Supported by the Faculty Research Committee of the University of the Witwatersrand and the Belgium Technical Cooperation Fellowship.
Presented at the PainSA Congress, Pretoria, June 22–24, 2012, and the 14th World Congress on Pain, Milan, August 27–31, 2012.
The authors have no conflicts of interest to disclose.
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Polymorphisms in the genes *KCNS1* and *GCH1* have been associated with differences in reported pain intensity in previous studies conducted in non-African populations. A particular single nucleotide polymorphism (SNP) in *KCNS1* (rs734784), a gene encoding a subunit of voltage-gated potassium ion channels that affects the excitability of sensory neurons, was found to be associated with increased sensitivity to pain in several neuropathic pain states.[7] *GCH1* encodes guanosine triphosphate cyclohydrolase 1, the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin, which is a cofactor in the synthesis of several algogenic molecules.[8] In a white cohort, individuals with a particular haplotype (15-SNP "pain-protective" haplotype) experienced reduced pain after a discectomy for persistent lumbar radiculopathy.[9] A later study showed that this haplotype could be represented by 3 SNPs alone (rs8007267, rs3783641, rs10483639).[10]

Few studies investigating associations between genes and pain intensity have been performed in non-European populations. The association between the valine encoding allele of rs734784 in *KCNS1* and increased pain intensity was detected in an Israeli cohort (Ashkenazi and non-Ashkenazi ethnicities) with postamputation pain,[7] but no association was found between polymorphisms in *GCH1* and pain sensitivity to experimental stimuli in African Americans, Hispanics, and Asian Americans.[11] Our previous study was the first to investigate genetic risk factors for pain in a black Southern African population.[12] In that study, we investigated whether 6 SNPs from the 15-SNP *GCH1* "pain-protective" haplotype that had been identified in a European cohort were associated with altered pain intensity and pain susceptibility in a black Southern African population with painful HIV-SN. We found that the 3-SNP "pain-protective" haplotype[10] was significantly associated with reduced pain intensity in our cohort on univariate analysis. However, the association was weak and did not withstand correction for other factors that may influence pain sensitivity (eg, sex and age). Given the weak association, we detected on univariate analysis and the greater genetic diversity in African populations compared with other populations,[13] we suggest that a more detailed study of *GCH1* be undertaken employing a more nuanced analysis that is using African-specific markers, rather than only those identified in a European population.

In this study, we reinvestigated the association between polymorphisms in *GCH1* and pain intensity in a black Southern African cohort with HIV-SN using population-specific tagging

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SNPs. Additionally, due to the strong association between rs734784 in *KCNS1* in various neuropathic pain conditions in European and Israeli cohorts, we have extended our assessment of "pain genes" in HIV-SN to include *KCNS1*. Here we investigate whether the previously identified SNP in *KCNS1* and population-specific tagging SNPs in *KCNS1* associate with pain intensity in a black Southern African population.

METHODS

Ethical approval for the study was obtained from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (protocol no M110754), and written informed consent was obtained from all the participants before sampling.

Population and DNA Samples

DNA samples were collected in a previous study from 342 adult (>18 years) black Southern African individuals attending the Virology Clinic at the Charlotte Maxeke Johannesburg Academic Hospital, South Africa.[12] The total number of individuals in the current analysis includes only those 158 patients who were deemed to have HIV-SN using the AIDS Clinical Trials Group Brief Peripheral Neuropathy Screen,[14] based on the bilateral presence of signs and symptoms of neuropathy, and who had a current pain score.[4] The group of 158 participants included 123 females (77.8%). The mean (SD) age of the group was 40.9 (8.1), and the median (interquartile ratio) CD4 T-cell count was 400 (291–557). There were no significant differences in age and CD4 T-cell count between males and females. All the patients had been on antiretroviral therapy for at least 6 months, and all but 8 individuals had been exposed to stavudine. The intensity of the pain experienced at the site of the neuropathy by each individual was rated on an 11-point numeric pain rating scale ("0" = no pain, "10" = worst pain imaginable).

SNP Selection

SNPs from *KCNS1* and *GCH1* that had a previous association with pain intensity were selected and supplemented with tagSNPs appropriate for an African population. tagSNPs were selected based on data from the Yoruba in Ibadan, Nigeria population (HapMap Data Release 27, Phase II + III, February 2009, on NCBI B36 assembly, dbSNP b126).[15] tagSNP selection employed a pairwise approach at $r^2 = 1.0$ and with minor allele frequency >0.01. All alleles are designated respective to the forward (+) strand of the genome assembly and were obtained using BioMart (Ensembl release 67, May 2012).[16,17] A final SNP list was compiled after assay design tool evaluation by Illumina,[18] which eliminated any SNPs that could not be genotyped using the method outlined below.

Genotyping and Data Cleaning

Genotyping was carried out using the GoldenGate genotyping assay with VeraCode microbeads (Illumina) and data were read on an Illumina BeadXpress Reader.[19] Data were loaded into BeadStudio Data Analysis Software From Illumina, Inc (Version 3.1.3.0, genotyping module version 3.2.32; Illumina) for a preanalysis data quality control, and SNPs and samples were excluded accordingly.

Data Analysis

Data were analyzed using PLINK software for association analysis.[20,21] All SNPs were assessed for Hardy–Weinberg equilibrium and were excluded from further analysis if $P < 0.01$ in all individuals. A minor allele frequency threshold of 0.01 was used throughout the analysis. Univariate analysis involved a *t*-test for assessment of the association between the alleles present and pain score. Multivariate analysis correcting for age, gender, and CD4 T-cell count was performed using linear regression. Haplotype analysis in *KCNS1* involved testing all possible combinations of the SNP alleles across the region, from 2-SNP through to 4-SNP haplotypes. The sliding window approach (specifying a fixed haplotype size of 2 and 3 SNPs across the region) was carried out in *GCH1*. Univariate and multivariate analyses correcting for age, gender, and CD4 T-cell count were also conducted for haplotype association analysis. The pointwise empirical *P* value (P_{EMP1}; based on 1000 iterations) was calculated for all statistical comparisons. SNPs were only carried forward to multivariate analysis if $P_{EMP1} < 0.1$ and significant associations after multivariate analysis were considered to be those with $P_{EMP1} < 0.05$. Familywise correction for multiple comparisons was not performed for these exploratory analyses.

RESULTS

SNP Selection

tagSNP selection for *KCNS1* produced 3 additional SNPs (in addition to the previously associated SNP rs734784) for investigation—rs4499491, rs6017486, and rs6073643 (Fig. 1A).

In *GCH1*, 12 of the 15 "pain-protective" haplotype SNPs were genotyped and included in the analysis. The 3 SNPs that were not included (rs2183081, rs7147286, and rs7492600) failed assay design tool evaluation when developing the SNP list. tagSNP selection produced a further 19 SNPs for genotyping and analysis (Fig. 1B).

All SNPs were in Hardy–Weinberg equilibrium (results not shown).

Associations With Pain Intensity

KCNS1

One SNP, rs4499491, which was associated with decreased pain intensity, passed the $P_{EMP1} < 0.1$ univariate analysis threshold ($P = 0.09$). No significant association was detected between the SNP and pain intensity after correcting for age, gender and CD4 T-cell count ($P = 0.08$).

Five haplotypes associated with pain intensity on univariate and multivariate analyses (Table 1). Three of the haplotypes associated with decreased pain intensity and 2 with increased pain intensity. All haplotypes contained the SNP rs6017486, with the A-allele associating with decreased

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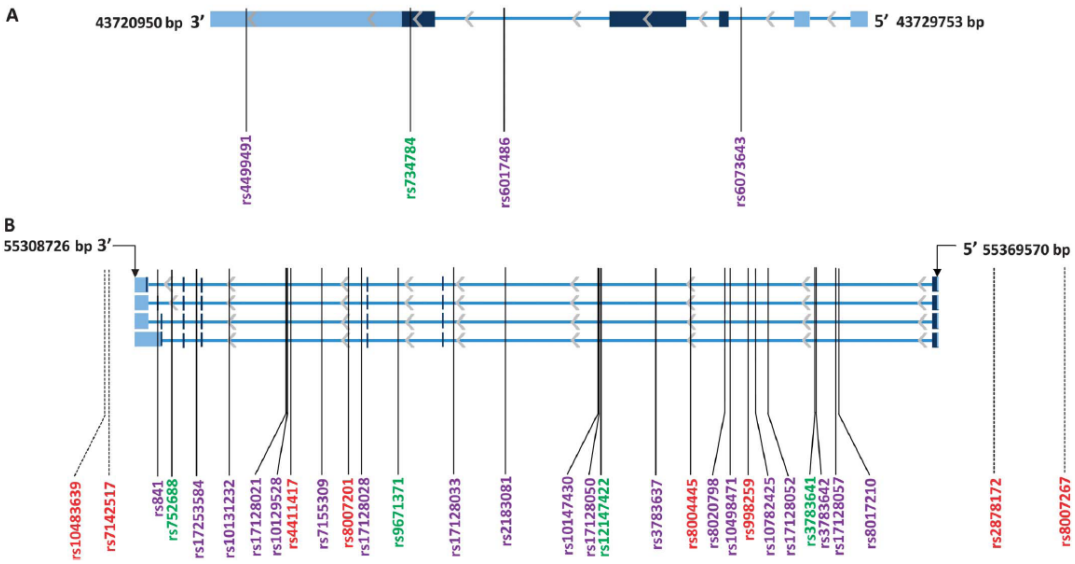

FIGURE 1. *KCNS1* and *GCH1* gene diagrams with the SNPs chosen for analysis annotated on the diagrams. A, *KCNS1*; B, *GCH1*; SNPs in red indicate the previously associated SNPs; SNPs in purple indicate the tagSNPs; and SNPs in green indicate SNPs that were both previously associated and resulted from tagSNP selection. [Information obtained from The National Center for Biotechnology Information Genome Browser (build 37.1); http://www.ncbi.nlm.nih.gov/gene.]

pain intensity and the G-allele with increased pain intensity. No haplotypes significantly associated with altered pain intensity contained rs734784, which had previously been identified in non-African populations.

GCH1

In addition to the lack of association we reported previously, no individual SNPs or haplotypes associated with pain intensity after correcting for age, gender or CD4 T-cell count (data not shown).

DISCUSSION

We investigated whether polymorphisms in *KCNS1* and *GCH1* were associated with the intensity of pain experienced by individuals with symptomatic HIV-SN. Marker selection was based on SNPs previously described in the literature in non-African populations, and additionally, population-specific tagging SNPs that allow a more nuanced investigation based on the underlying genetic structure of the population being studied. This is the second study to investigate the genetics of pain in a black Southern African population, and the first such study to employ strategies to attempt to tailor the investigation to the population under investigation.

Despite a strong indication for the association of polymorphisms in *GCH1* with reduced pain in individuals of European descent who had undergone discectomy for persistent lumbar root pain, a similar association was not detected when we assessed the same polymorphisms in a black Southern African population with HIV-SN.[12] In this study, we extended our initial study[12] by the inclusion of population-specific tagging SNPs, but nevertheless, the same conclusions were drawn as in our previous investigation, namely, no association between polymorphisms in *GCH1* and pain intensity in a black Southern African population with HIV-SN. Our failure to detect an association, even when using a population-tailored approach to SNP selection, could mean that polymorphisms in *GCH1* do not have a role in pain sensitivity in black Southern Africans as such, or that the polymorphisms in *GCH1* do not have a role in pain intensity in HIV-SN specifically.

Analysis of *KCNS1* revealed that no individual SNPs significantly associated with pain intensity after correcting for age, gender, and CD4 T-cell count. Five haplotypes, however,

TABLE 1. *KCNS1* Haplotype Association Analysis for Pain Intensity

Haplotypes				
rs4499491	rs6017486	rs6073643	*P**	Beta†
C	A	T	0.031	−1.72
C	G	T	0.031	0.89
A	A		0.005	−5.45
C	G		0.022	0.91
	A	T	0.009	−1.93

*P_{EMP1} after correction for age, gender, and CD4 T-cell count.
†A positive beta value indicates increased pain intensity, and a negative beta value indicates decreased pain intensity.

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did show a significant association with reported pain intensity on univariate analysis and multivariate analysis. Of the 5 haplotypes, 3 were associated with decreased pain intensity and 2 associated with increased pain intensity. The SNP rs6017486 seems to mark the direction of the relationship, with the presence of the A-allele being associated with the decreased pain intensity and the presence of the G-allele being associated with increased pain intensity, despite the SNP not associating itself, with changes in pain intensity. The fact that haplotypes, but not individual SNPs, associated with pain intensity in our cohort suggests that the actual causative SNP(s) could be incorporated in the region of the gene marked by the haplotypes. It is also noteworthy that the SNP (rs734784) originally described in the literature as being associated with increased pain sensitivity in a variety of neuropathic pain states in non-African populations[7] was not part of any of the haplotypes that we identified in the pain intensity analyses. This indicates the importance of using a population-based SNP selection process when assessing genetic associations.

Interpretation of our findings, both positive and negative, needs to occur in the context of the HapMap population used to select tagSNPs. We used the West African Yoruba population data from the HapMap database to select our population-specific tagging SNPs but conducted the study in a Southern African population. Population differences in genetic diversity, and hence linkage disequilibrium, between these 2 African groups may have weakened our power to detect small effects,[13] but at the time of the study, the Yoruban data were the best-matched dataset available.

In conclusion, polymorphisms in *GCH1* did not associate with pain intensity in HIV-positive individuals of black Southern African ancestry with HIV-SN, even though we used population-specific genetic markers. However, unique, population-specific haplotypes of *KCNS1* was associated with differences in pain intensity in the same group of patients. No individual SNPs we assessed in *KCNS1* were independently associated with differences in pain intensity suggesting that the haplotypes could incorporate the causative SNP(s). Our findings for *KCNS1* provide additional evidence supporting an important role for *KCNS1* in neuropathic pain across diverse population groups and neuropathic pain states. We believe that our study illustrates the importance of conducting association analyses in independent ethnic groups and using population-specific SNP selection.

ACKNOWLEDGMENTS

The authors 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.

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Appendix G – Full results set: Chapter 4

GCH1 – Single SNP analysis

SNP	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
	ALLELIC MODEL			GENOTYPIC MODEL		DOMINANT MODEL		RECESSIVE MODEL		ALLELIC MODEL		
	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	BETA
rs10483639	0.119	0.745	0.51	0.054	0.529	**0.050**	0.417	1.000	1.000	-	-	-
rs7142517	0.999	1.000	NA	1.000	1.000	1.000	1.000	1.000	1.000	0.696	1.000	0.52
rs841	0.197	0.892	0.46	0.102	0.838	0.064	0.633	1.000	1.000	0.299	0.991	-0.62
rs752688	0.104	0.691	0.39	**0.024**	0.312	0.021	0.279	0.989	1.000	0.290	0.987	-0.61
rs17253584	0.981	1.000	1.31	0.989	1.000	0.975	1.000	0.910	1.000	0.709	1.000	0.22
rs10131232	0.236	0.969	1.97	0.628	1.000	0.392	1.000	0.960	1.000	0.803	1.000	0.11
rs17128021	0.730	1.000	1.26	0.870	1.000	0.760	1.000	0.727	1.000	0.914	1.000	-0.04
rs10129528	1.000	1.000	1.01	1.000	1.000	1.000	1.000	1.000	1.000	0.841	1.000	0.08
rs4411417	0.162	0.894	0.46	0.163	0.961	0.126	0.880	0.939	1.000	0.416	0.998	-0.51
rs7155309	**0.034**	0.672	0.40	0.090	0.919	**0.021**	0.562	1.000	1.000	0.081	0.777	-1.08
rs8007201	0.110	0.786	2.36	0.407	0.998	0.168	0.936	0.535	1.000	0.948	1.000	0.03
rs17128028	0.658	0.999	0.84	0.553	1.000	1.000	1.000	0.283	0.958	0.602	1.000	-0.22
rs9671371	0.529	0.999	0.79	0.746	1.000	0.781	1.000	0.634	1.000	0.546	1.000	0.24
rs17128033	0.333	0.978	0.61	0.342	0.998	0.353	0.999	0.508	1.000	0.676	1.000	-0.19
rs2183081	0.063	0.625	3.12	0.198	0.978	0.085	0.723	0.521	1.000	0.458	1.000	0.29
rs10147430	1.000	1.000	1.68	0.835	1.000	0.821	1.000	0.617	1.000	0.863	1.000	0.14
rs17128050	0.496	0.999	2.60	0.696	1.000	0.674	1.000	1.000	1.000	0.917	1.000	0.09
rs12147422	1.000	1.000	1.01	1.000	1.000	1.000	1.000	1.000	1.000	0.742	1.000	-0.12
rs3783637	0.578	0.999	1.65	0.811	1.000	0.760	1.000	0.784	1.000	0.551	1.000	0.32
rs8004445	0.960	1.000	1.02	0.971	1.000	0.931	1.000	0.914	1.000	0.674	1.000	-0.15
rs8020798	0.816	1.000	0.89	0.974	1.000	0.796	1.000	0.918	1.000	0.291	0.990	-0.45
rs10498471	0.737	1.000	1.17	0.942	1.000	0.776	1.000	0.999	1.000	0.315	0.995	0.35
rs10782425	0.967	1.000	0.95	1.000	1.000	0.961	1.000	1.000	1.000	0.229	0.976	-1.29
rs17128052	0.164	0.937	0.50	0.195	0.990	0.083	0.765	0.388	1.000	0.090	0.792	-0.91
rs3783641	0.082	0.699	0.52	0.100	0.869	0.059	0.558	0.636	1.000	0.354	0.996	-0.37
rs3783642	0.118	0.782	2.49	0.352	0.998	0.165	0.934	0.594	1.000	0.670	1.000	0.16
rs17128057	1.000	1.000	1.20	1.000	1.000	0.827	1.000	1.000	1.000	0.810	1.000	-0.25
rs8017210	0.191	0.931	0.61	0.289	0.995	0.422	1.000	0.154	0.795	0.976	1.000	0.01
rs2878172	0.198	0.894	2.28	0.487	1.000	0.316	1.000	0.288	0.996	0.745	1.000	-0.12
rs8007267	**0.037**	0.397	2.85	0.115	0.885	0.084	0.745	0.122	0.709	0.916	1.000	-0.04

GCH1 – Haplotype analysis

HAPLOTYPE				UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
				OR	P_{EMP1}	P_{EMP2}	BETA	P_{EMP1}	P_{EMP2}
rs10483639	rs7142517	rs841	CCA	0.43	0.082	0.954	-	-	-
rs10483639	rs7142517	rs841	GCA	5.26	0.437	1.000	-	-	-

rs10483639	rs7142517	rs841	GAG	inf	0.942	1.000	-	-	-
rs10483639	rs7142517	rs841	CCG	0.74	0.464	1.000	-	-	-
rs10483639	rs7142517	rs841	GCG	1.56	0.249	0.999	-	-	-
rs7142517	rs841	rs752688	CAT	0.47	0.069	0.984	-0.78	0.194	1.000
rs7142517	rs841	rs752688	AGC	6.46E+25	0.934	1.000	0.66	0.643	1.000
rs7142517	rs841	rs752688	CGC	1.83	0.228	0.996	0.37	0.484	1.000
rs841	rs752688	rs17253584	GCC	1.57	0.581	1.000	0.29	0.607	1.000
rs841	rs752688	rs17253584	ATT	0.49	0.101	0.993	-0.76	0.206	1.000
rs841	rs752688	rs17253584	GTT	0.67	0.310	1.000	0.30	0.854	1.000
rs841	rs752688	rs17253584	GCT	1.37	0.478	1.000	0.16	0.695	1.000
rs752688	rs17253584	rs10131232	CCG	2.69	0.765	1.000	-0.44	0.863	1.000
rs752688	rs17253584	rs10131232	TTG	0.11	0.066	0.990	-1.43	0.507	1.000
rs752688	rs17253584	rs10131232	CTG	3.57	0.087	0.962	0.24	0.628	1.000
rs752688	rs17253584	rs10131232	CCA	1.43	0.656	1.000	0.36	0.560	1.000
rs752688	rs17253584	rs10131232	TTA	0.47	0.074	0.995	-0.62	0.326	1.000
rs752688	rs17253584	rs10131232	CTA	0.84	0.644	1.000	0.01	0.981	1.000
rs17253584	rs10131232	rs17128021	TAA	1.23	0.616	1.000	-0.08	0.844	1.000
rs17253584	rs10131232	rs17128021	CGG	0.28	0.211	1.000	-1.28	0.513	1.000
rs17253584	rs10131232	rs17128021	TGG	2.63	0.142	0.987	0.19	0.657	1.000
rs17253584	rs10131232	rs17128021	CAG	1.86	0.527	1.000	0.39	0.514	1.000
rs17253584	rs10131232	rs17128021	TAG	0.44	**0.013**	0.605	-0.17	0.668	1.000
rs10131232	rs17128021	rs10129528	AAT	1.00	0.910	1.000	0.11	0.780	1.000
rs10131232	rs17128021	rs10129528	AAC	3.12	0.396	1.000	-0.58	0.398	1.000
rs10131232	rs17128021	rs10129528	GGC	1.98	0.179	0.997	0.03	0.945	1.000
rs10131232	rs17128021	rs10129528	AGC	0.54	0.101	0.975	0.03	0.921	1.000
rs17128021	rs10129528	rs4411417	GCC	0.48	0.080	0.995	-0.49	0.444	1.000
rs17128021	rs10129528	rs4411417	ATT	1.00	0.986	1.000	0.11	0.780	1.000
rs17128021	rs10129528	rs4411417	ACT	3.11	0.397	1.000	-0.58	0.396	1.000
rs17128021	rs10129528	rs4411417	GCT	1.10	0.807	1.000	0.22	0.554	1.000
rs10129528	rs4411417	rs7155309	CCC	0.37	0.062	0.896	-1.02	0.129	0.996
rs10129528	rs4411417	rs7155309	CCT	2.44E+23	0.520	1.000	1.58	0.265	1.000
rs10129528	rs4411417	rs7155309	TTT	1.01	0.950	1.000	0.10	0.814	1.000
rs10129528	rs4411417	rs7155309	CTT	1.33	0.471	1.000	0.12	0.734	1.000
rs4411417	rs7155309	rs8007201	TTA	2.59	0.087	0.970	0.06	0.897	1.000
rs4411417	rs7155309	rs8007201	CCG	0.36	0.054	0.899	-1.06	0.132	0.997
rs4411417	rs7155309	rs8007201	CTG	1.13E+27	0.627	1.000	1.58	0.265	1.000
rs4411417	rs7155309	rs8007201	TTG	0.76	0.450	1.000	0.17	0.635	1.000
rs7155309	rs8007201	rs17128028	TGT	0.85	0.749	1.000	-0.19	0.665	1.000
rs7155309	rs8007201	rs17128028	TAC	2.58	0.101	0.970	0.13	0.782	1.000
rs7155309	rs8007201	rs17128028	CGC	0.38	0.074	0.955	-0.93	0.190	1.000
rs7155309	rs8007201	rs17128028	TGC	0.95	0.890	1.000	0.39	0.319	1.000
rs8007201	rs17128028	rs9671371	GCT	0.77	0.580	1.000	0.25	0.545	1.000
rs8007201	rs17128028	rs9671371	GTC	0.82	0.649	1.000	-0.14	0.767	1.000
rs8007201	rs17128028	rs9671371	ACC	2.30	0.144	0.987	0.14	0.755	1.000
rs8007201	rs17128028	rs9671371	GCC	0.79	0.607	1.000	-0.30	0.543	1.000
rs17128028	rs9671371	rs17128033	TCT	0.64	0.281	1.000	-0.26	0.596	1.000

rs17128028	rs9671371	rs17128033	CTC	0.79	0.600	1.000	0.31	0.455	1.000
rs17128028	rs9671371	rs17128033	TCC	2.74	0.300	1.000	0.09	0.894	1.000
rs17128028	rs9671371	rs17128033	CCC	1.48	0.404	1.000	-0.13	0.726	1.000
rs9671371	rs17128033	rs2183081	CCA	3.11	0.084	0.887	0.29	0.502	1.000
rs9671371	rs17128033	rs2183081	CTG	0.64	0.267	1.000	-0.13	0.803	1.000
rs9671371	rs17128033	rs2183081	TCG	0.76	0.537	1.000	0.27	0.516	1.000
rs9671371	rs17128033	rs2183081	CCG	0.90	0.818	1.000	-0.42	0.269	1.000
rs17128033	rs2183081	rs10147430	TGA	1.35	0.503	1.000	0.57	0.558	1.000
rs17128033	rs2183081	rs10147430	CGA	34.40	0.404	1.000	-1.07	0.528	1.000
rs17128033	rs2183081	rs10147430	CAG	3.14	0.064	0.891	0.20	0.646	1.000
rs17128033	rs2183081	rs10147430	TGG	0.59	0.214	0.999	-0.41	0.457	1.000
rs17128033	rs2183081	rs10147430	CGG	0.63	0.292	0.999	0.03	0.954	1.000
rs2183081	rs10147430	rs17128050	GAC	60.80	0.498	1.000	1.07	0.657	1.000
rs2183081	rs10147430	rs17128050	GGC	2.12	0.469	1.000	0.03	0.979	1.000
rs2183081	rs10147430	rs17128050	GAT	1.33	0.603	1.000	-0.03	0.971	1.000
rs2183081	rs10147430	rs17128050	AGT	3.03	0.100	0.935	0.31	0.475	1.000
rs2183081	rs10147430	rs17128050	GGT	0.35	**0.019**	0.690	-0.27	0.480	1.000
rs10147430	rs17128050	rs12147422	ATT	1.41	0.453	1.000	0.35	0.821	1.000
rs10147430	rs17128050	rs12147422	GTT	0.99	0.971	1.000	-0.11	0.748	1.000
rs10147430	rs17128050	rs12147422	GCC	2.46	0.353	1.000	0.02	0.975	1.000
rs10147430	rs17128050	rs12147422	ATC	1.56	0.598	1.000	-0.05	0.976	1.000
rs10147430	rs17128050	rs12147422	GTC	0.82	0.565	1.000	0.08	0.823	1.000
rs17128050	rs12147422	rs3783637	CCT	2.40	0.323	1.000	0.35	0.641	1.000
rs17128050	rs12147422	rs3783637	TCT	1.21	0.740	1.000	0.20	0.754	1.000
rs17128050	rs12147422	rs3783637	TTC	1.01	0.963	1.000	-0.13	0.725	1.000
rs17128050	rs12147422	rs3783637	TCC	0.80	0.474	1.000	0.01	0.956	1.000
rs12147422	rs3783637	rs8004445	TCG	0.99	0.913	1.000	-0.14	0.705	1.000
rs12147422	rs3783637	rs8004445	CTT	1.59	0.439	1.000	0.29	0.588	1.000
rs12147422	rs3783637	rs8004445	CCT	0.81	0.550	1.000	0.00	0.992	1.000
rs3783637	rs8004445	rs8020798	CGT	0.77	0.611	1.000	-0.55	0.257	1.000
rs3783637	rs8004445	rs8020798	CTT	1.55	0.743	1.000	-0.12	0.899	1.000
rs3783637	rs8004445	rs8020798	TGC	3.35	0.711	1.000	-0.72	0.777	1.000
rs3783637	rs8004445	rs8020798	CGC	1.24	0.652	1.000	0.20	0.650	1.000
rs3783637	rs8004445	rs8020798	TTC	1.65	0.405	1.000	0.36	0.516	1.000
rs3783637	rs8004445	rs8020798	CTC	0.74	0.439	1.000	0.03	0.947	1.000
rs8004445	rs8020798	rs10498471	TTA	1.63	0.661	1.000	-0.12	0.897	1.000
rs8004445	rs8020798	rs10498471	TCA	1.06	0.898	1.000	0.41	0.295	1.000
rs8004445	rs8020798	rs10498471	GTG	0.78	0.638	1.000	-0.56	0.247	1.000
rs8004445	rs8020798	rs10498471	GCG	1.21	0.708	1.000	0.21	0.632	1.000
rs8004445	rs8020798	rs10498471	TCG	0.57	0.417	1.000	-0.86	0.263	1.000
rs8020798	rs10498471	rs10782425	CGA	1.07	0.531	1.000	-1.35	0.272	1.000
rs8020798	rs10498471	rs10782425	TAG	1.44	0.681	1.000	0.01	0.992	1.000
rs8020798	rs10498471	rs10782425	CAG	1.13	0.782	1.000	0.38	0.341	1.000
rs8020798	rs10498471	rs10782425	TGG	0.81	0.702	1.000	-0.54	0.257	1.000
rs8020798	rs10498471	rs10782425	CGG	0.97	0.941	1.000	0.15	0.730	1.000
rs10498471	rs10782425	rs17128052	GGC	0.48	0.134	0.991	-0.96	0.090	0.982

rs10498471	rs10782425	rs17128052	GAG	0.94	0.238	1.000	-1.26	0.233	1.000
rs10498471	rs10782425	rs17128052	AGG	1.15	0.787	1.000	0.34	0.364	1.000
rs10498471	rs10782425	rs17128052	GGG	1.28	0.556	1.000	0.22	0.573	1.000
rs10782425	rs17128052	rs3783641	GCA	0.52	0.228	0.996	-0.91	0.100	0.991
rs10782425	rs17128052	rs3783641	GGA	0.71	0.470	1.000	0.11	0.819	1.000
rs10782425	rs17128052	rs3783641	AGT	2.06	0.454	1.000	-1.58	0.253	1.000
rs10782425	rs17128052	rs3783641	GGT	1.77	0.131	0.988	0.51	0.182	1.000
rs17128052	rs3783641	rs3783642	GAT	3.97	0.537	1.000	1.02	0.668	1.000
rs17128052	rs3783641	rs3783642	GTT	2.46	0.105	0.977	0.11	0.798	1.000
rs17128052	rs3783641	rs3783642	CAC	0.48	0.145	0.990	-0.93	0.092	0.983
rs17128052	rs3783641	rs3783642	GAC	0.69	0.459	1.000	0.04	0.928	1.000
rs17128052	rs3783641	rs3783642	GTC	1.03	0.948	1.000	0.27	0.477	1.000
rs3783641	rs3783642	rs17128057	ACT	1.26	0.572	1.000	-0.28	0.778	1.000
rs3783641	rs3783642	rs17128057	ATC	1.29	0.849	1.000	0.59	0.787	1.000
rs3783641	rs3783642	rs17128057	TTC	2.57	0.095	0.965	0.12	0.762	1.000
rs3783641	rs3783642	rs17128057	ACC	0.48	0.083	0.877	-0.40	0.365	1.000
rs3783641	rs3783642	rs17128057	TCC	1.02	0.957	1.000	0.24	0.513	1.000
rs3783642	rs17128057	rs8017210	CTA	1.24	0.622	1.000	-0.28	0.786	1.000
rs3783642	rs17128057	rs8017210	CCA	0.57	0.174	0.995	0.06	0.883	1.000
rs3783642	rs17128057	rs8017210	TCG	2.45	0.134	0.970	0.12	0.752	1.000
rs3783642	rs17128057	rs8017210	CCG	0.88	0.802	1.000	-0.12	0.765	1.000
rs17128057	rs8017210	rs2878172	CAA	4.10	0.754	1.000	-1.81	0.502	1.000
rs17128057	rs8017210	rs2878172	CGA	1.89	0.207	0.996	-0.11	0.774	1.000
rs17128057	rs8017210	rs2878172	TAG	1.24	0.528	1.000	-0.28	0.785	1.000
rs17128057	rs8017210	rs2878172	CAG	0.55	0.154	0.988	0.13	0.767	1.000
rs17128057	rs8017210	rs2878172	CGG	1.01	0.982	1.000	0.09	0.830	1.000
rs8017210	rs2878172	rs8007267	GAC	2.00	0.195	0.996	-0.11	0.811	1.000
rs8017210	rs2878172	rs8007267	GGC	4.05	0.133	0.995	0.07	0.895	1.000
rs8017210	rs2878172	rs8007267	GAT	10.70	0.264	1.000	0.42	0.737	1.000
rs8017210	rs2878172	rs8007267	AGT	0.56	0.181	0.995	0.06	0.880	1.000
rs8017210	rs2878172	rs8007267	GGT	0.64	0.291	0.999	0.00	0.998	1.000
rs10483639	rs7142517		GA	458.00	0.841	1.000			
rs10483639	rs7142517		CC	0.53	0.063	0.943			
rs10483639	rs7142517		GC	1.69	0.177	0.995			
rs7142517	rs841		CA	0.47	0.066	0.987	-0.54	0.387	1.000
rs7142517	rs841		AG	15.40	0.753	1.000	0.40	0.765	1.000
rs7142517	rs841		CG	1.65	0.330	1.000	0.34	0.536	1.000
rs841	rs752688		AT	0.48	0.095	0.987	-0.69	0.255	1.000
rs841	rs752688		GT	0.47	0.163	1.000	-0.11	0.966	1.000
rs841	rs752688		GC	2.14	0.061	0.960	0.49	0.382	1.000
rs752688	rs17253584		CC	1.35	0.587	1.000	0.25	0.661	1.000
rs752688	rs17253584		TT	0.46	0.053	0.947	-0.60	0.271	1.000
rs752688	rs17253584		CT	1.47	0.367	1.000	0.19	0.627	1.000
rs17253584	rs10131232		CG	4.08	0.414	1.000	-0.21	0.926	1.000
rs17253584	rs10131232		TG	2.10	0.211	0.996	0.14	0.770	1.000
rs17253584	rs10131232		CA	1.30	0.731	1.000	0.26	0.676	1.000

rs17253584	rs10131232	TA	0.55	0.188	0.996	-0.19	0.638	1.000
rs10131232	rs17128021	AA	1.24	0.656	1.000	-0.04	0.930	1.000
rs10131232	rs17128021	GG	1.99	0.199	0.996	0.02	0.983	1.000
rs10131232	rs17128021	AG	0.54	0.103	0.977	0.03	0.928	1.000
rs17128021	rs10129528	AT	1.00	0.987	1.000	0.08	0.842	1.000
rs17128021	rs10129528	AC	3.07	0.238	1.000	-0.61	0.367	1.000
rs17128021	rs10129528	GC	0.80	0.545	1.000	0.09	0.828	1.000
rs10129528	rs4411417	CC	0.47	0.068	0.993	-0.37	0.573	1.000
rs10129528	rs4411417	TT	1.00	0.921	1.000	0.07	0.858	1.000
rs10129528	rs4411417	CT	1.38	0.361	1.000	0.06	0.857	1.000
rs4411417	rs7155309	CC	0.34	**0.034**	0.827	-1.05	0.125	0.993
rs4411417	rs7155309	CT	6.81	0.865	1.000	1.56	0.274	1.000
rs4411417	rs7155309	TT	2.04	0.072	0.995	0.60	0.317	1.000
rs7155309	rs8007201	TA	3.42	0.066	0.874	0.11	0.801	1.000
rs7155309	rs8007201	CG	0.36	0.053	0.935	-1.10	0.119	0.991
rs7155309	rs8007201	TG	0.75	0.525	1.000	0.26	0.477	1.000
rs8007201	rs17128028	GT	0.85	0.754	1.000	-0.19	0.674	1.000
rs8007201	rs17128028	AC	2.34	0.129	0.986	0.09	0.821	1.000
rs8007201	rs17128028	GC	0.67	0.321	1.000	0.09	0.836	1.000
rs17128028	rs9671371	CT	0.78	0.577	1.000	0.36	0.405	1.000
rs17128028	rs9671371	TC	0.85	0.754	1.000	-0.19	0.671	1.000
rs17128028	rs9671371	CC	1.45	0.414	1.000	-0.16	0.675	1.000
rs9671371	rs17128033	CT	0.62	0.260	0.999	-0.15	0.765	1.000
rs9671371	rs17128033	TC	0.79	0.596	1.000	0.35	0.403	1.000
rs9671371	rs17128033	CC	1.74	0.178	0.995	-0.20	0.594	1.000
rs17128033	rs2183081	CA	3.12	0.064	0.882	0.22	0.613	1.000
rs17128033	rs2183081	TG	0.65	0.313	1.000	-0.17	0.743	1.000
rs17128033	rs2183081	CG	0.69	0.405	1.000	-0.06	0.880	1.000
rs2183081	rs10147430	GA	1.67	0.357	1.000	0.10	0.908	1.000
rs2183081	rs10147430	AG	3.12	0.070	0.882	0.17	0.693	1.000
rs2183081	rs10147430	GG	0.33	0.054	0.781	-0.17	0.671	1.000
rs10147430	rs17128050	AC	34.60	0.534	1.000	0.70	0.771	1.000
rs10147430	rs17128050	GC	2.38	0.263	1.000	-0.04	0.957	1.000
rs10147430	rs17128050	AT	1.12	0.591	1.000	-0.29	0.789	1.000
rs10147430	rs17128050	GT	0.50	0.320	1.000	0.06	0.913	1.000
rs17128050	rs12147422	TT	0.99	0.870	1.000	-0.14	0.705	1.000
rs17128050	rs12147422	CC	2.52	0.235	1.000	0.21	0.773	1.000
rs17128050	rs12147422	TC	0.86	0.640	1.000	0.09	0.809	1.000
rs12147422	rs3783637	CT	1.66	0.362	1.000	0.27	0.605	1.000
rs12147422	rs3783637	TC	1.01	0.946	1.000	-0.13	0.743	1.000
rs12147422	rs3783637	CC	0.81	0.500	1.000	7.65	1.000	1.000
rs3783637	rs8004445	TG	3.70	0.705	1.000	-0.81	0.753	1.000
rs3783637	rs8004445	CG	1.01	0.956	1.000	-0.13	0.724	1.000
rs3783637	rs8004445	TT	1.66	0.390	1.000	0.31	0.575	1.000
rs3783637	rs8004445	CT	0.81	0.532	1.000	0.02	0.975	1.000
rs8004445	rs8020798	GT	0.80	0.660	1.000	-0.51	0.283	1.000

rs8004445	rs8020798	TT	1.45	0.821	1.000	-0.26	0.772	1.000
rs8004445	rs8020798	GC	1.23	0.656	1.000	0.16	0.743	1.000
rs8004445	rs8020798	TC	0.93	0.859	1.000	0.20	0.616	1.000
rs8020798	rs10498471	TA	1.39	0.778	1.000	0.01	0.992	1.000
rs8020798	rs10498471	CA	1.13	0.802	1.000	0.40	0.318	1.000
rs8020798	rs10498471	TG	0.80	0.699	1.000	-0.65	0.185	1.000
rs8020798	rs10498471	CG	0.98	0.960	1.000	0.01	0.990	1.000
rs10498471	rs10782425	GA	0.96	0.360	1.000	-1.30	0.226	1.000
rs10498471	rs10782425	AG	1.16	0.653	1.000	0.35	0.339	1.000
rs10498471	rs10782425	GG	0.86	0.671	1.000	-0.22	0.578	1.000
rs10782425	rs17128052	GC	0.52	0.228	0.996	-0.83	0.139	0.998
rs10782425	rs17128052	AG	2.26	0.333	1.000	-1.38	0.247	1.000
rs10782425	rs17128052	GG	1.79	0.291	0.999	0.99	**0.049**	0.904
rs17128052	rs3783641	CA	0.48	0.173	0.995	-0.87	0.116	0.995
rs17128052	rs3783641	GA	0.76	0.529	1.000	0.11	0.814	1.000
rs17128052	rs3783641	GT	1.79	0.159	0.987	0.34	0.368	1.000
rs3783641	rs3783642	AT	1.74	0.801	1.000	0.59	0.789	1.000
rs3783641	rs3783642	TT	2.44	0.110	0.977	0.12	0.790	1.000
rs3783641	rs3783642	AC	0.52	0.100	0.963	-0.49	0.225	1.000
rs3783641	rs3783642	TC	1.02	0.994	1.000	0.30	0.433	1.000
rs3783642	rs17128057	CT	1.23	0.458	1.000	-0.30	0.760	1.000
rs3783642	rs17128057	TC	2.39	0.107	0.975	0.23	0.585	1.000
rs3783642	rs17128057	CC	0.42	0.089	0.955	-0.17	0.646	1.000
rs17128057	rs8017210	TA	1.14	0.434	1.000	-0.39	0.698	1.000
rs17128057	rs8017210	CA	0.56	0.176	0.995	0.13	0.785	1.000
rs17128057	rs8017210	CG	1.74	0.173	0.995	-0.06	0.907	1.000
rs8017210	rs2878172	AA	4.50	0.697	1.000	-1.99	0.460	1.000
rs8017210	rs2878172	GA	2.03	0.191	0.996	-0.11	0.816	1.000
rs8017210	rs2878172	AG	0.52	0.139	0.983	-0.03	0.943	1.000
rs8017210	rs2878172	GG	1.06	0.914	1.000	0.17	0.670	1.000
rs2878172	rs8007267	AC	1.93	0.236	0.999	-0.18	0.690	1.000
rs2878172	rs8007267	GC	3.76	0.180	0.996	0.00	0.994	1.000
rs2878172	rs8007267	AT	42.90	0.451	1.000	-0.02	0.991	1.000
rs2878172	rs8007267	GT	0.38	**0.035**	0.727	0.11	0.734	1.000

KCNS1 – Single SNP analysis

SNP	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
	ALLELIC MODEL			GENOTYPIC MODEL		DOMINANT MODEL		RECESSIVE MODEL		ALLELIC MODEL		
	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	BETA
rs4499491	0.178	0.448	0.59	0.207	0.500	0.090	0.247	0.996	1.000	0.092	0.248	-0.71
rs734784	0.164	0.496	0.61	0.363	0.772	0.332	0.698	0.267	0.485	0.667	0.973	0.19
rs6017486	0.925	1.000	1.08	1.000	1.000	1.000	1.000	1.000	1.000	0.266	0.617	-0.45
rs6073643	0.182	0.471	1.98	0.326	0.788	0.243	0.576	0.349	0.758	0.777	0.995	0.13

KCNS1 – Haplotype analysis

HAPLOTYPE					UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
					OR	P_{EMP1}	P_{EMP2}	BETA	P_{EMP1}	P_{EMP2}
rs4499491	rs734784	rs6017486	rs6073643	CCAC	1.81	0.271	0.929	0.09	0.837	1.000
rs4499491	rs734784	rs6017486	rs6073643	CCGC	2.54	0.278	0.981	0.34	0.661	1.000
rs4499491	rs734784	rs6017486	rs6073643	CTAT	0.62	0.620	1.000	-1.38	0.257	0.968
rs4499491	rs734784	rs6017486	rs6073643	CCAT	0.27	0.125	0.894	-2.17	0.120	0.799
rs4499491	rs734784	rs6017486	rs6073643	ATGT	0.45	0.162	0.839	-0.31	0.577	0.999
rs4499491	rs734784	rs6017486	rs6073643	CTGT	1.12	0.852	1.000	0.92	0.066	0.618
rs4499491	rs734784	rs6017486	rs6073643	ACGT	1.10	0.857	1.000	-1.01	0.149	0.855
rs4499491	rs734784	rs6017486	rs6073643	CCGT	1.19	0.758	1.000	0.58	0.335	0.992
rs4499491	rs734784	rs6017486		CTA	0.70	0.879	1.000	-1.23	0.350	0.992
rs4499491	rs734784	rs6017486		CCA	1.40	0.461	0.998	-0.22	0.592	0.999
rs4499491	rs734784	rs6017486		ATG	0.42	0.155	0.831	-0.33	0.581	0.999
rs4499491	rs734784	rs6017486		CTG	1.08	0.899	1.000	0.87	0.096	0.694
rs4499491	rs734784	rs6017486		ACG	1.06	0.930	1.000	-1.00	0.150	0.869
rs4499491	rs734784	rs6017486		CCG	1.77	0.378	0.981	0.79	0.156	0.872
rs4499491	rs734784	rs6073643		CCC	2.17	0.128	0.747	0.20	0.632	0.999
rs4499491	rs734784	rs6073643		ATT	0.34	**0.049**	0.440	-0.43	0.439	0.998
rs4499491	rs734784	rs6073643		CTT	1.04	0.910	1.000	0.60	0.209	0.943
rs4499491	rs734784	rs6073643		ACT	1.16	0.843	1.000	-0.99	0.160	0.875
rs4499491	rs734784	rs6073643		CCT	0.83	0.743	1.000	0.09	0.891	1.000
rs4499491	rs6017486	rs6073643		CAC	1.81	0.274	0.929	0.09	0.831	1.000
rs4499491	rs6017486	rs6073643		CGC	2.56	0.299	0.980	0.32	0.680	1.000
rs4499491	rs6017486	rs6073643		CAT	0.45	0.154	0.908	-1.47	0.079	0.678
rs4499491	rs6017486	rs6073643		AGT	0.65	0.290	0.956	-0.58	0.182	0.913
rs4499491	rs6017486	rs6073643		CGT	1.17	0.706	1.000	0.78	**0.050**	0.473
rs734784	rs6017486	rs6073643		CAC	1.81	0.244	0.923	0.01	0.975	1.000
rs734784	rs6017486	rs6073643		CGC	2.56	0.254	0.980	0.31	0.684	1.000
rs734784	rs6017486	rs6073643		TAT	0.33	0.098	0.789	-1.66	0.099	0.799
rs734784	rs6017486	rs6073643		CAT	0.26	0.128	0.892	-2.30	0.113	0.780
rs734784	rs6017486	rs6073643		TGT	0.69	0.387	0.981	0.48	0.241	0.967
rs734784	rs6017486	rs6073643		CGT	1.14	0.816	1.000	-0.15	0.749	1.000
rs4499491	rs734784			AT	0.27	**0.026**	0.272	-0.58	0.330	0.988
rs4499491	rs734784			CT	1.11	0.855	1.000	0.68	0.182	0.918
rs4499491	rs734784			AC	1.49	0.627	1.000	-1.13	0.107	0.764
rs4499491	rs734784			CC	1.74	0.239	0.897	0.24	0.558	0.999
rs4499491	rs6017486			AA	0.10	0.107	0.711	-5.08	**0.015**	0.301
rs4499491	rs6017486			CA	1.27	0.615	0.999	-0.30	0.468	0.999
rs4499491	rs6017486			AG	0.62	0.263	0.942	-0.61	0.175	0.910
rs4499491	rs6017486			CG	1.36	0.476	0.994	0.84	**0.028**	0.306
rs4499491	rs6073643			CC	2.12	0.128	0.759	0.23	0.599	0.999
rs4499491	rs6073643			AT	0.57	0.147	0.845	-0.70	0.114	0.753
rs4499491	rs6073643			CT	0.93	0.846	1.000	0.44	0.260	0.967

rs734784	rs6017486	TA	0.34	0.138	0.833	-1.59	0.139	0.872
rs734784	rs6017486	CA	1.43	0.450	0.993	-0.27	0.505	0.999
rs734784	rs6017486	TG	0.66	0.353	0.975	0.38	0.374	0.996
rs734784	rs6017486	CG	1.45	0.439	0.996	0.16	0.713	1.000
rs734784	rs6073643	CC	2.08	0.114	0.763	0.13	0.749	1.000
rs734784	rs6073643	TT	0.55	0.185	0.834	0.19	0.625	0.999
rs734784	rs6073643	CT	0.95	0.898	1.000	-0.39	0.377	0.997
rs6017486	rs6073643	AC	1.85	0.249	0.899	-0.03	0.963	1.000
rs6017486	rs6073643	GC	2.64	0.233	0.977	0.22	0.764	1.000
rs6017486	rs6073643	AT	0.33	0.060	0.536	-1.61	**0.039**	0.493
rs6017486	rs6073643	GT	0.77	0.551	0.999	0.36	0.368	0.996

Appendix H – Full results set: Chapter 5

IL1B – Single SNP analysis

SNP	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
	ALLELIC MODEL			GENOTYPIC MODEL		DOMINANT MODEL		RECESSIVE MODEL		ALLELIC MODEL		
	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	BETA
rs1143641	0.965	1.000	1.15	0.997	1.000	0.998	1.000	0.991	1.000	0.350	0.821	0.54
rs1143634	0.357	0.824	0.64	0.455	0.891	0.330	0.761	0.992	1.000	0.963	1.000	-0.03
rs1143633	0.964	0.999	1.15	0.119	0.375	0.426	0.802	0.206	0.539	0.058	0.226	-0.78
rs3136558	0.293	0.692	0.55	0.326	0.794	0.269	0.645	1.000	1.000	0.306	0.762	-0.69

IL1B – Haplotype analysis

HAPLOTYPE					UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
					OR	P_{EMP1}	P_{EMP2}	BETA	P_{EMP1}	P_{EMP2}
rs1143641	rs1143634	rs1143633	rs3136558	GGCG	0.41	0.114	0.712	-0.72	0.334	0.934
rs1143641	rs1143634	rs1143633	rs3136558	GGTA	1.14	0.807	1.000	-0.77	0.071	0.444
rs1143641	rs1143634	rs1143633	rs3136558	GACA	0.64	0.434	0.951	-0.06	0.911	1.000
rs1143641	rs1143634	rs1143633	rs3136558	AGCA	1.08	0.836	1.000	0.54	0.355	0.947
rs1143641	rs1143634	rs1143633	rs3136558	GGCA	1.40	0.403	0.949	0.55	0.113	0.645
rs1143641	rs1143634	rs1143633		GGT	1.15	0.752	1.000	-0.79	0.070	0.418
rs1143641	rs1143634	rs1143633		GAC	0.64	0.368	0.947	-0.06	0.915	1.000
rs1143641	rs1143634	rs1143633		AGC	1.16	0.847	1.000	0.53	0.346	0.942
rs1143641	rs1143634	rs1143633		GGC	1.07	0.911	1.000	0.42	0.268	0.885
rs1143641	rs1143634	rs3136558		GGG	0.41	0.105	0.712	-1.02	0.171	0.763
rs1143641	rs1143634	rs3136558		GAA	0.64	0.451	0.953	-0.02	0.970	1.000
rs1143641	rs1143634	rs3136558		AGA	1.04	0.928	1.000	0.49	0.421	0.974
rs1143641	rs1143634	rs3136558		GGA	1.60	0.244	0.816	0.05	0.904	1.000
rs1143641	rs1143633	rs3136558		GCG	0.40	0.097	0.685	-0.79	0.293	0.909
rs1143641	rs1143633	rs3136558		GTA	1.15	0.818	1.000	-0.78	0.071	0.418
rs1143641	rs1143633	rs3136558		ACA	1.05	0.913	1.000	0.53	0.372	0.954
rs1143641	rs1143633	rs3136558		GCA	1.13	0.791	1.000	0.54	0.136	0.655
rs1143634	rs1143633	rs3136558		GCG	0.55	0.218	0.885	-0.70	0.289	0.905
rs1143634	rs1143633	rs3136558		GTA	1.13	0.823	1.000	-0.75	0.082	0.480
rs1143634	rs1143633	rs3136558		ACA	0.64	0.443	0.946	-0.02	0.969	1.000
rs1143634	rs1143633	rs3136558		GCA	1.40	0.404	0.940	0.76	**0.032**	0.250
rs1143641	rs1143634			GA	0.65	0.427	0.959	-0.08	0.877	1.000
rs1143641	rs1143634			AG	1.13	0.861	1.000	0.49	0.386	0.966
rs1143641	rs1143634			GG	1.24	0.584	0.994	-0.23	0.605	0.999
rs1143641	rs1143633			GT	1.15	0.733	1.000	-0.81	0.055	0.373
rs1143641	rs1143633			AC	1.16	0.884	1.000	0.52	0.353	0.948
rs1143641	rs1143633			GC	0.83	0.658	0.995	0.43	0.281	0.883

rs1143641	rs3136558	AG	81.80	0.656	1.000	1.58	0.495	0.986
rs1143641	rs3136558	GG	0.39	0.098	0.635	-0.93	0.219	0.834
rs1143641	rs3136558	AA	1.01	0.869	1.000	0.41	0.509	0.992
rs1143641	rs3136558	GA	1.44	0.400	0.965	0.04	0.931	1.000
rs1143634	rs1143633	GT	1.14	0.804	1.000	-0.75	0.082	0.491
rs1143634	rs1143633	AC	0.65	0.428	0.959	-0.08	0.873	1.000
rs1143634	rs1143633	GC	1.14	0.753	1.000	0.66	0.076	0.532
rs1143634	rs3136558	GG	0.56	0.323	0.911	-0.67	0.320	0.921
rs1143634	rs3136558	AA	0.63	0.400	0.942	-0.08	0.886	1.000
rs1143634	rs3136558	GA	1.80	0.164	0.712	0.37	0.433	0.974
rs1143633	rs3136558	CG	0.53	0.128	0.869	-0.58	0.395	0.966
rs1143633	rs3136558	TA	1.15	0.784	1.000	-0.77	0.069	0.449
rs1143633	rs3136558	CA	1.15	0.717	1.000	0.80	**0.033**	0.258

IL6 – Single SNP analysis

	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
SNP	**ALLELIC MODEL**			**GENOTYPIC MODEL**		**DOMINANT MODEL**		**RECESSIVE MODEL**		**ALLELIC MODEL**		
	P_{EMP1}	**P_{EMP2}**	**OR**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**BETA**
rs2069834	1.000	1.000	1.38	1.000	1.000	1.000	1.000	1.000	1.000	0.325	0.884	0.90
rs1474347	0.467	0.952	0.68	0.499	0.960	0.449	0.910	1.000	1.000	0.416	0.952	-0.56
rs2066992	1.000	1.000	1.19	1.000	1.000	1.000	1.000	1.000	1.000	0.024	0.133	-1.47
rs1554606	0.271	0.766	0.61	0.306	0.832	0.403	0.873	0.224	0.495	0.567	0.998	-0.22
rs1548216	0.620	0.987	0.79	0.630	0.984	0.781	0.999	0.414	0.764	0.918	1.000	-0.05
rs2069844	0.635	0.991	0.77	0.578	0.983	0.767	0.999	0.466	0.799	0.991	1.000	-0.01
rs2069845	0.133	0.490	0.52	0.148	0.536	0.387	0.871	0.091	0.157	0.378	0.940	-0.35
rs2069849	0.676	0.999	0.86	0.446	0.936	1.000	1.000	0.132	0.612	0.930	1.000	0.04

IL6 – Haplotype analysis

				UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
HAPLOTYPE				**OR**	**P_{EMP1}**	**P_{EMP2}**	**BETA**	**P_{EMP1}**	**P_{EMP2}**
rs2069834	rs1474347	rs2066992	CAT	1.10	0.824	1.000	-1.45	**0.028**	0.402
rs2069834	rs1474347	rs2066992	TCG	1.31	0.779	1.000	1.01	0.276	0.986
rs2069834	rs1474347	rs2066992	CCG	0.38	0.103	0.907	-2.27	**0.025**	0.391
rs2069834	rs1474347	rs2066992	CAG	1.19	0.728	1.000	1.10	**0.037**	0.372
rs1474347	rs2066992	rs1554606	CGT	0.57	0.409	0.992	-0.38	0.599	1.000
rs1474347	rs2066992	rs1554606	AGT	0.66	0.400	0.979	-0.12	0.806	1.000
rs1474347	rs2066992	rs1554606	ATG	1.13	0.755	1.000	-1.46	**0.022**	0.374
rs1474347	rs2066992	rs1554606	AGG	1.46	0.335	0.976	0.73	0.059	0.593
rs1548216	rs2069844	rs2069845	CAG	0.86	0.766	1.000	0.09	0.864	1.000
rs1548216	rs2069844	rs2069845	GAG	0.71	0.595	1.000	-0.18	0.830	1.000
rs1548216	rs2069844	rs2069845	CCG	0.42	0.139	0.988	-0.68	0.631	1.000

rs1548216	rs2069844	rs2069845	GCG	0.23	0.051	0.491	-2.14	**0.048**	0.543
rs1548216	rs2069844	rs2069845	GCA	1.83	0.100	0.759	0.42	0.309	0.990
rs2069844	rs2069845	rs2069849	AGT	0.80	0.644	1.000	0.10	0.849	1.000
rs2069844	rs2069845	rs2069849	CAT	1.37E+26	0.169	0.984	-0.38	0.813	1.000
rs2069844	rs2069845	rs2069849	AGC	0.89	0.967	1.000	-0.17	0.816	1.000
rs2069844	rs2069845	rs2069849	CGC	0.33	**0.044**	0.445	-1.36	0.080	0.710
rs2069844	rs2069845	rs2069849	CAC	1.73	0.197	0.858	0.38	0.350	0.997
rs2069834	rs1474347		TC	1.40	0.459	1.000	0.90	0.320	0.994
rs2069834	rs1474347		CC	0.43	0.153	0.955	-2.26	**0.019**	0.308
rs2069834	rs1474347		CA	1.47	0.421	1.000	0.56	0.419	0.999
rs1474347	rs2066992		AT	1.11	0.710	1.000	-1.48	**0.025**	0.370
rs1474347	rs2066992		CG	0.53	0.266	0.980	-0.17	0.824	1.000
rs1474347	rs2066992		AG	1.25	0.673	1.000	1.01	0.061	0.560
rs2066992	rs1554606		GT	0.60	0.262	0.931	-0.23	0.601	1.000
rs2066992	rs1554606		TG	1.21	0.690	1.000	-1.47	**0.017**	0.313
rs2066992	rs1554606		GG	1.44	0.343	0.977	0.71	0.067	0.645
rs1548216	rs2069844		CA	0.78	0.575	1.000	0.12	0.838	1.000
rs1548216	rs2069844		GA	0.87	0.957	1.000	-0.10	0.889	1.000
rs1548216	rs2069844		CC	0.65	0.752	1.000	-1.20	0.351	0.995
rs1548216	rs2069844		GC	1.33	0.516	0.998	0.10	0.830	1.000
rs2069844	rs2069845		AG	0.77	0.575	1.000	-0.01	0.990	1.000
rs2069844	rs2069845		CG	0.33	**0.048**	0.489	-1.36	0.090	0.723
rs2069844	rs2069845		CA	1.88	0.105	0.684	0.38	0.363	0.997
rs2069845	rs2069849		GT	0.75	0.539	1.000	0.09	0.862	1.000
rs2069845	rs2069849		AT	2.5E+12	0.581	1.000	-0.43	0.783	1.000
rs2069845	rs2069849		GC	0.63	0.337	0.992	-0.59	0.337	0.996
rs2069845	rs2069849		AC	1.39	0.479	0.992	0.24	0.571	1.000

CCL2 – Single SNP analysis

	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
SNP	**ALLELIC MODEL**			**GENOTYPIC MODEL**		**DOMINANT MODEL**		**RECESSIVE MODEL**		**ALLELIC MODEL**		
	P_{EMP1}	**P_{EMP2}**	**OR**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**P_{EMP1}**	**P_{EMP2}**	**BETA**
rs4586	1.000	1.000	0.95	0.609	0.840	0.998	0.999	0.358	0.590	0.599	0.856	-0.22
rs13900	0.601	0.830	0.82	0.590	0.819	0.553	0.754	0.998	0.998	0.670	0.900	0.23

CCL2 – Haplotype analysis

			UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
HAPLOTYPE			**OR**	**P_{EMP1}**	**P_{EMP2}**	**BETA**	**P_{EMP1}**	**P_{EMP2}**
rs4586	rs13900	CT	0.80	0.638	0.868	0.15	0.768	0.961
rs4586	rs13900	TC	0.95	0.875	0.985	-0.23	0.561	0.834
rs4586	rs13900	CC	1.18	0.656	0.900	0.09	0.792	0.968

CCR2 – Single SNP analysis

SNP	UNIVARIATE - PAIN									UNIVARIATE - PAIN INTENSITY		
	ALLELIC MODEL			GENOTYPIC MODEL		DOMINANT MODEL		RECESSIVE MODEL		ALLELIC MODEL		
	P_{EMP1}	P_{EMP2}	OR	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	P_{EMP1}	P_{EMP2}	BETA
rs3762823	0.094	0.231	NA	0.194	0.577	0.079	0.201	1.000	1.000	0.178	0.525	0.83
rs3918378	0.294	0.589	2.61	**0.047**	0.145	0.094	0.267	0.449	0.718	0.579	0.946	0.27
rs3092963	0.431	0.802	0.70	0.342	0.714	0.794	0.994	0.150	0.334	0.441	0.862	-0.31
rs3918385	0.103	0.281	0.39	0.122	0.341	**0.037**	0.104	0.841	1.000	0.133	0.423	-0.89

CCR2 – Haplotype analysis

HAPLOTYPE					UNIVARIATE - PAIN			UNIVARIATE - PAIN INTENSITY		
					OR	P_{EMP1}	P_{EMP2}	BETA	P_{EMP1}	P_{EMP2}
rs3762823	rs3918378	rs3092963	rs3918385	3333	0.36	**0.031**	0.281	-0.89	0.150	0.652
rs3762823	rs3918378	rs3092963	rs3918385	1331	1.90E+08	0.963	1.000	0.94	0.154	0.648
rs3762823	rs3918378	rs3092963	rs3918385	3331	0.61	0.367	0.880	-0.53	0.329	0.874
rs3762823	rs3918378	rs3092963	rs3918385	3411	2.49	0.241	0.787	0.33	0.511	0.972
rs3762823	rs3918378	rs3092963	rs3918385	3311	0.97	0.977	1.000	0.09	0.818	1.000
rs3762823	rs3918378	rs3092963		133	1.89E+08	0.997	1.000	0.83	0.204	0.730
rs3762823	rs3918378	rs3092963		333	0.42	**0.026**	0.186	-0.74	0.080	0.455
rs3762823	rs3918378	rs3092963		341	2.53	0.251	0.767	0.29	0.546	0.980
rs3762823	rs3918378	rs3092963		331	0.96	0.916	1.000	0.11	0.752	1.000
rs3762823	rs3092963	rs3918385		333	0.36	**0.031**	0.281	-0.89	0.150	0.652
rs3762823	rs3092963	rs3918385		131	1.90E+08	0.963	1.000	0.94	0.154	0.648
rs3762823	rs3092963	rs3918385		331	0.61	0.361	0.886	-0.52	0.332	0.874
rs3762823	rs3092963	rs3918385		311	1.47	0.367	0.908	0.31	0.426	0.951
rs3762823	rs3918378	rs3918385		333	0.36	0.054	0.277	-0.88	0.163	0.663
rs3762823	rs3918378	rs3918385		341	2.48	0.256	0.788	0.30	0.543	0.979
rs3762823	rs3918378	rs3918385		131	2.67E+08	0.999	1.000	0.85	0.196	0.721
rs3762823	rs3918378	rs3918385		331	0.70	0.365	0.939	-0.14	0.717	0.998
rs3918378	rs3092963	rs3918385		333	0.36	**0.032**	0.281	-0.89	0.150	0.652
rs3918378	rs3092963	rs3918385		331	1.19	0.772	0.997	0.07	0.876	1.000
rs3918378	rs3092963	rs3918385		411	2.49	0.246	0.787	0.33	0.511	0.972
rs3918378	rs3092963	rs3918385		311	0.97	0.932	1.000	0.08	0.816	1.000
rs3762823	rs3918378			34	2.35	0.253	0.807	0.28	0.581	0.985
rs3762823	rs3918378			13	2.75E+09	0.944	1.000	1.05	0.127	0.597
rs3762823	rs3918378			33	0.24	**0.031**	0.249	-0.59	0.163	0.661
rs3762823	rs3092963			13	1.91E+08	0.959	1.000	0.95	0.154	0.642
rs3762823	rs3092963			33	0.42	**0.041**	0.195	-0.76	0.075	0.425
rs3762823	rs3092963			31	1.46	0.332	0.912	0.31	0.429	0.953
rs3762823	rs3918385			33	0.36	**0.031**	0.281	-0.89	0.150	0.652
rs3762823	rs3918385			11	1.90E+08	0.963	1.000	0.94	0.154	0.648
rs3762823	rs3918385			31	1.11	0.913	1.000	0.02	0.957	1.000

rs3918378	rs3092963	33	0.71	0.397	0.928	-0.25	0.536	0.978
rs3918378	rs3092963	41	2.29	0.244	0.818	0.30	0.556	0.981
rs3918378	rs3092963	31	0.96	0.982	1.000	0.04	0.894	1.000
rs3918378	rs3918385	33	0.37	**0.038**	0.281	-0.81	0.192	0.735
rs3918378	rs3918385	41	2.34	0.218	0.794	0.31	0.543	0.977
rs3918378	rs3918385	31	1.12	0.751	1.000	0.14	0.746	0.999
rs3092963	rs3918385	33	0.37	**0.042**	0.281	-0.89	0.158	0.656
rs3092963	rs3918385	31	1.19	0.699	0.997	0.07	0.873	1.000
rs3092963	rs3918385	11	1.46	0.332	0.912	0.31	0.429	0.953

Appendix I – rs3918378 BeadStudio SNP graph scatter plots