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Genetic Predictors of Antiretroviral Response and Toxicity

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Genetic Predictors of Antiretroviral Response and Toxicity

by

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DISSERTATION

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Dedication

I would like to dedicate this thesis to Rock, who is not with us anymore, TR, General Jack D. Ripper, and Page. Thank you for sitting with me while I worked for countless hours over the years.

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I would like to express my special appreciation and thanks to my advisor Dr. Deanna Kroetz, you have been a superb mentor for me. I would like to thank you for encouraging my research and for helping me to grow as a research scientist. Your advice on both research, as well as on my career have been priceless.

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Special thanks to my parents, Dr. Robert Micheli (the *real* Dr. Micheli) and Edie Micheli and my sister Jill Micheli who have supported and encouraged me to continue my education. Thank you to my husband John Jazdzewski who has put up with me on a day to day basis. I would also like to thank all of my friends (Dana!) who supported me in writing, and encouraged me to strive towards my goal.

Abstract

Since the emergence of the HIV epidemic it has been recognized that complications to HIV infection and variations in drug response and toxicity are influenced by patient genetics. Identification of genetic predictors of HIV infection complications and variation in drug response and toxicity will lead to better treatment options for patients and reduce HIV-related mortality and morbidity. This dissertation contains research that uses candidate gene and genome-wide approaches to identify and characterize novel genetic predictors of nevirapine pharmacokinetics, nucleoside reverse transcriptase inhibitor-induced peripheral neuropathy and HIV-induced peripheral neuropathy. This research demonstrates that nevirapine pharmacokinetic properties are heritable in European and African patients and characterizes the significant effects of *CYP2B6* 516G>T, *CYP2B6* 983T>C and *ABCC10* rs2125739 on nevirapine C_{min} concentrations in a Ugandan HIV+ population. It also highlights the importance of considering all three polymorphisms for prediction of nevirapine C_{min}. This dissertation also explores the genetic predictors of NRTI-SN using whole genome and candidate gene approaches in a Ugandan HIV+ population. A polymorphism in *VAMP4*, rs188298690, was identified in the whole genome study and bioinformatic analyses found that this marker is in an active regulatory region and also a population specific eQTL locus. The candidate gene analysis found that polymorphisms in *SLC28A1* and *ABCC4* are predictive of the development of NRTI-SN. Finally, this dissertation describes research to identify genetic predictors of HIV-SN. Several polymorphisms in the *FOLH1* region were identified in a whole genome study and bioinformatic analyses support a role for these polymorphisms in determining *FOLH1* expression. Analysis of the top *FOLH1*

polymorphism in additional samples showed a trend towards significance and a meta-analysis of the discovery and replication cohorts had improved statistical significance. The research obtained in this dissertation increases the understanding of the role of genetic variation in determining antiviral pharmacokinetics and toxicity and in complications to HIV infection.

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Introduction

1.1 History of HIV/AIDS: Initial Epidemic and Virus Discovery

From October 1980 to May 1981, Dr. Michael Gottlieb and colleagues observed five unusual cases of *Pneumocystis carinii* pneumonia (PCP), an infection most commonly seen in immunosuppressed patients, in previously healthy young homosexual men. This prompted the Centers for Disease Control (CDC) to issue a report on these cases in their Morbidity and Mortality Weekly Report (MMWR) on June 5, 1981[1]. Two of the five patients had already died at the time the report was issued. Soon after this report was issued the CDC began to receive accounts from other locations of other cases of opportunistic infections and Kaposi's Sarcoma[2]. By July 1981 the CDC had identified 108 cases of Kaposi's Sarcoma and PCP with an onset between January 1976 and July 1981. The Case Fatality Rate (CFR) was 40%, on par with yellow fever[2,3]. The first published description of the patients suffering from this new immunodeficiency disease appeared in the *Lancet* in September 1981[4]. Hymes *et.al.* described eight previously healthy patients suffering from Kaposi's Sarcoma. What made these cases unique was that the patients were relatively young (median age 34 years old), homosexual and suffered from a type of aggressive Kaposi's Sarcoma more commonly seen in Africa rather than Europe and North America. Five out of eight of those patients had died at the time of publication. In December 1981, several reports were published in *The New England Journal of Medicine* detailing outbreaks of Kaposi's Sarcoma and PCP in

California and New York[5-8]. These reports also detailed abnormalities in immune function in patients with Kaposi's Sarcoma or other opportunistic infections.

By January 1982 the newly created CDC Task Force on Kaposi's Sarcoma and Opportunistic Infections had published a special report detailing the state of the outbreak of this unknown immune disease[9]. They identified 216 cases of Kaposi's Sarcoma or other opportunistic infections with a mortality rate of 40%. The report highlighted the rapid increase in cases and high mortality rate of those with the disease (Figure 1)[9]. This report also discussed that the outbreak seemed to be confined geographically around New York, Los Angeles and San Francisco. The outbreak also was most common in young (median age 35 years), Caucasian (70%) homosexual men (92%)[9].

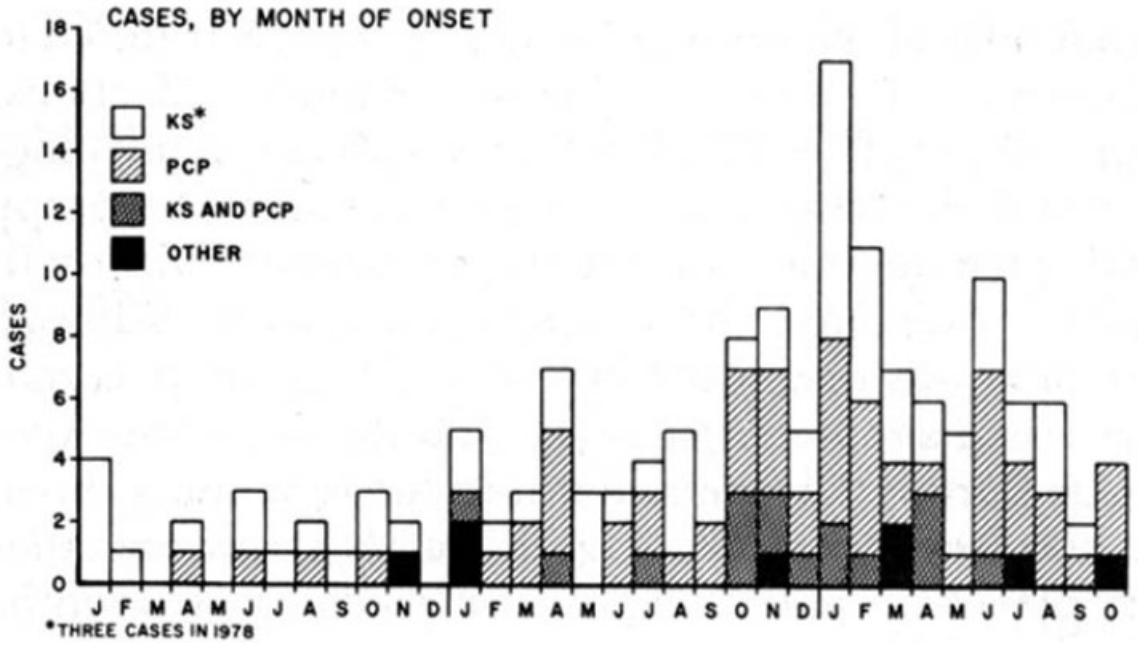

Figure 1.1 Taken from the CDC's Special Report - Epidemiologic Aspects of the Current Outbreak of Kaposi's Sarcoma and Opportunistic Infections showing the incidence of cases by month of onset[9]. Fill within the bars represent cases of Kaposi's Sarcoma (white), Pneumocystis carinii Pneumonia (diagonal hashes), both (grey) or other infections (black**).**

In September of 1982, the CDC issued an update on what was now being called acquired immune deficiency syndrome (AIDS), highlighting that in addition to homosexual men, cases were being identified in intravenous drug users, Haitians and two patients with hemophilia A[10]. The CDC then conducted a large scale case-control epidemiologic study to identify risk factors for acquiring AIDS and to characterize laboratory abnormalities in persons with AIDS[11,12]. These studies suggested that the infectious agent for AIDS would be found in blood or sexual fluid secretions and also characterized differences in immune function in patients compared to controls, specifically differences in T-cell populations and mitogen response (Figure 1.2)[12].

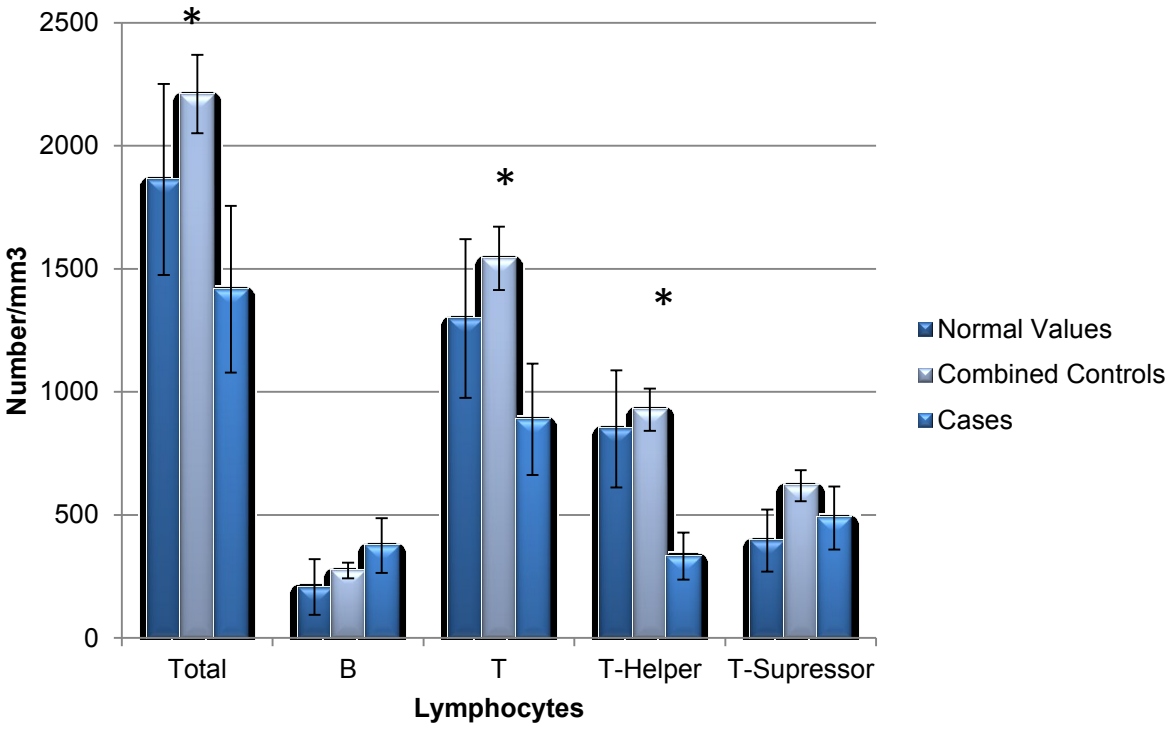

Figure 1.2. Taken from the National Case-Control Study of Kaposi's Sarcoma and Peumocystis carinii Pneumonia in Homosexual Men: Part 2, Laboratory Results. The graph shows the absolute number of lymphocytes per mm^3. Bars denote mean ± SE. *Combined controls are significantly different from cases.

Once the CDC and the medical community realized an epidemic was emerging, efforts began to identify the infectious agent responsible for AIDS. In 1983 and 1984 three independent groups isolated what they believed to be the virus responsible for causing AIDS[13-15]. While there is some controversy surrounding what group isolated the virus first, the viruses they identified all belonged to a family of retroviruses known as Human T-cell Leukemia Virus (HTLV) and were designated by the discoverers HTLV-III, Lymphadenopathy Associated Virus (LAV) and AIDS associated retroviruses . Figure 1.3 is an image of a T-cell infected with HIV, showing viral particles (in yellow) budding off the host cell[16].

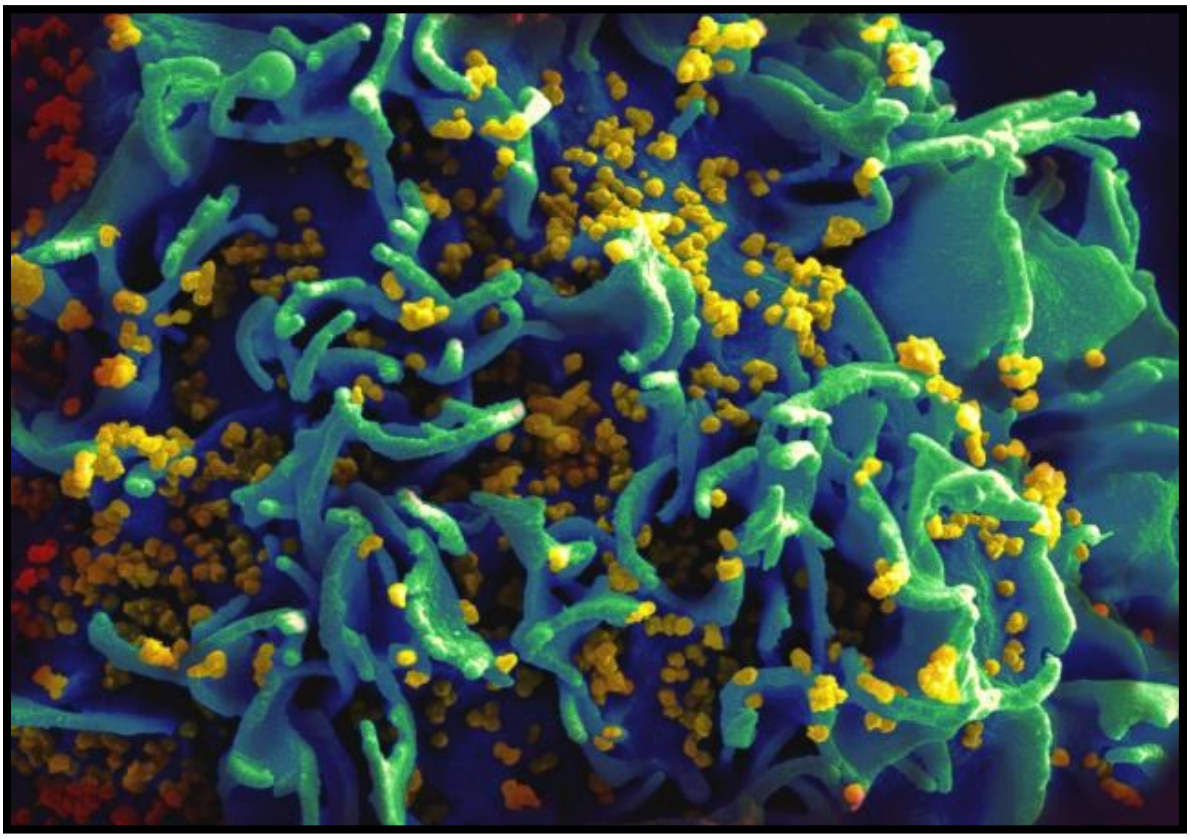

Figure 1.3. Scanning electron micrograph of an HIV infected H9 T-cell. Viral particles can be seen in yellow budding off the infected host cell[16]**.**

The discovery of the virus that causes AIDS, which would be named Human Immunodeficiency Virus (HIV) in 1986, allowed researchers to begin focusing on developing diagnostic tests and treatments for HIV. Developing a diagnostic test was critical to protect the blood supply and to identify new cases. Researchers developing diagnostics for the detection of HIV focused on finding antibodies reactive to HIV antigens with favorable sensitivity and specificity profiles[17,18]. The first diagnostic test approved by the FDA was an ELISA developed by Abbott in March 1985; the CDC issued a recommendation for universal testing of the blood supply shortly thereafter to prevent transmission from tainted blood transfusions (Figure 1.4)[19,20]. Efforts to create a vaccine against HIV were also undertaken, however, none to date have been shown to be effective against HIV infection[21].

Figure 1.4. Abbott HTLV-III EIA. This is the first diagnostic test approved by the FDA for the detection of HIV. From the National Museum of American History, Kenneth E. Behring Center. ID# 2007.0060.001
Courtesy of the National Museum of American History, Smithsonian Institution, Division of Medicine and Science. http://americanhistory.si.edu/collections/search/object/nmah_1322289

1.2 Overview of HIV: Origins, Life Cycle and Disease Natural History

Since the first reports of AIDS in 1981, over 25 million people worldwide have died from HIV infections[22]. Currently, 35.3 million people are living with HIV infections, mostly in resource poor settings such as sub-Saharan Africa[22].

Genetic studies have determined that the most common strain worldwide of HIV, group M, originated from a zoonotic transmission from chimpanzee *Pan troglodytes troglodytes* to humans sometime around 1920 in West-Central Africa[23]. The factors that allowed HIV to become a pandemic are varied and somewhat disputed, with leading theories including the prevalence of unsterile injections in colonial Africa in the early 20th century, increased urbanization in early 20th century Africa and increased exposure to primates due to increased hunting of bushmeat[23-25]. HIV is a genetically diverse virus; to date four groups of HIV-1 and eight groups of HIV-2 have been discovered which arose independently in separate zoonotic transmissions with different prevalence rates worldwide[23]. Within each of those groups there are multiple subtypes and recombinant strains consisting of multiple subtypes[23]. Globally in HIV-1, subtype C of group M is the most prevalent, whereas in the United States subtype B of group M is the most prevalent[23]. It is important to note that amongst the different strains of HIV, there are varying rates of disease progression and resistance to drug therapy[26,27].

HIV belongs to the genus Lentivirus which is part of the family Retroviridae[28]. Lentiviruses are single stranded, positive sense, enveloped RNA viruses. The HIV-1 HXB2 genome, which is considered the reference genome, consists of 9719 base pairs (bp) and encodes 11 proteins (Figure 1.5 and Table 1)[29].

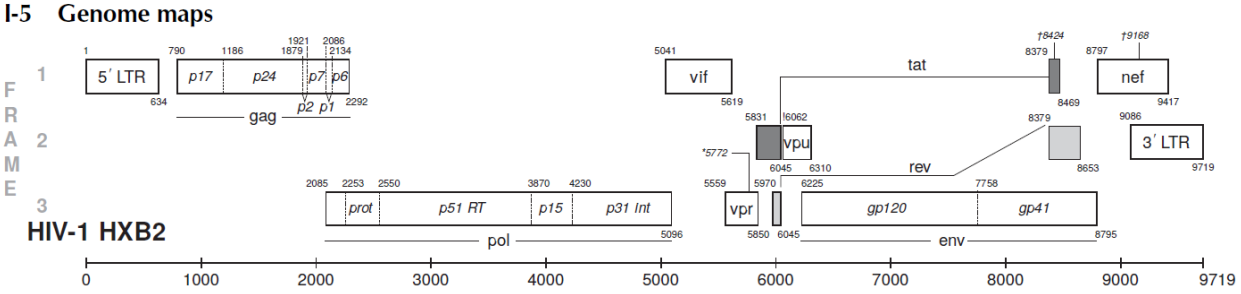

Figure 1.5. Structure of the HIV-1 HXB2 genome[29].

Table 1.1 HIV proteins organized by their size function and location. Proteins that are drug targets are in bold. Adapted from Leitner et al.[29]

Name	Size	Function	Localization
Gag			
MA	p17	membrane anchoring; env interaction; nuclear transport of viral core	virion
CA	p24	core capsid	virion
NC	p7	nucleocapsid, binds RNA	virion
	p6	binds Vpr	virion
Pol			
Protease (PR)	p15	Gag/Pol cleavage and maturation	virion
Reverse Transcriptase (RT)	p66,p51	reverse transcription, RNAse H activity	virion
RNase H	p15		virion
Integrase (IN)	p31	DNA provirus integration	virion
Env	gp120/gp41	external viral glycoproteins bind to CD4 and secondary receptors	plasma membrane, virion envelope
Tat	p16/p14	viral transcriptional transactivator	primarily in nucleolus/nucleus
Rev	p19	RNA transport, stability and utilization factor (phosphoprotein)	primarily in nucleolus/nucleus shuttling between nucleolus and cytoplasm
Vif	p23	promotes virion maturation and infectivity	cytoplasm, virion
Vpr	p10-15	promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M	virion nucleus
Vpu	p16	promotes extracellular release of viral particles; degrades CD4 in the ER; (protein only present in HIV-1 and SIV_{cpz})	integral membrane protein
Nef	p27-p25	CD4 and class I downregulation	plasma membrane, cytoplasm
Vpx	p12-p16	Vpr homolog present in HIV-2 and some SIVs, absent in HIV-1	virion
Tev	p28	tripartite tat-env-rev protein	primarily in the nucleolus/nucleus

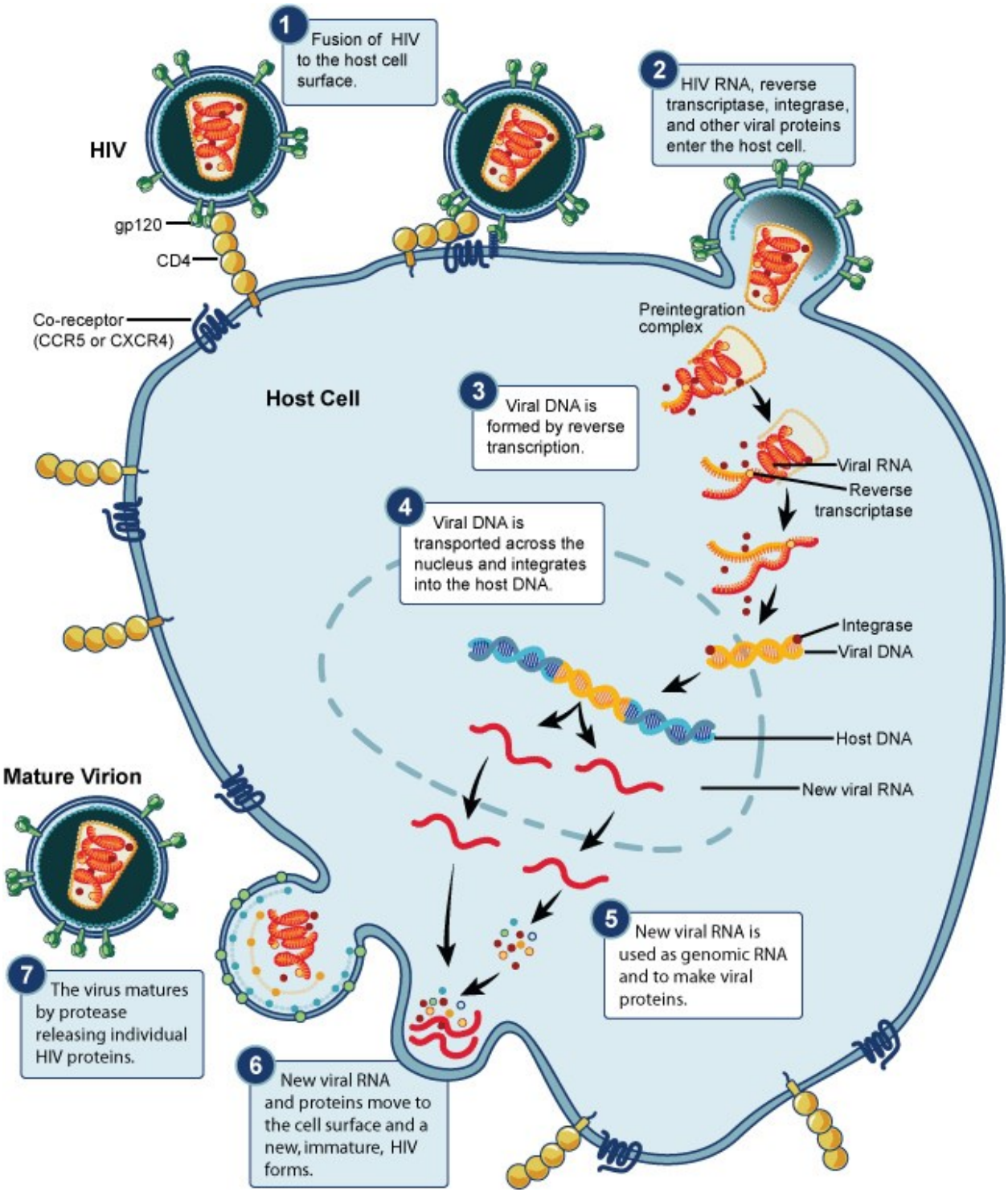

Figure 1.6. HIV life cycle with descriptions of each step[30].

The HIV life cycle consists of seven stages: (1) binding, (2) fusion, (3) reverse transcription, (4) integration, (5) transcription and translation, (6) assembly and (7) budding (Figure 1.6)[31]. During binding, the HIV envelope protein, gp120, binds to host cell CD4 receptors, which is followed by binding to either CCR5 or CXCR4 co-receptors. This induces a conformational change in gp120 which allows for the release of gp41, which brings the viral membrane and the host cell membranes to close proximity and leads to membrane fusion[32]. Once fusion has occurred, the virus matrix and capsid are digested and viral enzymes and viral genomes are released into the cell[31]. When the viral single stranded RNA genomes are released into the cell they are reverse transcribed into double stranded DNA by viral reverse transcriptase[31]. Many of the mutations in HIV are introduced in this step as HIV reverse transcriptase has poor fidelity and is prone to errors during transcription[33]. After the viral DNA has been generated it associates with the HIV protein, integrase, which mediates integration of the viral DNA into the host genome[34]. The integrase/DNA complex preferentially targets transcriptionally active sites, which is thought to promote efficient viral gene expression after integration into the host genome[34]. After integration of the viral DNA into the host genome, viral transcription and translation begins. This is a multi-step process that involves host and viral proteins that is largely controlled by the viral proteins, *tat* and *rev,* which are the first proteins that the viral genome produces[35]. HIV transcription is mediated by *tat* by promoting efficient elongation of viral primary mRNA transcripts[35]. After viral primary mRNA transcripts are produced, they undergo complex post transcriptional processing to produce RNA transcripts of all viral proteins[35]. The *rev*

protein then exports mRNA transcripts out of the nucleus into the cytoplasm where the HIV *gag* protein promotes viral translation[35]. After translation, the viral protease cleaves viral polyproteins, specifically the *gag* and *pol* proteins, into their mature forms[29,36]. If this step is inhibited the new HIV particles are rendered uninfectious[36]. Two copies of the viral ssRNA and the mature proteins are then assembled into a new viral particle at the host cell membrane where they bud off and are released into the host[36].

The pathology of HIV infection has three distinct clinical phases: acute, latent and development of AIDS[37]. During the acute phase of HIV infection, patients experience symptoms similar to influenza or mononucleosis with symptoms occurring one to four weeks after infection and lasting for one to two weeks[38]. During the acute phase there is a large increase in plasma viral load and a sharp decrease in CD4+ T-cell count[37]. After the acute phase of HIV infection, the virus and T-cell counts stabilize and the infection enters the chronic or latent stage of infection[37]. This stage is usually asymptomatic, although lymphadenopathy and Kaposi's sarcoma can been seen, and can last from one to twenty years before progressing[37]. The final stage of HIV infection is progression to AIDS which is defined by the CDC as *"CD4+ T-lymphocyte count of <200 cells/μL or CD4+ T-lymphocyte percentage of total lymphocytes of <14 or documentation of an AIDS-defining condition."*[39,40] As the T-cell population plummets and the viral load increases, patients experience increases in the incidence of opportunistic infections, cancers, nervous system disorders and wasting. Patients that progress to AIDS and are untreated will generally die within three years[37,41].

1.3 AZT Discovery and Approval

In the 1960s the National Cancer Institute (NCI) undertook efforts to identify novel drugs to treat cancer[42]. During this time, Jerome Horwitz at the Barbara Ann Karmanos Cancer Institute and Wayne State University School of Medicine, first synthesized a thymidine analog which would later be called 3'-azido-3'-deoxythymidine (AZT; Figure 1.7)[43]. Unfortunately, this drug did not prove to be effective in treating cancer in preclinical studies, so no further testing was conducted on AZT at the time[42].

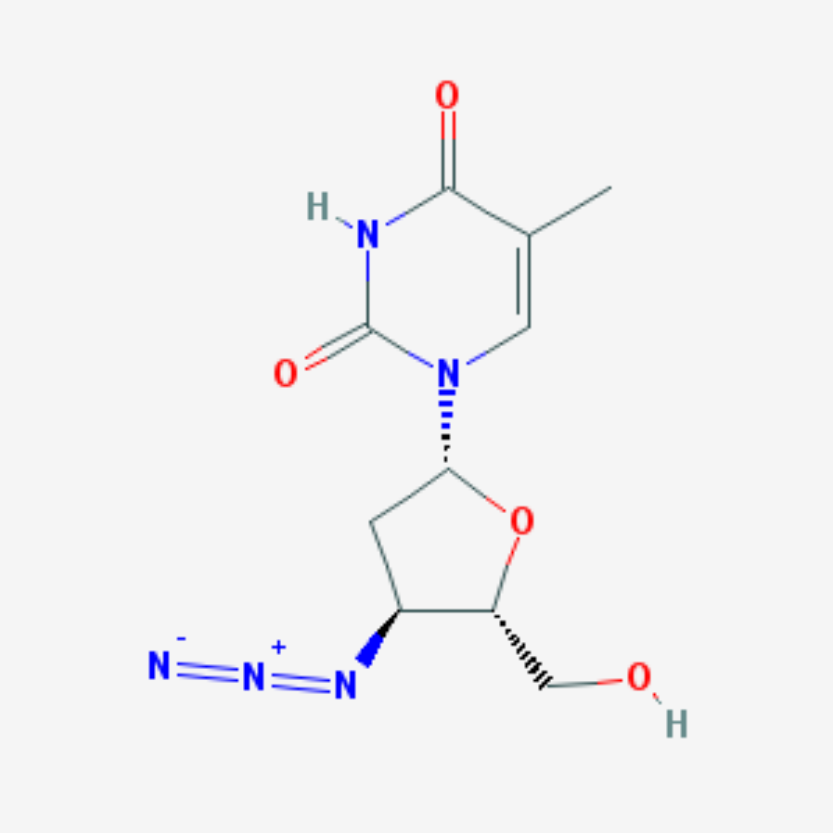

Figure 1.7. Structure of AZT.
Note the azide group replacing the hydroxyl group at the 3' position of the sugar moiety. Image from PubChem, Compound ID: 35370.
http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=35370

In the 1970s, Wolfram Ostertag of the Max Planck Institute demonstrated that AZT suppressed the replication of the Friend murine leukemia virus, a retrovirus that causes cancer in murine models[44]. With the advent of the AIDS epidemic and the identification of the HIV virus as its cause, efforts at the NCI were undertaken to find compounds that had the ability to suppress HIV replication *in vitro* and would have pharmacologic properties that made them amenable for clinical studies[42]. Compounds that could inhibit the synthesis of nucleic acids were the focus of many of these studies, as they had already proved useful in the treatment of virus-induced cancers, notably 6-mercaptopurine for the treatment of leukemia and Non-Hodgkin's lymphoma[45]. Mitsuya *et.al.* demonstrated in early 1985 that AZT potently suppressed HIV replication and abrogated HIV's cytotoxic effects *in vitro*[46].

This led the corporate sponsor of AZT, Burroughs-Wellcome, to file an Investigational New Drug Application (IND) with the FDA to begin clinical trials of AZT in humans[42]. Amazingly, the FDA approved the IND in seven days[42]. This trial was a Phase I efficacy and dosing trial to determine the appropriate dose of AZT for efficacious treatment of AIDS without undue toxicity[47]. The results from the study showed that doses of 2.5 mg/kg IV followed by 5 mg/kg orally at either 8 or 4 hour intervals showed the maximum benefit of AZT treatment, with all patients increasing T-cell counts and four out of eight patients showing reactivity to antigenic skin testing, an indication of immune system function[47].

Based on the results of the Phase I AZT clinical trial, a double-blind, placebo-controlled Phase II trial to evaluate AZT efficacy and toxicity was initiated in February 1986 and enrolled 282 patients with AIDS or AIDS-related complex (ARC) which were

recruited into either the AZT (n=145) arm or the placebo (n=137) arm. One hundred ninety four patients were still participating when the trial was halted by its Drug Safety Monitoring Board (DSMB) in September 1986[48]; the study was halted due to 19 patients in the placebo arm dying versus one in the AZT arm. The AZT arm of the study showed clear benefit to survival ($p < 0.001$) and additional endpoints used to evaluate efficacy, regardless of subgrouping by diagnosis or CD4+ T-cell counts [48].

The results of the companion toxicity study discovered that AZT patients had increased incidence of anemia ($p < 0.001$) and neutropenia ($p < 0.001$) compared to the placebo group [49]. Compared to the severity of AIDS, these toxicities were viewed as acceptable, although studies were conducted shortly thereafter that determined a reduced dose was equally efficacious and reduced toxicity[50].

Three weeks after the Phase II AZT trial was halted, the FDA issued a treatment IND, an exceedingly rare waiver that allowed patients to access AZT prior to approval by the FDA[42]. Burroughs-Wellcome submitted a New Drug Application (NDA) for the approval of AZT in the treatment of AIDS in late 1986 and was approved by the FDA in March of 1987; AZT is still widely used today (Figure 1.8) [42].

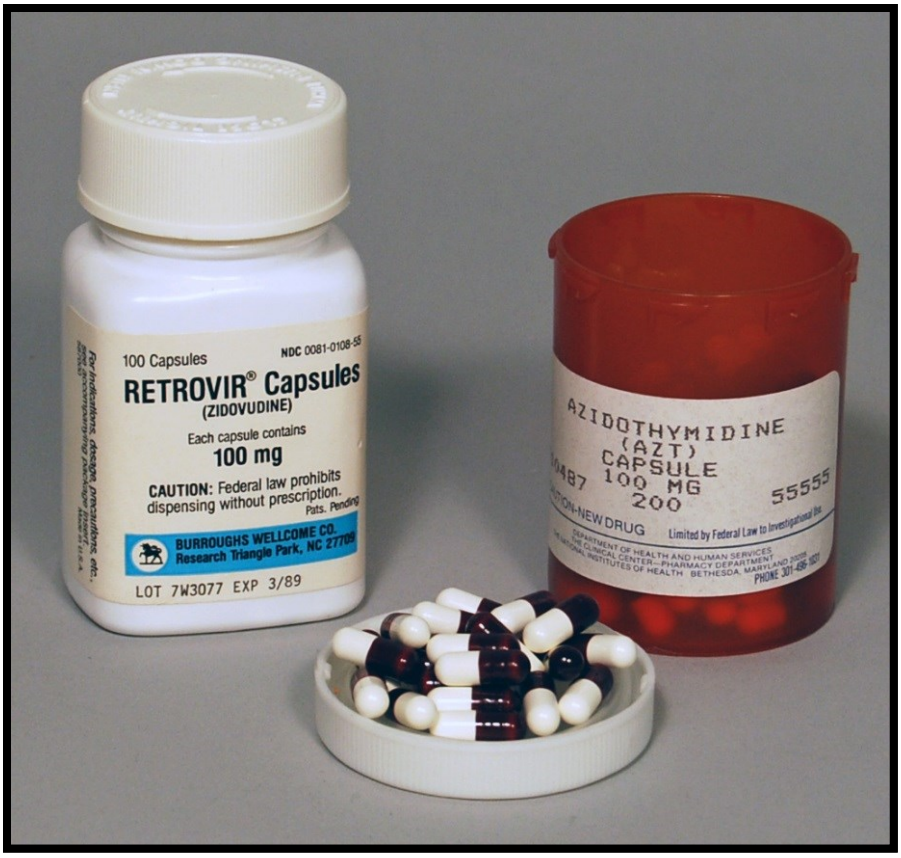

Figure 1.8. Vials of AZT, first marketed as Retrovir.
"AZT drug vials ," *HIV and AIDS 30 Years Ago*, accessed March 18, 2014, Courtesy of the National Museum of American History, Smithsonian Institution, Division of Medicine and Science.
http://hivaids.omeka.net/items/show/35.

1.4 Antiretroviral (ARV) Pharmacology

1.4.1 ARV Overview

With the advent of Highly Active Antiretroviral Therapy (HAART), AIDS related deaths have plummeted, changing HIV infection and AIDS from a fatal disease to a chronic condition (Figure 1.9)[51].

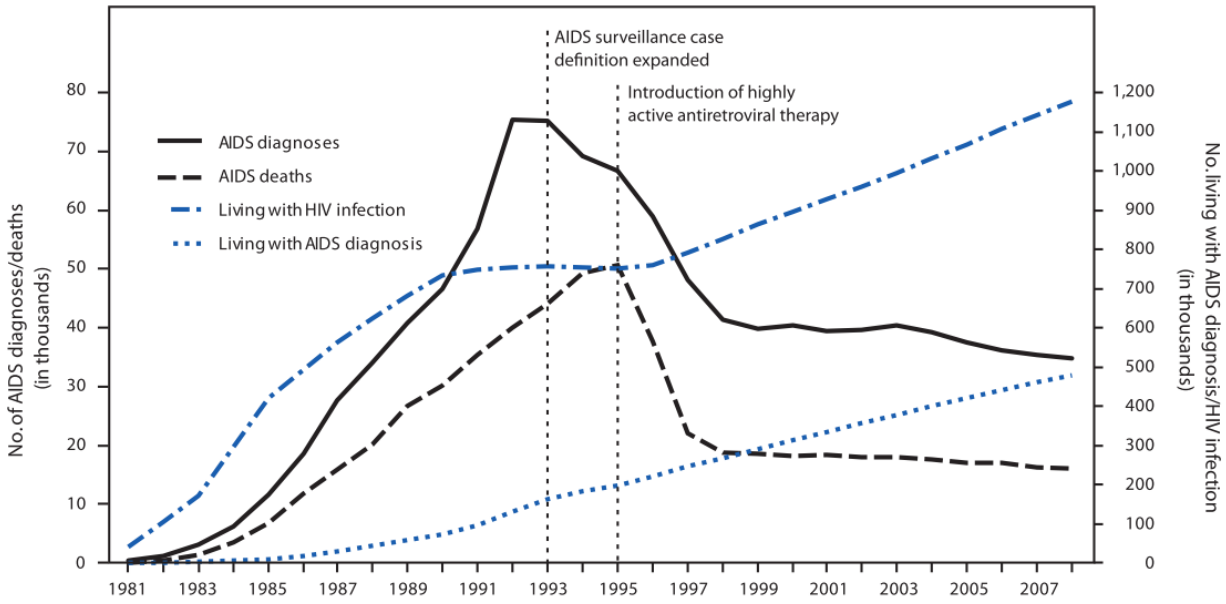

Figure 1.9. Numbers of persons infected with HIV, AIDS diagnoses and deaths from 1981-2008[51].

Since the approval of AZT in 1987, the FDA has approved 38 drugs in seven different drug classes for the treatment of HIV (Table 1.2)[52]. Each of these classes, with the exception of combination therapies, targets a different and specific stage in the HIV replication cycle. Current World Health Organization (WHO) and the U.S. Department of Health and Human Services treatment guidelines recommend multiple classes of drugs be used in combination as first line and second line treatments in adults infected with HIV, with variations for special populations such as pregnant women and children[53,54].

First line treatment for adults usually consists of two nucleoside reverse transcriptase inhibitors (NRTI) along with one non-nucleoside reverse transcriptase inhibitor (NNRTI); in second line therapy the NNRTI is most commonly replaced with a ritonavir boosted protease inhibitor (PI)[53,54]. If toxicities develop that are specific to a

particular drug, then a similar drug in the same class may be substituted; for example if a patient develops renal toxicity due to tenofovir (TDF) use, AZT may be substituted in the treatment regimen[53,54].

In resource poor settings compared to developed countries, cost and access to treatment are the primary reasons for differences in treatment regimens. For example, in a resource poor setting, an AZT based regimen may be selected over TDF as TDF costs three times as much per non-generic pill as AZT ($33.95 per 300 mg Viread vs. $9.10 per 300 mg Retrovir[55]). There also are large variations in access to medical care, societal customs and access to treatment drugs that present specific challenges to obtaining HIV treatment in undeveloped countries[22].

While HAART regimens have changed HIV infection to a chronic disease state, there are limitations to HIV treatments. There are two main limitations to HAART: 1) incomplete immune reconstitution and 2) drug toxicities[56]. While virtually all patients that receive HAART experience viral loads <500 copies/mL, most patients do not have full immune system reconstitution[57,58]. This leads to inflammatory diseases and complications to HIV infection such as increased risk of cardiovascular, hepatic, renal and neurological disorders[56,59]. Now that HIV infected patients live life spans close to the non-infected population, patients are taking ARVs for decades[59]. Many of these drugs' have side effects that are not immediately apparent, such as tenofovir and renal toxicity, and ultimately lead to treatment regimen changes or less than optimal adherence[56,59,60].

Table 1.2. Antiretroviral (ARV) drugs approved by the FDA for the treatment of HIV infection. Data modified from FDA[52].

Drug Class	Brand Name	Generic Name/ Abbreviation	Manufacturer	Approval Date	Time to Approval (Months)
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Emtriva	emtricitabine, FTC	Gilead Sciences	2-Jul-03	10
	Epivir	lamivudine, 3TC	GlaxoSmithKline	17-Nov-95	4.4
	Hivid	zalcitabine, dideoxycytidine, ddC (no longer marketed)	Hoffmann-La Roche	19-Jun-92	7.6
	Retrovir	zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline	19-Mar-87	3.5
	Videx	didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9-Oct-91	6
	Viread	tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01	5.9
	Zerit	stavudine, d4T	Bristol Myers-Squibb	24-Jun-94	5.9
	Ziagen	abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98	5.8
Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Edurant	rilpivirine	Tibotec Therapeutics	20-May-11	10
	Intelence	etravirine, ETR	Tibotec Therapeutics	18-Jan-08	6
	Rescriptor	delavirdine, DLV	Pfizer	4-Apr-97	8.7
	Sustiva	efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98	3.2
	Viramune	nevirapine, NVP	Boehringer Ingelheim	21-Jun-96	3.9
Protease Inhibitors (PIs)	Agenerase	amprenavir, APV (no longer marketed)	GlaxoSmithKline	15-Apr-99	6
	Aptivus	tipranavir, TPV	Boehringer Ingelheim	22-Jun-05	6
	Crixivan	indinavir, IDV,	Merck	13-Mar-96	1.4
	Fortovase	saquinavir (no longer marketed)	Hoffmann-La Roche	7-Nov-97	5.9
	Invirase	saquinavir mesylate, SQV	Hoffmann-La Roche	6-Dec-95	3.2
	Kaletra	lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15-Sep-00	3.5
	Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03	10
	Norvir	ritonavir, RTV	Abbott Laboratories	1-Mar-96	2.3
	Prezista	darunavir	Tibotec, Inc.	23-Jun-06	6
	Reyataz	atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03	6
	Viracept	nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-Mar-97	2.6
Fusion Inhibitors	Fuzeon	enfuvirtide, T-20	Hoffmann-La Roche & Trimeris	13-Mar-03	6
Entry Inhibitors	Selzentry	maraviroc	Pfizer	6-Aug-07	8
HIV Integrase Inhibitors	Isentress	raltegravir, RAL	Merck & Co., Inc.	12--Oct-07	6
	Tivicay	dolutegravir	GlaxoSmithKline	13-Aug-13	6

1.4.2 NRTI Pharmacology

NRTIs are the backbone of most antiretroviral (ARV) regimens and are most frequently prescribed in pairs with the most efficacious and least toxic combination being TDF/FTC (or 3TC)[53,54]. To date eight NRTIs (Table 1.2) have been approved by the FDA to treat HIV, with several other combination forms also approved and many more drugs in the development pipeline[52,61]. NRTIs are analogs of 2'-deoxy-nucleosides and nucleotides that all lack the 3'-OH group and require multiple intracellular phosphorylation steps for conversion to their active deoxynucleoside triphosphate (dNTP) analogs[61,62]. They inhibit HIV replication by competing with endogenous dNTPs for incorporation by HIV reverse transcriptase into replicating HIV proviral DNA[61,62]. When NRTIs are incorporated into the elongating DNA strand, they cause chain termination and stop proviral replication[62].

NRTIs gain entry into cells through passive diffusion or active transport facilitated by several members of the solute-carrier (SLC) family and are actively effluxed out of cells by members of the ATP-binding cassette (ABC) transporter family[61-63]. Because NRTIs persist intracellularly in their active triphosphorylated form, their efficacy is correlated with intracellular concentrations of drug rather than plasma concentrations[64,65]. Drug pharmacokinetics may be influenced by polymorphisms in drug transporter or catabolic genes. An example of this is the *ABCC4* 3463A>G polymorphism (rs1751034) and TDF, where the intracellular concentration of TDF is increased and the fraction of TDF excreted in the urine is decreased in patients with the rs1751034 polymorphism[66,67].

There are two major limitations to the use of NRTIs, resistance and toxicity. The major mechanisms of resistance are exclusion and excision. Resistance due to

exclusion occurs when the HIV-RT gains mutations that allow it to favor endogenous dNTPs over the triphosphorylated NRTIs[61]. Excision based resistance occurs when the HIV-RT gains a mutation that allows it to efficiently excise the monophosphate form of the drug (primarily AZT) from the end of the elongating viral DNA strand and allows viral elongation to continue[61]. Generally, resistance mutations are specific to a particular drug or dNTP analog, thus when resistance to one NRTI occurs, patients can be switched to another NRTI to maintain viral suppression[53]. For example, resistance mutations to abacavir (ABC), a guanosine analog, do not affect viral susceptibility to thymidine analogs such as AZT[68].

The other main limitation to NRTI use is NRTI toxicity. The most common toxicities associated with all NRTIs are lipodystrophy and lactic acidosis. Some toxicities specific to a particular drug are: AZT and anemia, TDF and renal toxicity, and ABC and hypersensitivity reactions[69-71]. The majority of NRTI toxicities, with the exception of ABC hypersensitivity, are related to mitochondrial damage induced by NRTIs[72-75]. The mechanism of NRTI induced mitochondrial toxicity is the affinity that the mitochondrial polymerase, polγ, has for NRTIs[76]. The mitochondria incorporate NRTIs into their genomes during replication and this leads to depletion of mtDNA, resulting in oxidative stress, mitochondrial dysfunction and ultimately cellular apoptosis[77].

1.4.3 NNRTI Pharmacology

NNRTIs are also a common component of ARV regimens[53,54]. In 1996, nevirapine (NVP) was the first NNRTI approved and NNRTIs were the second class of drug to be approved by the FDA for the treatment of HIV infection (Table 1.2). To date, five

NNRTIs have been approved for use in the treatment of HIV-1 (Table 1.2). Interestingly, HIV-2 is naturally resistant to NNRTIs, since the site of NNRTI binding on HIV-RT is not present in HIV-2[78]. Like NRTIs, NNRTIs inhibit the function of HIV-RT, however, they work by allosterically binding to the active site of HIV-RT in the NNRTI binding pocket (NNIBP)[61]. When NNRTIs bind in the NNIBP, they force a conformational change in the active site of the HIV-RT which halts DNA polymerization[61].

NNRTIs are passively absorbed into cells and undergo hepatic metabolism, primarily by CYP2B6 and CYP3A4[79]. The hydroxylated metabolites undergo glucoronidation and may be effluxed by ABC transporters[79]. Because NNRTIs undergo extensive Cytochrome P450 (CYP450) metabolism and can induce expression of CYP450 enzymes, there are numerous drug-drug interactions associated with their use[54]. Multiple studies have demonstrated the effect of *CYP2B6* polymorphisms on the pharmacokinetics of NVP and EFV, most notably the *CYP2B6* 516G>T polymorphism (rs3745274) that has been shown repeatedly to increase plasma concentrations of both drugs in patients with the variant allele[80-82]. There is some controversy surrounding the effect of the *ABCB1* 3435C>T polymorphism (rs1045642) on the pharmacokinetics of EFV and NVP, with one study showing an effect on EFV C_{min} concentrations and several studies showing no effect[79,83-87].

Like NRTIs, drug toxicity and resistance mutations are the main limitations to the use of NNRTIs in the treatment of HIV. The most common dose limiting side effects with NVP are rash and hepatotoxicity[88]. Decreased risk of developing NVP hepatotoxicity has been associated with the presence of *ABCB1* 3435C>T (rs1045642)[89,90].

Additionally, NVP induced rash has been associated with the *HLA-DRB1**0101 allele[79]. Efavirenz is mainly associated with central nervous system disorders such as insomnia and vivid dreams and these effects have been associated with increased plasma concentrations associated with *CYP2B6* polymorphisms[79,91].

1.4.4 PI Pharmacology

Protease inhibitors are commonly prescribed along with NRTIs for the treatment of HIV[53,54]. Their utility as a monotherapy in virally suppressed patients is also being studied[92]. There are ten PIs approved by the FDA for the treatment of HIV infection (Table 1.2). Protease inhibitors are peptidomimetic molecules that have a nonhydrolyzable hydroxyethylene core that cannot be cleaved by HIV protease and inhibit its function[93,94]. When the HIV protease is inhibited, viral polyproteins cannot be processed and the newly synthesized HIV viral particle is rendered uninfectious[94]. PIs are extensively metabolized by CYP450 metabolic enzymes, specifically CYP3A4, and are substrates for transporters in the SLC and ABC drug transporter families[93]. Many PIs have short half-lives due to CYP3A4 metabolism[93] and are commonly boosted by ritonavir, a CYP3A4 inhibitor, to allow once-daily dosing. Because many drugs are substrates of CYP3A4 and ABC transporters, there is a large risk of drug-drug interactions with ritonavir boosted PI regimens[93]. There have been many *in vitro* studies suggesting that polymorphisms in ABC transporters, OATPs, OCTs and CYP3As affect the transport and metabolism of PIs. However, many of these effects (particularly for *CYP3A4* and *CYP3A5*) have not been shown to have clinical implications, possibly due to ritonavir modulation and phenotypic effects[93,95]. One exception to this is *SLCO1B1*

521T>C (rs4149056), which has been shown to increase plasma area under the curves (AUC) and clearance (CL) of lopinavir/ritonavir and affect viral loads in children[96].

1.4.5 Integrase Inhibitor and Entry Inhibitor Pharmacology

There are two relatively new classes of drugs to treat HIV, integrase inhibitors and fusion/entry inhibitors (Table 1.2), with two drugs approved in each class. Integrase inhibitors block integration of the viral DNA complex into the host genome by inhibiting the strand transfer process mediated by HIV integrase[97,98]. Integrase inhibitors are primarily hepatically metabolized via glucuronidation by UGT1A1[99,100]. There are relatively few side effects and drug-drug interactions associated with the use of integrase inhibitors due to their specificity to HIV integrase and lack of CYP450 metabolism[98,101].

Fusion/entry inhibitors block the first step in HIV infection, the fusion and entry of the virus into the host cell[102]. Maraviroc is a CCR5 receptor agonist that prevents binding of the HIV virus to the host cell. Maraviroc is metabolized by CYP3A4, is a substrate of P-glycoprotein and has very few side effects[95]. Due to *CYP3A4* metabolism, maraviroc does interact with other drugs using the same metabolic pathway, but these effects can be overcome with dose adjustments[103].

Enfuvirtide is a fusion inhibitor that prevents the viral *gp41* protein from fusing with the host cell membrane[102]. Enfuvirtide is a synthetic peptide that is expected to undergo catabolism into its respective amino acids[104]. Enfuvirtide has been relatively well tolerated; there have been sporadic reports of hypersensitivity reactions but they have

been unconfirmed to date[105]. These new classes of drugs have given hope to patients that have experienced treatment failure or drug toxicities with the older drug classes.

1.5 Pharmacogenetics of ARV Therapy

While great progress has been made in the treatment of HIV, there still remains large interindividual variation in drug response and toxicity between patients. Pharmacogenetics is the study of how patients' genetic backgrounds influence drug efficacy and toxicity. The field of pharmacogenetics truly began in the 1950s when the widely known effects of polymorphisms in N-acetyltransferase on isoniazid metabolism were observed[106]. Also during the 1950s, many studies on the heritability of drug toxicity and response were conducted using twin studies, which greatly expanded the field of pharmacogenetics[106]. With the advent of molecular cloning and genetic sequencing technologies, it has become possible to thoroughly investigate the effect of genetic variation on drug response and toxicity[106]. While much of the interindividual variation in drug response and toxicity has been explained, there still are many questions that remain to be answered. The majority of pharmacogenetic studies conducted have been in Caucasian populations, however, with the advent of large scale sequencing it has become clear that there are wide variations in the frequency of polymorphisms across differing populations[107].

Pharmacogenetic studies related to ARV use have largely focused on drug metabolizing enzymes, drug transporters and drug targets[79]. Many clinically relevant genotype-phenotype associations have been identified and additional associations are

still being discovered[79]. Table 1.3 highlights some clinically relevant associations that have been discovered to date.

Drug metabolizing enzymes play an important role in the elimination of many drugs. ARV drugs, specifically NNRTIs, are primarily metabolized by CYP3A4 and CYP2B6, and to a lesser extent by CYP2A6 (Table 1.3). Polymorphisms in these enzymes impact drug plasma concentrations and toxicity. Several missense mutations in *CYP2B6*, 785A>G (rs2279343, Lys262Arg), 983T>C (rs28399499, Ile328Thr), and 516G>T (rs3745274, Gln172His), have been associated with increased plasma levels of NVP and EFV as a result of a decrease in enzyme function[80-82,108-111]. The *CYP2B6* 516G>T polymorphism has also been associated with increased incidence of NVP induced hepatotoxicity[112,113]. The *CYP3A5* 6986A>G (rs776746) polymorphism causes an alternate RNA spice site which leads to a nonfunctional protein[114]. As a consequence of this, carriers of the variant allele of this polymorphism have a decreased NVP AUC[115]. There are two *CYP2A6* variants that impact the metabolism of NNRTIs, *CYP2A6* 47441C>T (rs28399454, Val365Met) and 6857G>T (rs8192726) (Table 1.3). Both of these polymorphisms have been shown to increase EFV plasma concentrations, however only the 47441C>T polymorphism has been shown to increase NVP plasma levels[116]. Several *UGT1A1* polymorphisms are associated with increased PI-induced hyperbilirubinemia, specifically with atazanavir and indinavir[117]. Additionally, the *UGT2B7* 802T>C (rs7439366, Tyr268His) polymorphism is associated with increased EFV plasma concentrations[116].

The role that drug transporters play in the disposition of many ARVs has been well documented and polymorphisms in these genes can impact drug pharmacokinetics and

toxicity. ABC transporters have demonstrated the most clinically significant effects on drug pharmacokinetics and toxicity, most notably *ABCC2* and *ABCC4*, with emerging data to support the role of *ABCC10* and controversial data on the role of *ABCB1* (Table 1.3). Multiple polymorphisms in the *ABCC2* and *ABCC4* genes are associated with increased risk of tenofovir induced renal toxicity[67]. The role of *ABCC10* is just beginning to be recognized in the disposition of ARVs, with one polymorphism, 1791+526G>A (rs9349256) associated with increased risk of tenofovir renal toxicity and another polymorphism, 2759T>C (rs2125739), associated with increased nevirapine plasma concentrations[118,119]. The role of P-gp in the disposition of ARVs remains unclear at this date and there is significant controversy surrounding the impact, if any, of the *ABCB1* 3435C>T (rs1045642, Ile1145Ile) and 2677G>T/A (rs2032582, Ser893Ala/Ser893Thr) on ARV pharmacokinetics and toxicity[120].

Several genes that are not part of the pharmacokinetic pathways of ARVs have been demonstrated to effect ARV toxicity (Table 1.3). The most important of these is the increased risk of abacavir hypersensitivity reaction associated with the *HLA-B*57:01:01* allele. The risk of abacavir hypersensitivity is greatly increased in individuals with the HLA-B*57 allele and the Clinical Pharmacogenetics Implementation Consortium (CPIC) discourages prescribing of abacavir to patients with the allele[71]. Variants in several other genes are associated with increased ARV toxicity including: *HTR2A*, *NT5C2* and *XDH* (Table 1.3). Polymorphisms in *HTR2A*, a serotonin receptor, are associated with increased EFV central nervous system side effects and polymorphisms in *NT5C2* and *XDH* are associated with increased risk of didanosine induced noncirrhotic portal hypertension[91,121].

Table 1.3. Selected polymorphisms in drug metabolizing enzymes and drug transporters associated with ARV response and toxicity.

Gene	SNP	Allele	Drugs	Function	Amino Acid Change	Clinical Effect
ABCB1	rs1045642	3435C>T	efavirenz lamivudine lopinavir nelfinavir nevirapine ritonavir tenofovir zidovudine	Synonymous	Ile1145Ile	Controversial[120]
ABCB1	rs2032582	2677G>A, 2677G>T	efavirenz lamivudine nevirapine zidovudine	Missense	Ser893Ala; Ser893Thr	Controversial[120]
ABCC10	rs9349256	1791+526G>A	tenofovir	Intronic		Risk of kidney toxicity[118]
ABCC10	rs2125739	2759T>C	nevirapine	Missense	Ile948Thr	TC/CC ↑ Plasma levels; EFV, NVP[119]
ABCC2	rs17222723	3563T>A	tenofovir	Missense	Val1188Glu	Risk of kidney toxicity[67]
ABCC2	rs2273697	26353G>A	tenofovir	Missense	Val417Ile	Risk of kidney toxicity[67]
ABCC2	rs717620	-24C>T	tenofovir	5' UTR		Risk of kidney toxicity[67]
ABCC2	rs8187710	4544G>A	tenofovir	Missense	Cys1515Tyr	Risk of kidney toxicity[67]
ABCC4	rs11568695	3609G>A	tenofovir	Synonymous	Ala1203Ala	Risk of kidney toxicity[67]
ABCC4	rs1751034	3348A>G	tenofovir	Synonymous	Lys1116Lys	Risk of kidney toxicity[67]

Gene	SNP	Allele	Drugs	Function	Amino Acid Change	Clinical Effect
CYP2A6	rs28399454	47441C>T	efavirenz nevirapine	Missense	Val365Met	Variant alleles ↑ Plasma levels[116]
CYP2A6	rs8192726	6857G>T	efavirenz	Intronic		CA/AA ↑ Plasma levels[116]
CYP2B6	rs2279343	785A>G, CYP2B6*4	efavirenz nevirapine	Missense	Lys262Arg	AG/GG ↑ Plasma levels; EFV, NVP[80]
CYP2B6	rs28399499	983T>C, part of CYP2B6*18	efavirenz nevirapine	Missense	Ile328Thr	CT/CC ↑ Plasma levels; EFV, NVP[109,110]
CYP2B6	rs3745274	516G>T, CYP2B6*6	efavirenz nevirapine	Missense	Gln172His	GT/TT ↑ Plasma levels; EFV, NVP; ↑NVP Hepatotoxicity [80-82,111,122]
CYP3A5	rs776746	6986A>G	nevirapine	Acceptor		↓NVP Plasma levels[115]
HLA-B	-	*57:01:01	abacavir	-	-	↑ hypersensitivity[71]
HTR2A	rs6313	102C>T	efavirenz	Intronic	Ser34Ser	↑CNS Side effects[91]
NT5C2	rs11191561	104869531C>G	didanosine	Intronic		↑ Noncirrhotic portal hypertension [121]
NT5C2	rs11598702	104897985T>C	didanosine	Intronic		↑ Noncirrhotic portal hypertension[121]
UGT1A1	rs4148323	211G>A	indinavir	Intronic	Gly71Arg	↑ hyperbilirubinemia[117]
UGT1A1	rs8175347	UGT1A1*28, UGT1A1*36, UGT1A1*37	atazanavir indinavir	Not Available		↑ hyperbilirubinemia;ATZ, IDV[117]
UGT2B7	rs7439366	802T>C, UGT2B7*2	efavirenz	Missense	Tyr268His	↑EFV plasma concentration[116]
XDH	rs1429376	10410448A>C	didanosine	Intronic		↑ Noncirrhotic portal hypertension[121]
XDH	rs1594160	10400856A>C	didanosine	Intronic		↑ Noncirrhotic portal hypertension[121]

1.6 Dissertation Aims

The aim of this dissertation is to characterize the impact of host genetics on complications due to HIV infection and ARV pharmacology and toxicity. Since the advent of HAART, many of the complications to HIV infection have not been frequently observed in the developed world. However, these complications are still a significant problem in the developing world and much is still unknown about the effect that host genetics play in the risk of developing these complications. Additionally, while numerous studies detailing environmental and genetic factors have explained a portion of the interpatient variability of ARV PK and toxicity, there is still substantial unaccounted for variability in patient populations. The main questions addressed in the studies of this dissertation are as follows:

1. Are NVP pharmacokinetics heritable in different ethnic populations?

2. What are the genetic predictors of NVP pharmacokinetics, both known and novel?

3. What are the genetic predictors of HIV induced peripheral neuropathy?

4. What are the genetic predictors of NRTI induced peripheral neuropathy?

The following studies were conducted to determine the answers to these questions.

1. To determine if NVP pharmacokinetics is heritable the relative genetic contribution to nevirapine pharmacokinetics was characterized in African and European Americans. Repeated dose data was used to estimate heritability of NVP exposure and selected polymorphisms in *CYP2B6* and *ABCB1* were also examined.

2. To identify and characterize genetic predictors of NVP pharmacokinetics the relationship between nevirapine C_{min} concentrations and polymorphisms in candidate genes was examined in treatment naïve HIV+ Ugandans.

3 .To identify novel genetic predictors of HIV induced peripheral neuropathy a genome-wide association study was conducted in a Ugandan HIV+ population. Bioinformatic analyses were conducted to identify biologically plausible genetic loci associated with HIV-induced peripheral neuropathy.

4. To identify genetic predictors of NRTI induced peripheral neuropathy a genome-wide association study was conducted in a Ugandan HIV+ population. The biological plausibility of candidate genomic loci was investigated using bioinformatic tools.

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Chapter 2

Measuring the Overall Genetic Component of Nevirapine Pharmacokinetics and the Role of Selected Polymorphisms: Towards Addressing the Missing Heritability in Pharmacogenetic Phenotypes?*

2.1 Abstract

Nevirapine is an important component of highly active antiretroviral therapy used in the treatment of human immunodeficiency virus infection. There is considerable variation in the pharmacokinetics of nevirapine and this variation can impact the efficacy and toxicity of nevirapine. While some of this variation can be attributed to environmental factors, the degree to which heritability influences nevirapine pharmacokinetics is unknown. This study aims to estimate how much variation in nevirapine pharmacokinetics is due to genetic factors and to investigate the contribution of selected polymorphisms to this variability. Two doses of immediate-release nevirapine were administered to European (n=11) and African American (n=6) subjects recruited from the Research in Access to Care in the Homeless (REACH) cohort. A repeated-dose drug administration (RDA) method was used to determine the relative genetic contribution (rGC) to variability in nevirapine $AUC_{0\text{-}6h}$. Nevirapine plasma levels were quantified using LC-MS/MS. Patients were also genotyped for selected polymorphisms in candidate genes that may influence nevirapine pharmacokinetics. A significant rGC for nevirapine $AUC_{0\text{-}6h}$ was found in Europeans (p = 0.02) and African Americans (p = 0.01). A trend towards higher nevirapine $AUC_{0\text{-}6h}$ for the *CYP2B6*

* The text of this dissertation chapter is a reprint of the material as it appears in *Pharmacogenetics*[1]. The co-author Dr. Deanna Kroetz listed in this publication directed and supervised the research that forms the basis for the chapter.

516TT (rs3745274; Q172H) genotype was observed in European Americans (p = 0.19). This study demonstrates that there is a significant genetic component to variability in nevirapine pharmacokinetics. While genetic variants such as *CYP2B6* polymorphisms may contribute to some of this variation, these data suggest that there are additional genetic factors that influence nevirapine pharmacokinetics.

2.2 Introduction

The importance of understanding the role of genetics in variation in pharmacokinetics and pharmacodynamics has been recognized since the 1950s[2-5]. Twin studies have historically been used to determine the heritability of genetic diseases and traits; these studies have also been used to determine the heritability of pharmacodynamic and pharmacokinetic parameters[6]. While twin studies are a useful technique to determine genetic contributions to pharmacokinetic variation, it can be impractical to use twins in pharmacogenetic studies due to difficulty in recruitment and the need to expose them to drugs. A statistical technique that was specifically developed to address this issue is the repeated drug administration (RDA) method, which uses repeated administrations of a drug to the same individuals to compare the within subject and between-subject variation in pharmacokinetic parameters[7]. This comparison can be used to quantify the relative genetic contribution to variations in pharmacokinetic parameters of a drug. While the RDA method is useful in determining whether pharmacokinetic or pharmacodynamic parameters of a drug have strong genetic components, it may vary with the route of administration or patient population studied[7]. Additionally, while one pharmacokinetic parameter for a given drug may have a strong relative genetic component, other parameters may not due to the genes involved in the absorption, metabolism and excretion of a drug[8]. Repeated drug administration has successfully been employed to characterize the genetic contribution to variability in pharmacokinetic parameters of several drugs, including erythromycin, midazolam and metformin[9,10]. However, the genetic contribution to pharmacokinetic parameter variability for many drugs is still unknown.

Nevirapine is a non-nucleoside reverse transcriptase inhibitor widely used as a component of antiretroviral therapy in the treatment of human immunodeficiency virus (HIV)[11]. Nevirapine exhibits considerable variability in its pharmacokinetic properties; however, only part of this variability can be explained by environmental factors and concomitant conditions[12]. Variation in nevirapine pharmacokinetics can lead to reduced efficacy, increased viral resistance and increased toxicities[13]. Nevirapine is metabolized to its primary metabolite 3-hydroxynevirapine by CYP2B6[14]. The *CYP2B6* 516G>T (rs3745274) and *CYP2B6* 983T>C (rs28399499) variant alleles have a significant effect on nevirapine plasma levels and the *CYP2B6* 516T allele has also been associated with increased recovery of CD4+ T-cell populations in pediatric patients following initiation of nevirapine-containing antiretroviral therapy [15-17]. Additionally, *ABCB1* 3435C>T (rs1045642) has been associated with protection against nevirapine-induced hepatotoxicity and increased nevirapine concentrations in cerebral spinal fluid[18,19]. Despite evidence that nevirapine pharmacokinetics are influenced by specific polymorphisms, there has not been a study conducted to quantify the relative genetic contribution to variability in nevirapine pharmacokinetics.

This study uses the repeated drug administration method to quantify the relative genetic contribution to variability in nevirapine pharmacokinetics. A significant relative genetic contribution to variation in nevirapine exposure was shown in two ethnic populations. The contribution of *CYP2B6* 516G>T and *ABCB1* 3435C>T to variability in nevirapine pharmacokinetics was also investigated.

2.3 Materials and Methods

2.3.1 Study Design and Subjects:

Subjects were recruited from the Research in Access to Care in the Homeless (REACH) cohort as previously described[20]. Study participants are marginally housed HIV positive individuals living in San Francisco. Seventeen patients were recruited to participate in a pharmacokinetic study where subjects receiving 200 mg nevirapine twice daily consented to pharmacokinetic blood sampling. All subjects were on therapy at least four months and were concomitantly receiving two nucleoside reverse transcriptase inhibitors. Subjects were presumed to have reached steady state concentrations. Blood samples were drawn at 0, 1, 2, 3 and 6 hr post-dose. The time between time courses varied from 13 days to 173 days. European American (n=11) and African American (n=6) patients were included in this study. Ethnicity was self-reported and verified through genotyping of 112 ancestry informative markers and analysis using the STRUCTURE program[21-23]. The study was approved by the University of California San Francisco Institutional Review Board and all subjects provided written informed consent prior to participation.

2.3.2 Nevirapine Quantification:

Plasma was prepared from blood samples by centrifugation and stored at -80°C until analysis. Nevirapine was extracted using Oasis HLB SPE columns (Waters Corp., Milford, MA) and plasma concentrations were quantified by LC/MS/MS analysis as described by Mistri *et. al*[24]. Briefly, each 0.5 mL plasma aliquot was heated for 1.5 hr at 56°C to inactivate HIV-1 virus and then spiked with 25 μl of 20 μM metaxolone (Toronto

Research Chemicals, Toronto, Ontario) in methanol, which served as an internal standard. SPE columns were equilibrated with 1 mL methanol followed by 1 mL distilled water. Samples were then loaded on the column and washed with 1 mL of 2 mM ammonium acetate followed by 1 mL of water. Samples were eluted in 1 mL mobile phase (80:20 acetonitrile:water, 0.1% acetic acid) and a 5 µl aliquot was injected onto a 5 µm Hypersil BDS C18 column, 50 x 4.6 µm (Thermo Fisher Scientific, Waltham, MA). The flow rate into the API4000 mass spectrometer (AbSciex, Framingham, MA) was 0.2 mL/min and nevirapine retention time was 1.7 min. The parent ion (267.2 m/z, amu) and product ion (226.2 m/z, amu) were monitored at Q1 and Q3, respectively. Nevirapine standard curves were linear from 50 - 5000 ng/mL ($r^2 > 0.9$). Assay accuracy was between 100.3% and 112.9% relative standard deviation. Assay precision ranged from 8.2 – 18.5% CV.

2.3.3 Genotyping:

Genomic DNA was extracted from whole blood samples. Genotyping of polymorphisms of interest (*CYP2B6* 516G>T and *ABCB1* 3435C>T) was accomplished using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). TaqMan assays were used to genotype *CYP2B6* 516G>T (rs3745274, Assay ID: C___7817765_60) and *ABCB1* 3435C>T (rs1045642, Assay ID: C___7586657_20). Genotypes were called with ABI Sequence Detection System software (version 2.1; Applied Biosystems, Foster City, CA).

2.3.4 Calculation of Pharmacokinetic Parameters:

Due to the long half-life of nevirapine (45 hr), only $AUC_{0\text{-}6h}$ was calculated[25]. $AUC_{0\text{-}6h}$ was calculated for each dose administration using the trapezoidal rule.

2.3.5 Calculation of Relative Genetic Component:

The genetic contribution to the variability in nevirapine $AUC_{0\text{-}6h}$ was assessed with a modified ANOVA formula for estimating the relative genetic component or r_{GC} and 95% confidence intervals proposed by Kalow et.al.[26]:

$r_{GC} = (SD_b^2 - SD_w^2)/SD_b^2$

which can be rearranged as

$r_{GC} = 1-(1/F)$ where $F = SD_b^2/SD_w^2$

Upper and Lower Confidence intervals can be calculated using:

Lower 95% confidence interval = $F_{observed}/F_{0.025,b.d.f,w.d.f}$

Upper 95% confidence interval = $F_{observed}*F_{0.025,b.d.f,w.d.f}$

where r_{GC} represents the estimated relative genetic component, SD_b^2 is the between subjects variation, SD_w^2 is the within subject variation, b.d.f is the between subjects degrees of freedom, w.d.f. is the within subject degrees of freedom and $F_{0.025}$ is the tabulated F statistic at the 2.5% significance level at the appropriate degrees of freedom. Due to well characterized differences in allele frequency and linkage disequilibrium patterns, European Americans (n=11) and African Americans (n=6) were analyzed separately in this study.

2.3.6 Statistical Methods:

Statistical significance for genetic contribution to $AUC_{0\text{-}6h}$ variability was calculated using an F-test, α=0.05, to determine if the inter- and intra-individual variation was significantly different. One-way ANOVA, α=0.05, was used to determine significance for the effect of genetic polymorphisms on $AUC_{0\text{-}6h}$ values. All other calculations of *p*-values were obtained using two-sided *t*-tests or one-way ANOVA as appropriate[27]. Calculations were performed using R and Microsoft Excel[28]. All figures were produced in Prism Version 5.01 (GraphPad Software Inc., San Diego, CA).

2.4 Results*Ethnicity does not play a role in nevirapine AUC0-6h variability*

Since there are well characterized differences in the genetic structure and linkage disequilibrium patterns in different ethnic populations, a statistical analysis to examine any overall differences in nevirapine $AUC_{0\text{-}6h}$ between African and European Americans was conducted. A total of 17 subjects were included in this study, 11 European Americans and six African Americans (Table 2.1). Median ages and concomitant medications were similar in the two ethnic groups, while the African American group had a higher proportion of females than the European American group.

Analysis of nevirapine plasma concentrations indicated very little intrasubject variability in concentrations during the six hours following drug administration, consistent with the long terminal half-life of this drug (see Figures 2.1A and 2.1B). In contrast, there is considerable variation in nevirapine concentrations between individuals; three individuals in the African American and two in the European American groups never reach plasma concentrations above the minimum effective concentration

(MEC) for nevirapine of 3000 μg/L[29]. Average $AUC_{0\text{-}6h}$ did not differ between the two visits, although there was significant interpatient variability in these values (Table 2.1). For example, the mean $AUC_{0\text{-}6h}$ was 22.5 mg nevirapine/L*hr (SEM = 3.81 mg nevirapine/L*hr) and 18.3 mg nevirapine/L*hr (SEM = 2.69 mg nevirapine/L*hr) for European and African Americans, respectively. There was not a significant difference in $AUC_{0\text{-}6h}$ between ethnicities (*t*-test, $p = 0.45$).

Table 2.1 Patient demographics and relative genetic contribution (rGC) to nevirapine $AUC_{0\text{-}6h}$

		European Americans	African Americans
Sample Size	n	11	6
Gender	Male (%)	4 (36)	1 (17)
	Female (%)	7 (64)	5 (83)
Age (years)	Median	45	49
	Range	29 - 57	33 - 74
Nevirapine $AUC_{0\text{-}6h}$ (mg/L*hr)[1]	SD_w^2	2.39	5.34
	SD_b^2	24.9	54.7
Estimated Relative Genetic Component	r_{GC}^2 (95% CI)	0.904 (0.64-0.97)	0.902 (0.42-0.98)
	F	10.4	10.2
	p	0.02	0.01

[1] SD_w^2 is within individual variation and SD_b^2 is between subject variation.
[2] Estimated relative genetic component

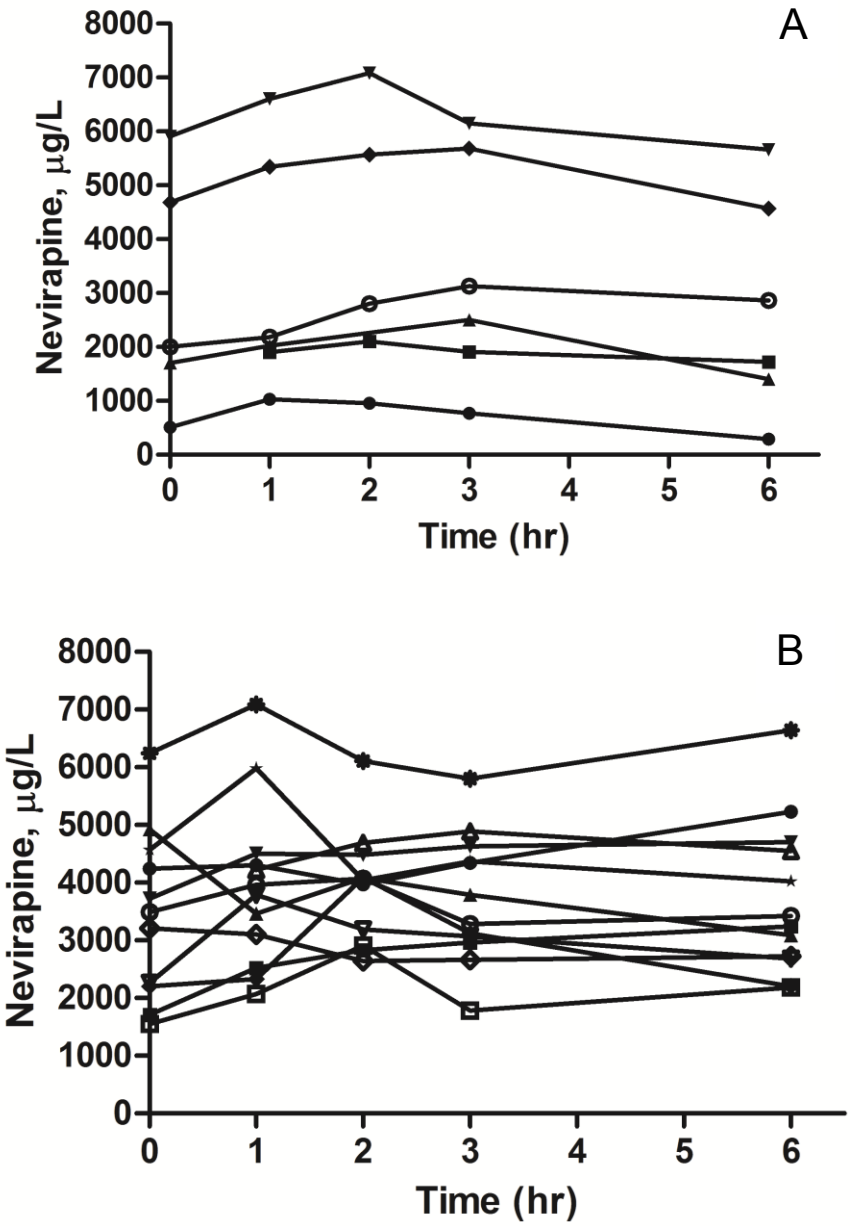

Figure 2.1. Interindividual variation in nevirapine plasma levels.
Plasma concentrations of A) African American subjects and B) European American subjects 0-6 hours after nevirapine dose administration. Each line represents one individual.

2.4.2 Age and sex do not play a role in the variability of nevirapine AUC_{0-6h}

To ensure further analyses were not confounded by demographic factors, the effects of age and sex on nevirapine AUC_{0-6h} were examined by linear regression and t-tests, respectively. Age had no effect on nevirapine AUC_{0-6h} with an r^2 of 0.04. Males tended to have slightly lower AUC_{0-6h} (16.2 mg nevirapine/L*hr, SEM = 37.0 mg nevirapine/L*hr) than females (23.0 mg nevirapine/L*hr, SEM=24.2 mg nevirapine/L*hr) however, this difference was not statistically significant (p = 0.14).

2.4.3 There is a genetic contribution to variation in nevirapine AUC_{0-6h}

The relative genetic contribution to nevirapine pharmacokinetics was calculated using the repeated drug administration method described previously[7,9]. The between-subject (SD_b^2) variation in AUC_{0-6h} was about 10-fold greater than the within subject variation (SD_w^2) in both ethnic groups (Table 2.1). The calculated r_{GC} and upper and lower 95% confidence intervals for the European Americans and African Americans was 0.902 (0.64 - 0.97) and 0.904 (0.42 - 0.98), respectively. F-tests indicate there is a significant genetic contribution to the variability in AUC_{0-6h} in both Europeans (p = 0.02) and African Americans (p = 0.01).

2.4.4 CYP2B6 516G>T may influence nevirapine AUC0-6h

Considering the evidence for a significant genetic contribution to the variability in nevirapine exposure, polymorphisms in candidate genes implicated in the metabolism and transport of nevirapine were tested for association with nevirapine pharmacokinetics. In African Americans, there is a trend for increased plasma nevirapine levels in individuals carrying the CYP2B6 516G>T allele or the ABCB1

3435C>T allele (Figures 2.2A and 2.3A); however, the sample sizes are too small for formal statistical analysis (Table 2.2). A similar trend was observed for the CYP2B6 516G>T allele in European Americans, but these differences did not reach statistical significance (Figure 2.2B and Table 2.2). There was no indication of an association between the ABCB1 3435C>T polymorphism and nevirapine pharmacokinetics in European Americans (Figure 2.3B and Table 2.2).

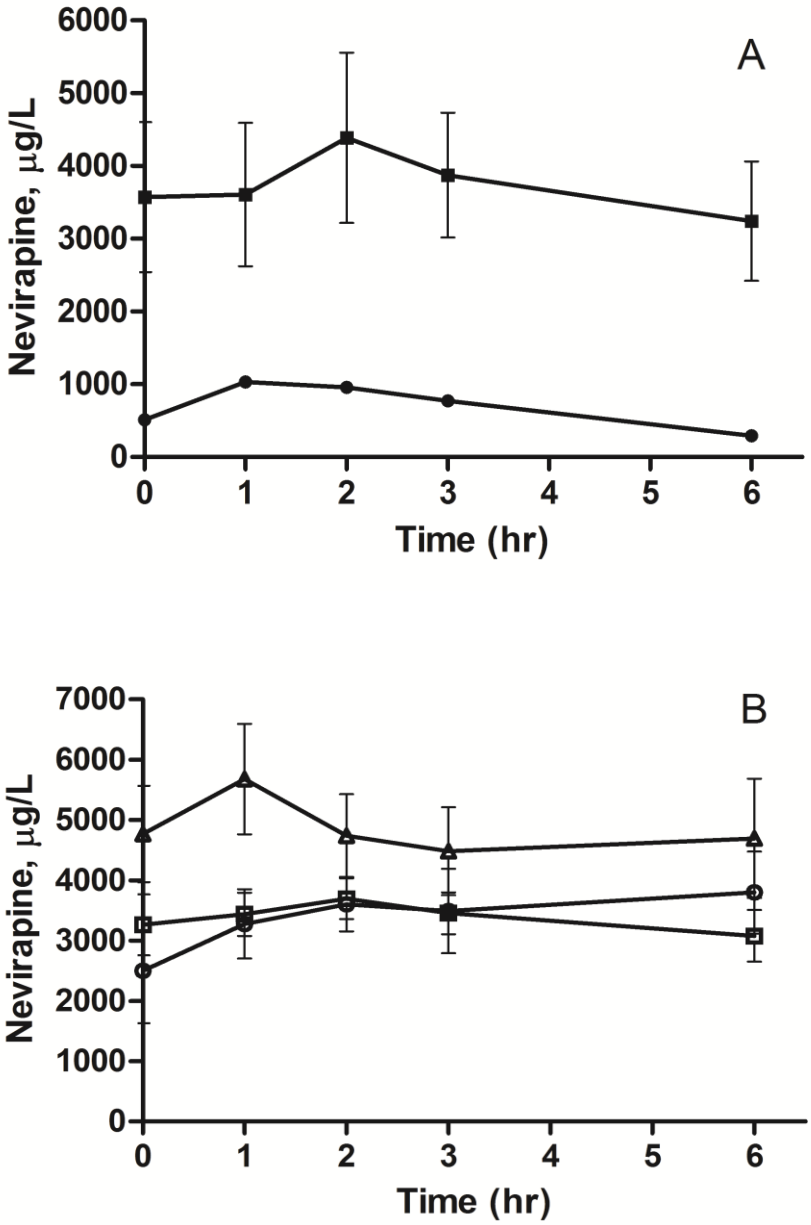

Figure 2.2. Nevirapine plasma concentrations following a single oral dose.
A 200 mg dose of nevirapine was administered to A) African Americans and B) European Americans and concentrations were measured over six hours. The concentrations (mean ± SEM) are stratified by *CYP2B6* 516G>T genotype: circles GG, squares GT and triangles TT.

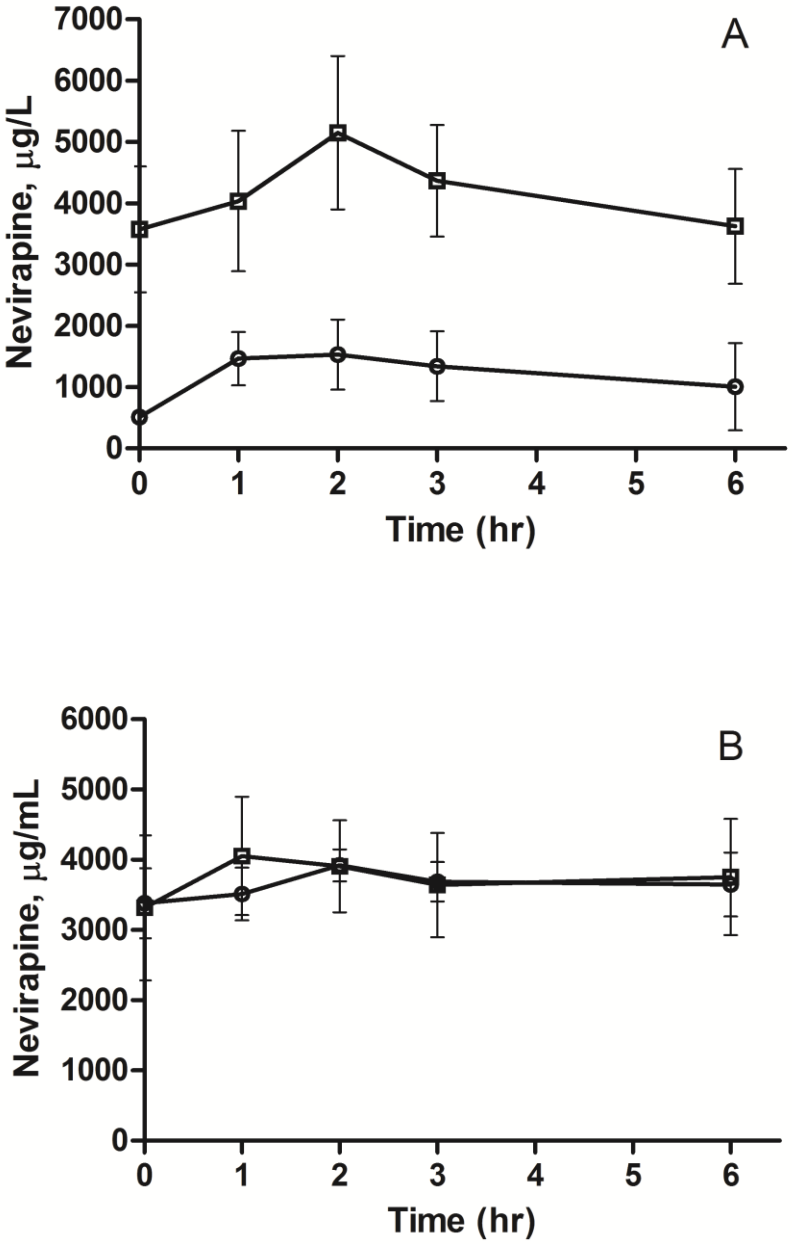

Figure 2.3. Nevirapine plasma concentrations following a single oral dose.
A 200 mg dose of nevirapine was administered to A) African Americans and B) European Americans and concentrations were measured over six hours. The concentrations (mean ± SEM) are stratified by *ABCB1* 3435C>T genotype: circles CC and squares CT.

Table 2.2. The effect of ethnicity and genotype on nevirapine exposure

	Ethnicity	n	Nevirapine $AUC_{0\text{-}6h}$ (mg/L*h)[1]	*p*
	African American	6	18.3 ± 3.81	0.45
	European American	12	22.5 ± 2.69	
CYP2B6 516G>T	**African Americans**			
	GG	1	4.23	ND
	GT	5	21.2 ± 5.63	
	TT	0	-	
	European Americans			
	GG	4	20.5 ± 3.16	0.19
	GT	5	20.3 ± 2.82	
	TT	3	28.8 ± 3.64	
ABCB1 3435C>T	**African Americans**			
	CC	2	7.31 ± 7.97	0.17
	CT	4	23.8 ± 12.8	
	European Americans			
	CC	6	22.0 ± 3.03	0.96
	CT	5	22.2 ± 3.32	

[1] Mean ± SEM

2.5 Discussion

While there have been many candidate gene association studies of nevirapine pharmacokinetics, this is the first study to determine the overall relative genetic influence on nevirapine exposure. A significant relative genetic contribution to the variability in nevirapine pharmacokinetics was demonstrated in European and African Americans. This supports previous findings that have implicated polymorphisms in drug metabolism and transport genes in nevirapine pharmacokinetic variability and toxicity[16-18]. A trend consistent with previous studies of elevated plasma concentrations in subjects homozygous for the *CYP2B6* 516G>T allele was also observed[15,17].

Variability in nevirapine pharmacokinetics and toxicity has been observed since its approval for the treatment of HIV. Many candidate gene studies have confirmed that a portion of pharmacokinetic variability is due to polymorphisms in *CYP2B6*[16,30,31]. However, the variation in pharmacokinetics due to genetic versus environmental factors has never been examined. The current study demonstrates that there is a significant genetic component to nevirapine pharmacokinetics in African and European Americans.

While the population examined here is small, one advantage of the RDA method is the ability to use small populations to estimate relative genetic components of drugs[8]. In our European population, we have the required number of subjects to estimate a 95% Lower Confidence limit of ~0.65 for an r_{GC} of 0.9[8]. This suggests that interindividual variation in nevirapine drug levels could be reduced through knowledge of a patient's genetic background. The importance of this is reflected in the observation that several patients did not reach the MEC of nevirapine. The RDA method has been successfully employed to identify drugs whose renal clearance has a strong genetic component and

could also be used to identify antiretroviral drugs that are good candidates for pharmacogenomics research[10]. Employing the RDA method in pharmacogenomic research could lead to decreased efficacy against HIV and increased viral resistance to nevirapine and other antiretroviral drugs.

To further investigate the influence of genetics on nevirapine pharmacokinetics, two candidate polymorphisms were selected for study. A trend was observed towards elevated $AUC_{0\text{-}6h}$ of nevirapine in both European and African Americans homozygous for the *CYP2B6 516G>T* polymorphism. This polymorphism is associated with a slight decrease in hepatic protein expression and function, therefore increases in $AUC_{0\text{-}6h}$ are expected[32]. While the results in European Americans did not reach statistical significance, the analysis was limited by a small sample size and may have been confounded by unidentified environmental factors. The trend observed is consistent with other published work, which supports the need for a larger study population[16,17,33].

No association of *ABCB1* 3435C>T with nevirapine exposure was observed in our study. The effect of this polymorphism on nevirapine pharmacokinetics remains controversial, with many studies not showing an effect on nevirapine plasma pharmacokinetics[15-17,31,34]. $AUC_{0\text{-}6h}$ may not be the most appropriate pharmacokinetic parameter to observe the effects of these polymorphisms; however, due to the long half-life of nevirapine, it was not possible to calculate other pharmacokinetic parameters such as half-life or oral clearance.

2.6 Conclusions

The current study demonstrates that there is a significant relative genetic component to nevirapine pharmacokinetics. While there are genetic variants such as *CYP2B6* polymorphisms that have been attributed to some of this variation[16,17,33], this study suggests that there may be additional genetic factors that influence nevirapine pharmacokinetics. This study supports additional research to discover novel genetic factors influencing nevirapine variability. Furthermore, the RDA method could also be used to study endpoints of antiretroviral drugs other than pharmacokinetic and pharmacodynamic parameters, such as metabolomic endpoints[35]. Additional knowledge of genetic factors that affect nevirapine pharmacokinetics may help increase the efficacy of nevirapine in the treatment of HIV and lead to less viral resistance over time.

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Chapter 3

CYP2B6 and *ABCC10* Polymorphisms Influence Nevirapine Exposure in HIV+ Ugandans

3.1 Abstract:

Nevirapine is an important component of highly active antiretroviral therapy used in the treatment of human immunodeficiency virus infection. There is considerable variation in the pharmacokinetics of nevirapine and this variation can impact its efficacy and toxicity. Some of the variation in nevirapine pharmacokinetics can be attributed to polymorphisms in *CYP2B6*, but other genes may also play a role in this variation. This study examined the effect of polymorphisms in *CYP2B6*, *CYP2C19*, *CYP2C9*, *ABCC10*, *NR1L2*, *CYP2D6*, *CYP3A4* and *CYP3A5* on nevirapine pharmacokinetics. Patients on antiretroviral therapy regimens containing nevirapine were recruited from the Uganda AIDS Rural Treatment Outcomes cohort. Plasma samples were taken before patients received their daily dose of nevirapine. Nevirapine trough levels were quantified using LC-MS/MS. DNA samples were genotyped for selected polymorphisms in candidate genes that may influence nevirapine pharmacokinetics. The combined effect of multiple polymorphisms on nevirapine exposure was also explored. Several polymorphisms significantly influence nevirapine trough levels. *CYP2B6 516G>T* (rs3745274; $p = 0.03$), *CYP2B6 983T>C* (rs28399499; $p = 0.003$) and *ABCC10* rs2125739 ($p = 0.001$) were associated with higher nevirapine trough levels. Additionally, the number of variants in the composite of *CYP2B6 516/983* ($p = 0.0002$) was associated with increases in nevirapine plasma concentrations. Finally, the variant load in a *CYP2B6/ABCC10* composite ($p = 2.5 \times 10^{-6}$) was strongly associated with an increase in nevirapine

concentration. This study demonstrates the importance of *CYP2B6* and *ABCC10* in nevirapine pharmacokinetics. The results also support consideration of the combined effects of multiple polymorphisms on nevirapine trough levels.

3.2 Introduction:

Sub-Saharan Africa accounts for 69% of worldwide HIV infections and it is important to understand the consequences of common polymorphisms in African populations on the pharmacokinetics of commonly used antiretroviral (ARV) drugs[1]. ARV treatment for HIV infections in sub-Saharan Africa generally consists of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleotide reverse transcriptase inhibitor (NNRTI), usually in a fixed dose combination form[2]. Nevirapine (NVP) is a NNRTI commonly used in sub-Saharan Africa to treat HIV and is also the recommended first line treatment to prevent mother to child transmission of HIV[3,4]. While NVP is an effective treatment for HIV, it has variable pharmacokinetic properties that can affect its efficacy and toxicity[5,6]. There have been many studies exploring what genetic and environmental factors contribute to NVP pharmacokinetics, however, there still is unaccounted for variability in NVP disposition.

NVP pharmacokinetics has been shown to be heritable and several genetic variants contribute to this variability[7-11]. NVP is hepatically metabolized primarily by CYP3A4 and CYP2B6 with the latter being a major metabolic enzyme upon autoinduction[12,13]. *CYP2B6* 516G>T (rs3745274) and *CYP2B6* 983T>C (rs28399499) influence the pharmacokinetics of NVP and efavirenz[9-11,14,15]. NVP is also a substrate of the efflux pump MRP7 (*ABCC10*) and polymorphisms in *ABCC10* are associated with the pharmacokinetics of NVP[16].

CYP2B6 516G>T is a missense mutation that causes an amino acid change from a glutamine to a histidine at the 172 position in the protein. This leads to decreased expression and function of CYP2B6[17,18]. *CYP2B6 983T>C* is also a missense mutation

that causes an amino acid change from an isoleucine to a tyrosine at the 328 position in the protein. The CYP2B6 328Tyr variant is associated with decreased expression and function[18]. *CYP2B6 516G>T* and *CYP2B6 983T>C* have minor allele frequencies of 42% and 12%, respectively, in the African Yoruban (YRI) population. The consequences of both *CYP2B6* polymorphisms have been extensively studied for the pharmacokinetics of efavirenz, but have been less studied for their impact on NVP pharmacokinetics[8,9,19-21]. *ABCC10* rs2125739 has not been well studied and has only been implicated in NVP pharmacokinetics in Caucasian populations[16]. A survey of the literature was used to identify genes important in the biotransformation and transport of NVP (Figure 3.1). Genes selected for inclusion in the exploratory analysis were *CYP2B6*, *CYP2C19*, *CYP2C9*, *ABCC10*, *NR1L2*, *CYP2D6*, *CYP3A4* and *CYP3A5*[12,13,22]. *CYP2C19*, *CYP2C9*, *CYP2D6* and *CYP3A5* were included in the candidate gene study because of their role in NVP metabolism[12]. PXR, encoded by *NR1L2*, was included because of its ability to regulate basal expression levels of CYP3A4/5[23].

This study examines the impact of individual polymorphisms in candidate genes on NVP trough plasma concentrations. It also considers the combined effect of selected polymorphisms as a predictor of NVP exposure.

3.3 Materials and Methods:

3.3.1 Study Design and Patients:

Patients were recruited from the Uganda AIDS Rural Treatment Outcomes cohort. Study participants are treatment naïve HIV+ patients living in Mbarra, Uganda. Patients

enrolled in the study receive a treatment regimen consisting of two NRTI and one NNRTI. Patients routinely have blood drawn to monitor $CD4^+$ cell counts. Plasma was obtained from 121 patients receiving NVP as a component of their ARV therapy. All samples were collected prior to the administration of the morning dose as a measure of C_{min}. The study was approved by the University of California San Francisco Institutional Review Board and all subjects provided written informed consent prior to participation. In the event that there are cultural literacy reasons why a signature is not appropriate, participants are allowed to mark consent forms with a thumbprint.

3.3.2 Nevirapine Quantification:

Blood samples were centrifuged for plasma isolation and samples were stored at -80°C until analysis. NVP was extracted using Oasis HLB SPE columns (Waters Corp., Milford, MA) and plasma concentrations were quantified by LC/MS/MS analysis as described by Mistri et al[24]. Briefly, each 0.5 mL plasma aliquot was heated for 1.5 hrs at 56°C to inactivate HIV-1 virus and then spiked with 25 µl of 20 µM metaxolone (Toronto Research Chemicals, Toronto, Ontario) in methanol, which served as an internal standard. SPE columns were equilibrated with 1 mL methanol followed by 1 mL distilled water. Samples were then loaded on the column and washed with 1 mL of 2 mM ammonium acetate followed by 1 mL of water. Samples were eluted in 1 mL mobile phase (80:20 acetonitrile:water, 0.1% acetic acid) and a 5 µl aliquot was injected onto a 5 µm Hypersil BDS C18 column, 50 x 4.6 µm (Thermo Fisher Scientific, Waltham, MA). The flow rate into the API4000 mass spectrometer (AbSciex, Framingham, MA) was 0.2 mL/min and NVP retention time was 1.7 min. The parent ion (267.2 m/z, amu) and product ion (226.2 m/z, amu) were monitored at Q1 and Q3, respectively. NVP standard

curves were linear from 50 - 5000 ng/mL ($r^2 > 0.9$). Assay accuracy was between 100.3% and 112.9% relative standard deviation. Assay precision ranged from 8.2 – 18.5% CV.

3.3.3 Genotyping:

Genomic DNA was extracted from saliva samples. Genotyping of *CYP2B6 516G>T* was accomplished using a Taqman assay (Applied Biosystems, Assay ID: C___7817765_60) and the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Genotypes were called with ABI Sequence Detection System software (version 2.1; Applied Biosystems). All other genotypes were determined using an Illumina OmniExpress Bead Chip as outlined in Chapter 4.3.2. Genotypes were called with Illumina Bead Studio Software. A X^2 test of observed vs. expected genotypes was used to determine Hardy-Weinberg equilibrium.

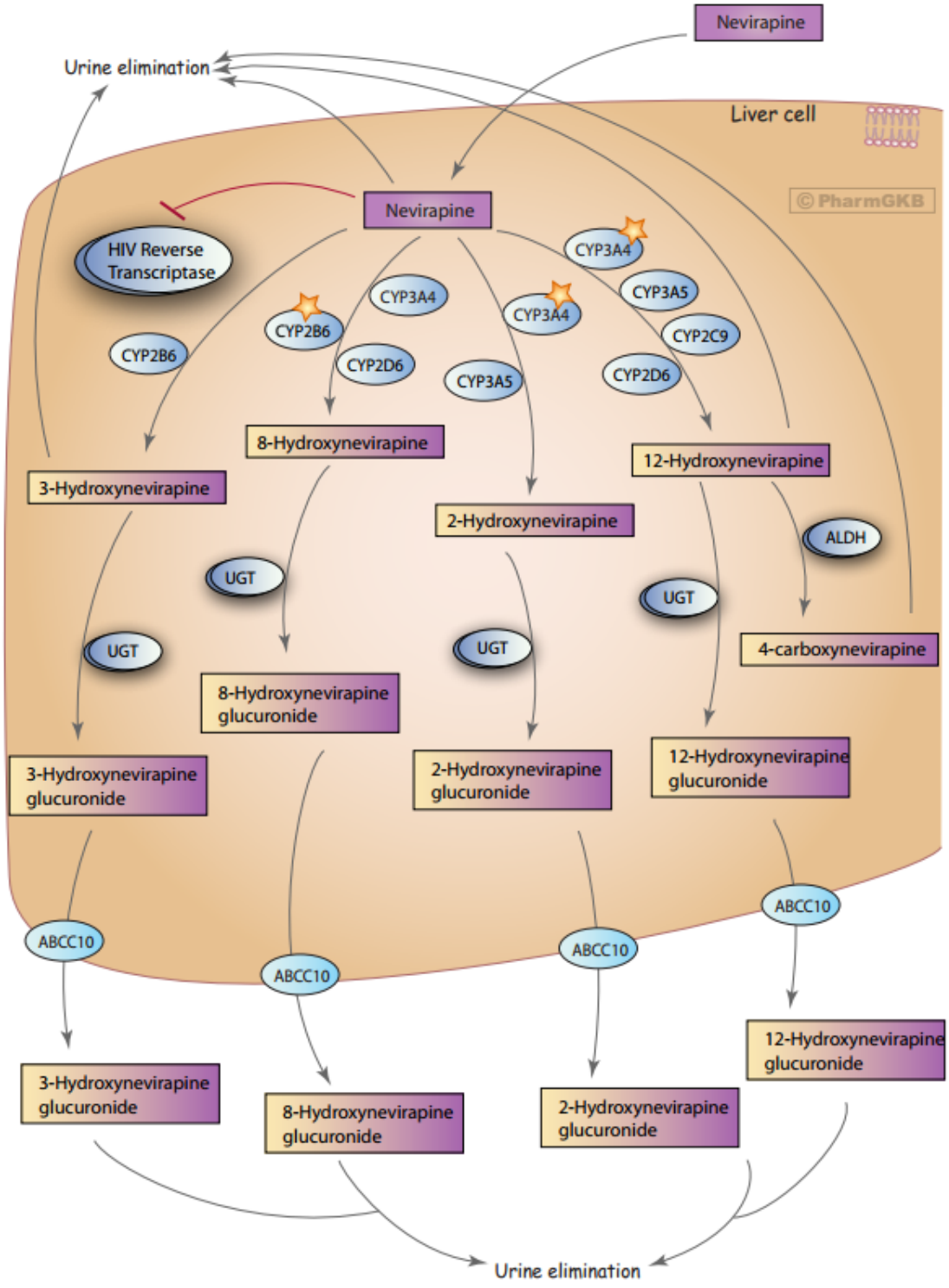

Figure 3.1. PharmGKB.org NVP pharmacokinetic pathway[23].
PharmGKB©. Permission to reproduce this has been granted by Stanford University and PharmGKB. Stars indicate genes that have genetic variants which have been shown to have a significant impact on NVP pharmacokinetics.

3.3.4 Statistical Methods:

3.3.4.1 Univariate Analysis

Nevirapine C_{min} values were log transformed for statistical analyses to better approximate a normal distribution. SNPs within ±10 kb of the selected genes were included in the analysis. Only SNPs that had minor allele frequencies greater than 5% in the study population were included in the analysis. Univariate analyses using linear regression or ANOVA, α=0.05, were used to determine significance for the effect of continuous or categorical demographic covariates on C_{min} values. Linear regression was used to estimate the effect of imputed dosage genotype on NVP C_{min} values with a significance level of α = 0.05. SNPs in high linkage disequilibrium ($r^2 > 0.8$) were collapsed with the highest *p*-value SNP retained. Scatter plots showing log NVP concentration vs. genotype were constructed and examined for Hardy-Weinberg Equilibrium (HWE) and were removed from further analysis if a Chi-Squared test comparing the observed versus expected genotypes was $p < 0.001$. To reduce the amount of false positives generated by a sparse number of samples in genotype groups, genotypes were converted from dosage form to allelic genotypes. SNPs that had less than five subjects in the variant homozygote category were combined with the heterozygote category. SNPs that were significant at the level of 0.05 in the linear regression analysis were then reanalyzed using the allelic genotype calls and an ANOVA test, α=0.05, to confirm the validity of the results. *CYP2B6* 516G>T was directly genotyped using a Taqman assay and only an allelic ANOVA test was performed for this SNP. Bonferroni multiple testing corrections were performed for each

gene separately based on the number of haplotype blocks per gene. SNPs with ANOVA adjusted *p* values < 0.05 were included in the multivariate analyses (Figure 3..2).

Bioinformatic analyses were conducted on all SNPs with ANOVA adjusted *p* values ≤ 0.05 to determine the potential for functional or regulatory consequences and linkage disequilibrium patterns were examined using SCANdb (University of Chicago, http://www.scandb.org/), RegulomeDB and HaploReg[25,26]. Analyses were performed using R[27].

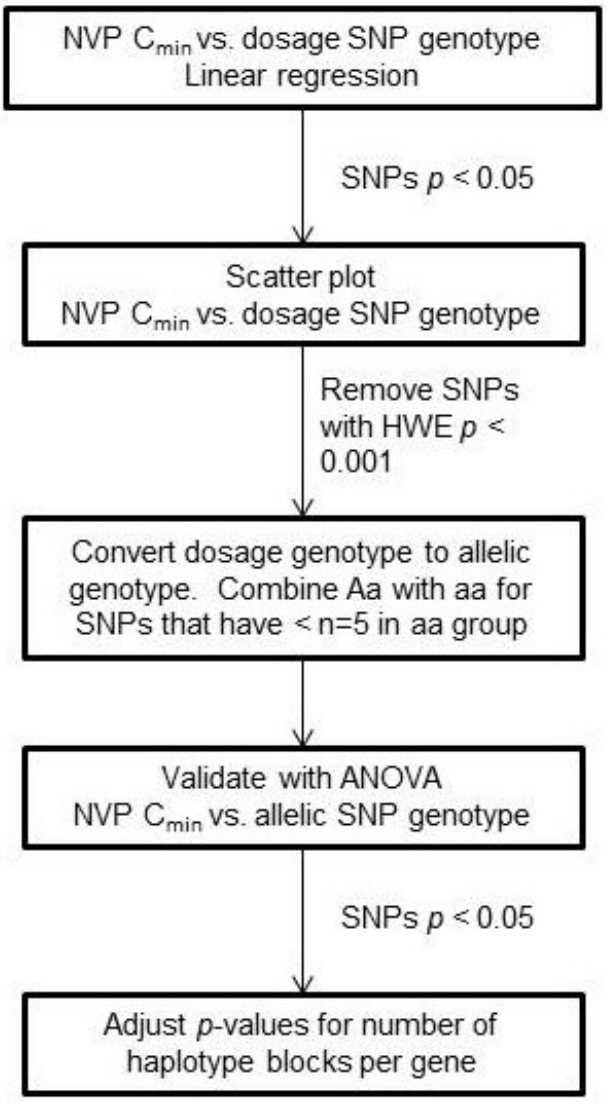

Figure 3.2. Flow chart of statistical analyses.

3.3.4.2 Multivariate Analysis:

Multiple linear regression was used to estimate the effect of demographic and genetic variables on NVP C_{min} values in multivariate analyses. Only demographic variables with a $p \leq 0.2$ and SNPs with an adjusted *p* value of ≤ 0.05 in the univariate analysis were included in multivariate analyses. Analyses were performed using R[27]. Figures were produced in R and Prism Version 5.01 (GraphPad Software Inc., San Diego, CA).

3.4 Results

3.4.1 Characteristics of Study Participants and Analysis of the Effect of Demographic Characteristics on NVP C_{min}:

A total of 121 subjects were included in the analysis. Table 3.1 describes the demographics of the patient population. The patient population was 76% female and the median age of study participants was 35 years (range, 21 - 75 years). The patients were extremely adherent to their treatment regimens, with a median adherence of 100% (range, 50 - 100%). Since all but five patients were > 80% adherent and because adherence data were unavailable for all patients in the study group it was not included in further statistical analyses. Patients were on a standard regimen of two NRTIs along with NVP. The majority (77%) of patients had an NRTI regimen containing zidovudine (AZT) and lamivudine (3TC).

Univariate analyses were performed to assess the impact of demographic covariates on NVP C_{min}. Age (linear regression, $p = 0.102$) and gender (t-test, $p = 0.163$) had $p \leq$

0.2, and were included in multivariate analyses. Concomitant NRTI therapy (t-test, p = 0.53) did not have significant effects on NVP C_{min}.

Table 3.1. Patient Demographics and Effect on NVP C_{min}

Characteristic			p^1
Gender n (%)	Male	29 (24)	0.163
	Female	92 (76)	
Age (years)	Median	35	0.102
	Range	21-75	
Adherence (%)	Median	100	-
	Range	50-100	
Concomitant NRTIs n (%)	AZT+3TC	98 (77)	0.53
	D4T+3TC	23 (23)	

[1] p denotes the effect of each variable on NVP C_{min} concentration. Age and adherence are linear regression p values. Gender and Concomitant NRTIs are ANOVA p values.

3.4.2 Several polymorphisms are associated with NVP C_{min}:

The initial univariate analysis examined 1804 SNPs for association with NVP C_{min} plasma concentrations. Of those SNPs, 55 were associated (unadjusted $p < 0.05$) with NVP C_{min} plasma concentrations. There were 33 unique loci after filtering for LD (Table 3.2). After recoding the genotypes from dosage to allelic format and reexamining the SNPs using ANOVA, four SNPs had adjusted p values < 0.05 (Table 3.2).

Table 3.2. Linkage Disequilibrium Filtered Top Variants Associated with NVP C_{min}

SNP	CHR	Gene	MAF	Beta	Upper 95% CI	Lower 95% CI	*p*	ANOVA *p*[1]	Adjusted *p*
rs1987236	19	*CYP2B6*	0.27	-0.16	-0.061	-0.259	0.002	NS	
rs2253635	10	*CYP2C9*	0.24	0.13	0.209	0.051	0.004	NS	
rs4917623	10	*CYP2C19*	0.22	0.13	0.209	0.051	0.004	0.050	0.050
rs7903917	10	*CYP2C9*	0.25	0.14	0.239	0.041	0.005	NS	
rs4388808	10	*CYP2C19*	0.18	0.12	0.219	0.021	0.008	0.028	0.028
rs2475376	10	*CYP2C9*	0.20	0.12	0.219	0.021	0.011	Removed	
rs60618718	19	*CYP2B6*	0.29	0.19	0.348	0.032	0.014	NS	
rs11188082	10	*CYP2C19*	0.36	-0.13	-0.031	-0.229	0.015	NS	
rs2096069	10	*CYP2C9*	0.27	0.11	0.209	0.011	0.015	Removed	
rs954356	3	*NR1I2*	0.09	-0.14	-0.021	-0.259	0.016	0.040	0.360
rs73933721	19	*CYP2B6*	0.28	0.19	0.348	0.032	0.016	NS	
rs9332209	10	*CYP2C9*	0.07	0.35	0.647	0.053	0.019	NS	
rs2125739	6	*ABCC10*	0.20	0.11	0.209	0.011	0.020	0.002	0.009
rs2224566	10	*CYP2C19*	0.22	0.20	0.358	0.042	0.020	NS	
rs8105382	19	*CYP2B6*	0.28	0.17	0.328	0.012	0.026	NS	
rs11188091	10	*CYP2C19*	0.22	0.18	0.338	0.022	0.027	NS	
rs12721652	19	*CYP2B6*	0.30	0.17	0.328	0.012	0.027	NS	
rs75249760	10	*CYP2C19*	0.08	0.30	0.577	0.023	0.028	NS	
rs2472682	3	*NR1I2*	0.16	0.10	0.199	0.001	0.029	NS	
rs74699808	19	*CYP2B6*	0.20	-0.20	-0.022	-0.378	0.029	NS	
rs4918690	10	*CYP2C19*	0.23	0.17	0.328	0.012	0.031	NS	
rs79460985	3	*NR1I2*	0.09	-0.25	-0.012	-0.488	0.035	0.040	0.360
rs28399499	19	*CYP2B6*	0.08	0.23	0.448	0.012	0.038	0.003	0.008
rs6956305	7	*CYP3A5*	0.11	0.12	0.239	0.001	0.039	NS	
rs73933726	19	*CYP2B6*	0.08	0.23	0.448	0.012	0.039	NS	
rs12721612	3	*NR1I2*	0.07	0.14	0.279	0.001	0.040	0.030	0.270
rs61557439	19	*CYP2B6*	0.32	0.16	0.318	0.002	0.041	NS	
rs11528090	10	*CYP2C19*	0.19	0.18	0.358	0.002	0.047	Removed	
rs16974790	19	*CYP2B6*	0.31	0.15	0.308	-0.008	0.047	NS	
rs57830676	7	*CYP3A5*	0.14	0.22	0.438	0.002	0.048	NS	
rs60549239	19	*CYP2B6*	0.11	-0.28	-0.003	-0.557	0.048	NS	
rs4688035	3	*NR1I2*	0.28	0.09	0.189	-0.009	0.048	Removed	

[1] NS not significant; adjusted *p* value > 0.05. Removed denotes SNPs that were removed after failing HWE testing as outlined in the Statistical methods section.

3.4.3 Polymorphisms in CYP2B6, ABCC10 and CYP2C19 have significant effects on NVP C_{min}

Table 3.3 describes the effect of the four significant SNPs on NVP C_{min}. *CYP2B6* 516G>T was included in the table due to its unadjusted significance and its reported effects on NNRTI concentration, however it is not known to be a predictor of NVP concentrations in African populations.

Table 3.3. Relationship Between Genotypic Variants and NVP C_{min}

SNP	Genotype	NVP C_{min} (mg/L) Mean ± St. Dev.	n (%)	*p*	*p* adjusted*
ABCC10 rs2125739	TT	2.4 ± 0.9	81 (67)	**0.002**	**0.009[a]**
	TC	3.1 ± 1.4	36 (30)		
	CC	3.5 ± 1.3	4 (3)		
CYP2B6 516G>T rs3745274	GG	2.3 ± 1.2	49 (41)	**0.03**	0.09
	GT	2.6 ± 1.0	54 (45)		
	TT	3.3 ± 1.4	16 (14)		
CYP2B6 983T>C rs28399499	TT	2.5 ± 1.1	98 (81)	**0.003**	**0.008[a]**
	TC/CC	3.2 ± 1.3	23 (18)		
CYP2C19 rs4917623	TT	2.4 ± 0.9	74	**0.03**	**0.03[a]**
	TC/CC	2.9 ± 1.4	47		
CYP2C19 rs4388808	AA	2.4 ± 0.9	83	**0.05**	**0.05[a]**
	AG/GG	2.9 ± 1.5	38		

* *P* values adjusted for the number of haplotype blocks per gene: *ABCC10* = 4; *CYP2B6* = 3; *CYP2C19* = 1.
[a] Variables that have significant adjusted *p* values

CYP2B6 983T>C (adjusted *p* = 0.008, Figure 3.) and *ABCC10* rs2125739 (adjusted *p* = 0.009, Figure 3.4) genotype were associated with higher NVP C_{min} values in individuals carrying the variant allele for either polymorphism. *CYP2B6 516G>T* (adjusted *p* = 0.09, Figure 3.5) genotype did not meet statistical significance when corrected for the number of haplotype blocks in the gene but did show a trend toward

higher NVP C_{min} values. Two polymorphisms in *CYP2C19* (rs4917623, $p = 0.03$; rs4388808, $p = 0.05$) were also associated with higher NVP C_{min} values (Figure 3.6, Figure 3.7).

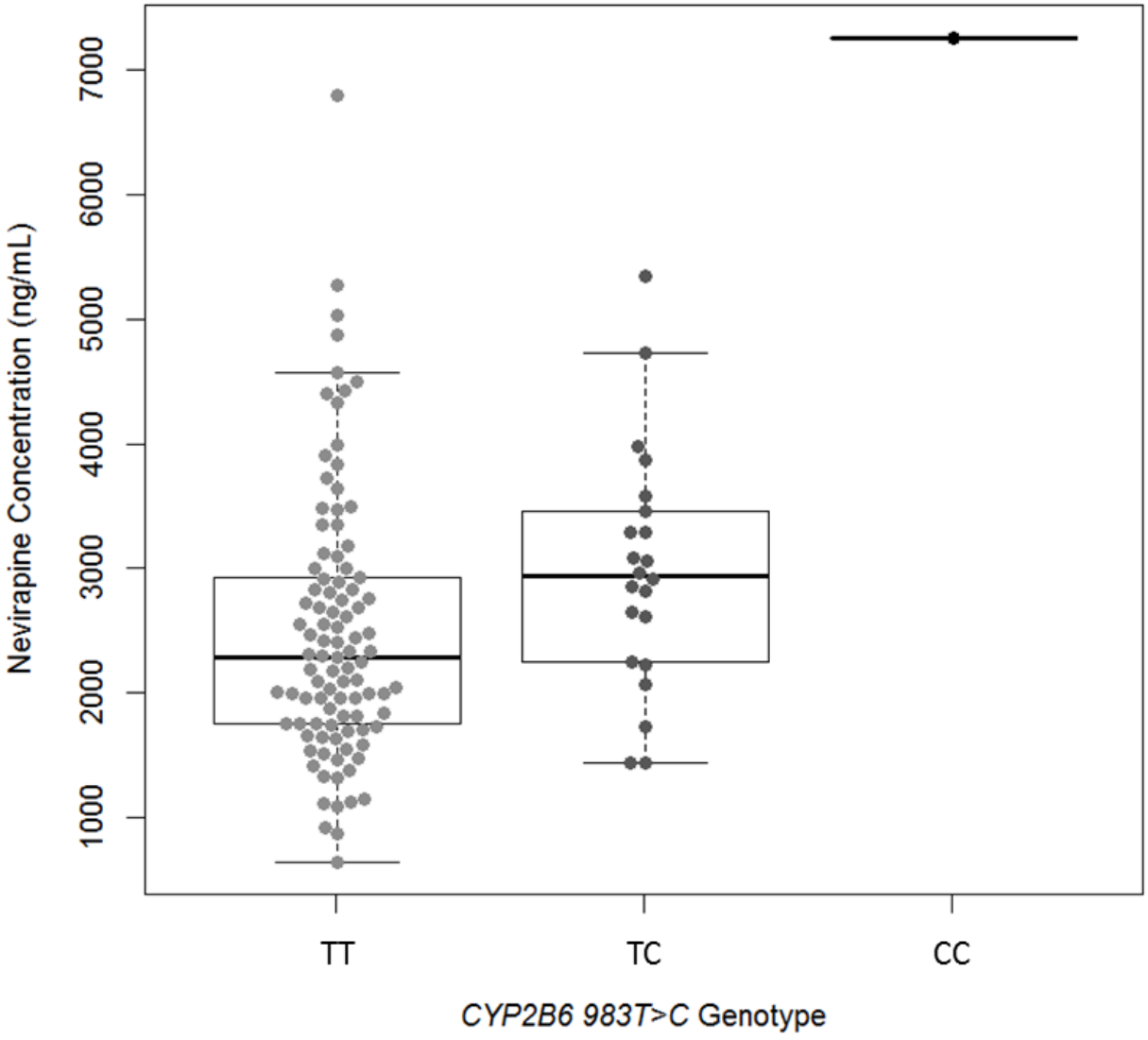

Figure 3.3. Nevirapine C_{min} as a function of *CYP2B6* 983T>C genotype.
Concentrations (mean ± SEM) are stratified by *CYP2B6* 983T>C genotype (adjusted $p = 0.008$).

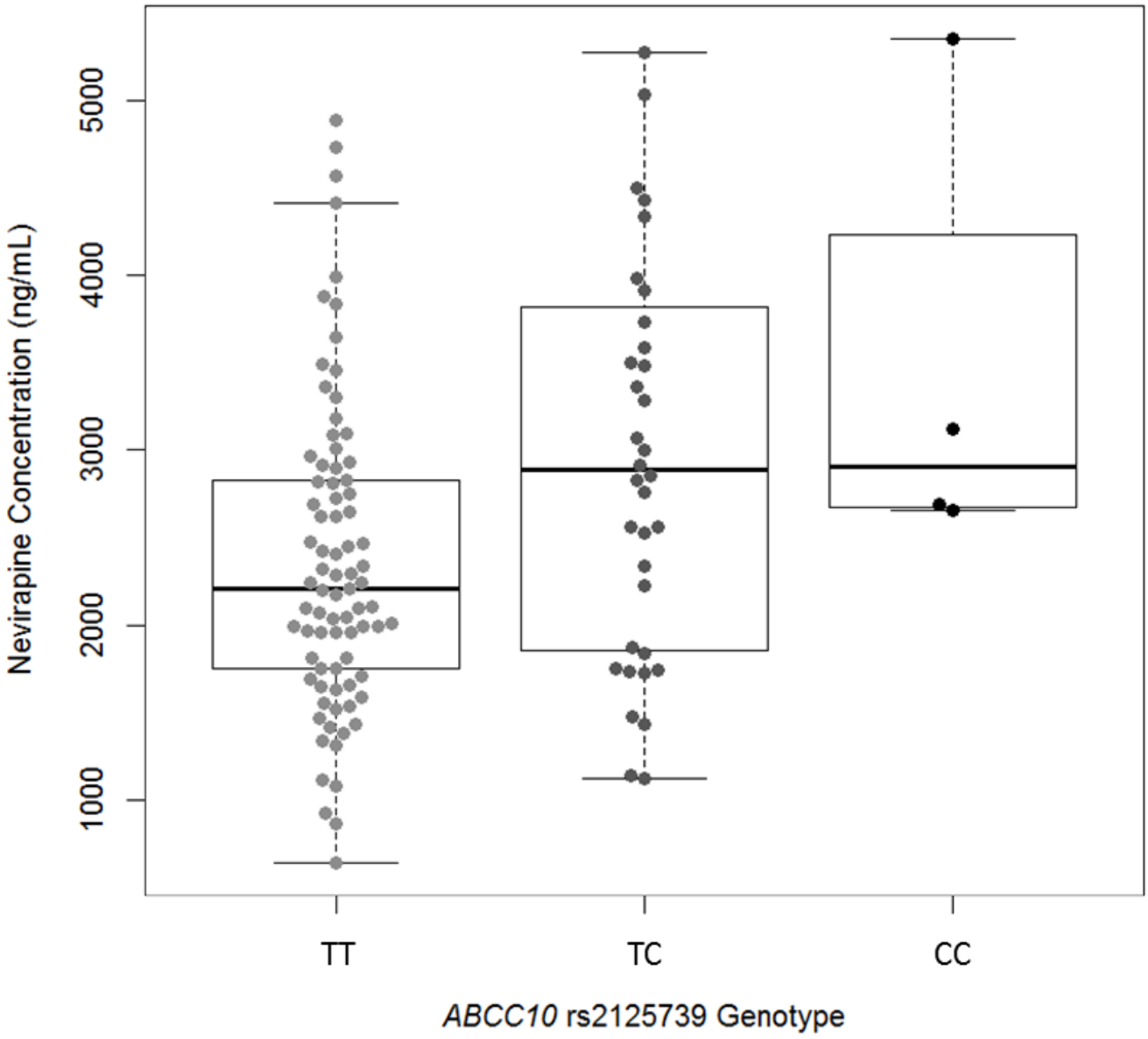

Figure 3.4. Nevirapine C_{min} as a function of *ABCC10* rs2125739.
Concentrations (mean ± SEM) are stratified by *ABCC10* rs2125739 genotype (adjusted p = 0.009).

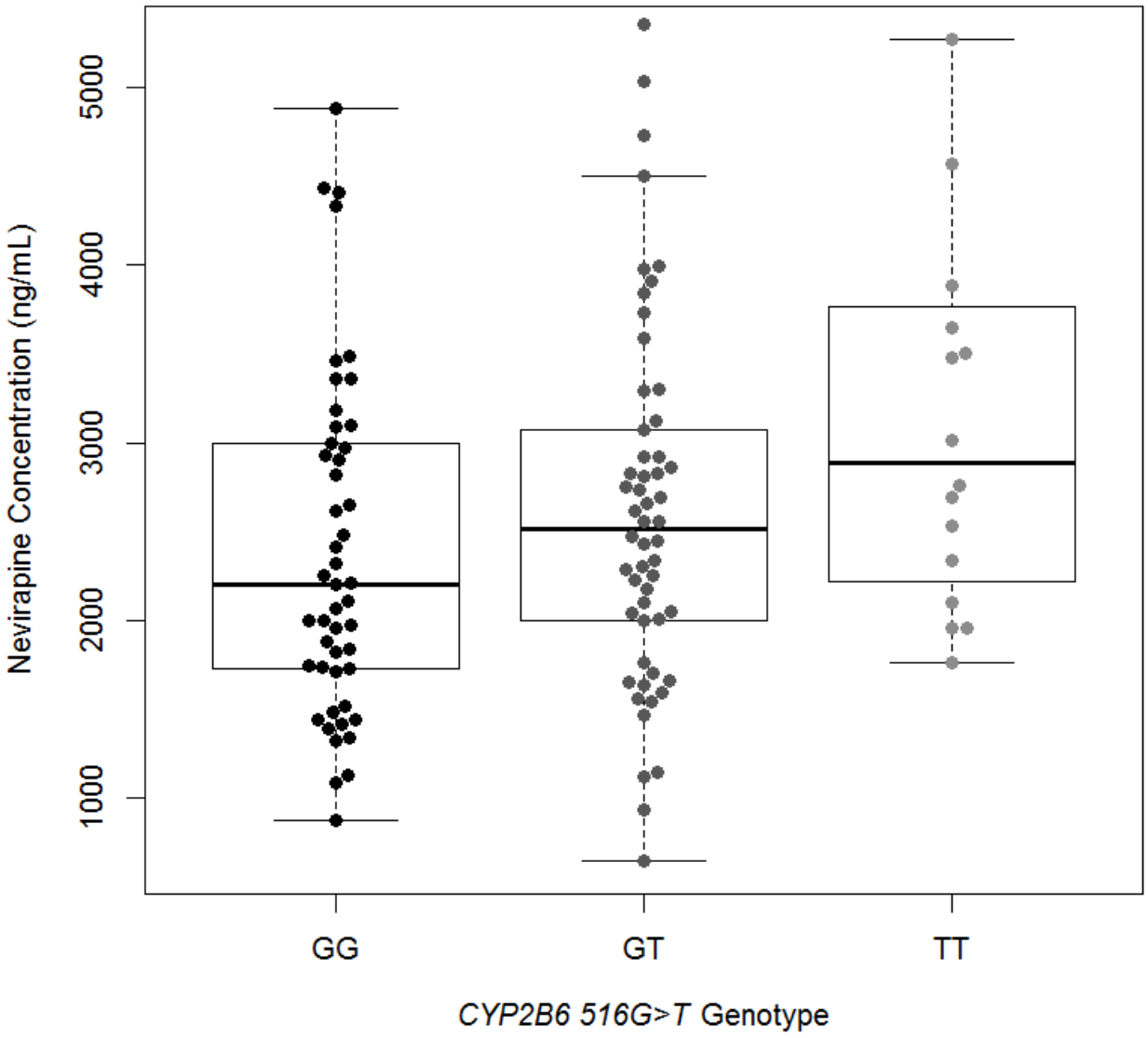

Figure 3.5. Nevirapine C_{min} as a function of *CYP2B6* 516G>T genotype.
Concentrations (mean ± SEM) are stratified by *CYP2B6* 516G>T genotype (adjusted p = 0.08).

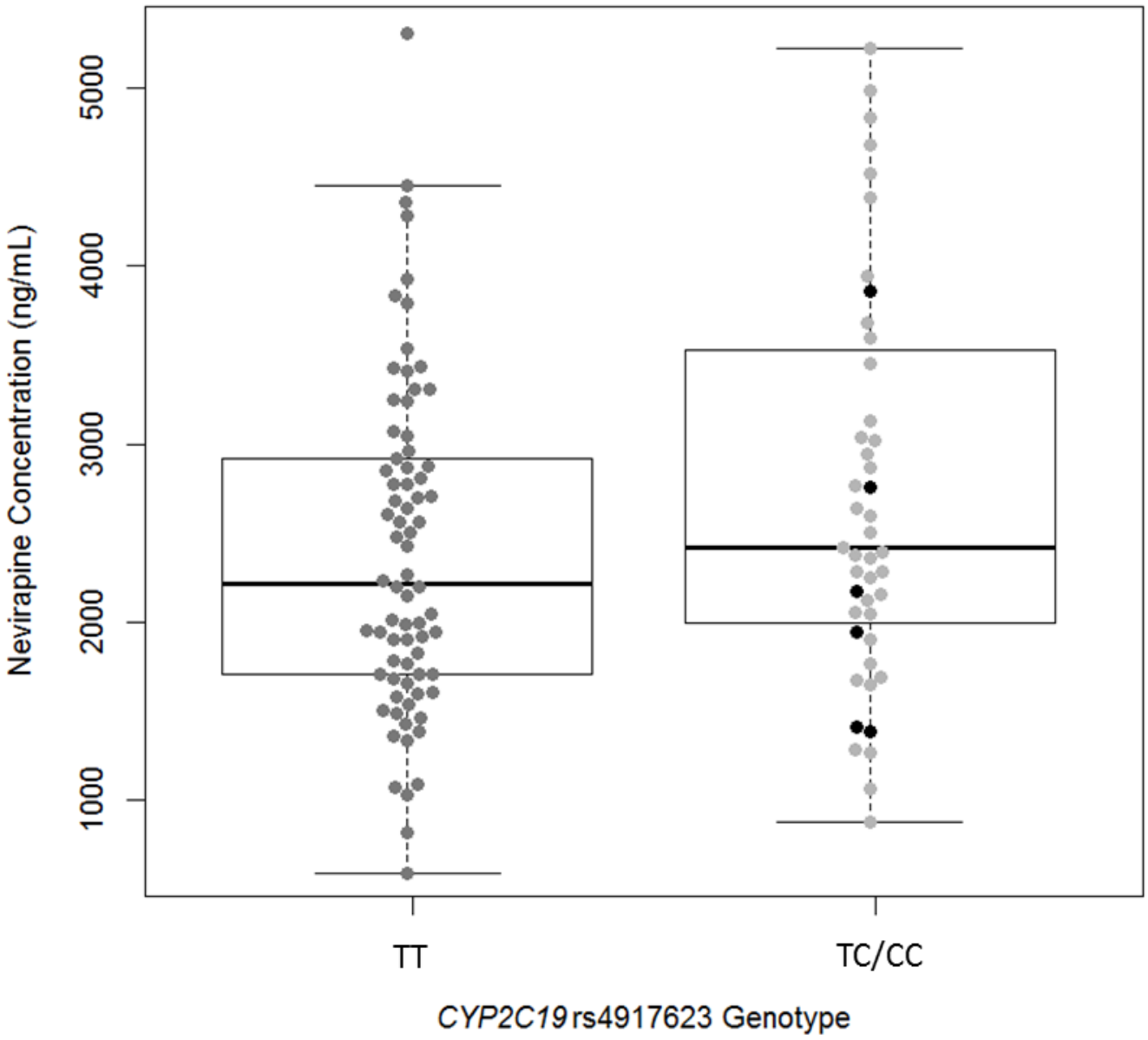

Figure 3.6. Nevirapine C_{min} as a function of *CYP2C19* rs491623 genotype. Concentrations (mean ± SEM) are stratified by *CYP2C19* rs491623 genotype (adjusted p = 0.03). In the TC/TT genotype group grey circles = TC, black circles = CC.

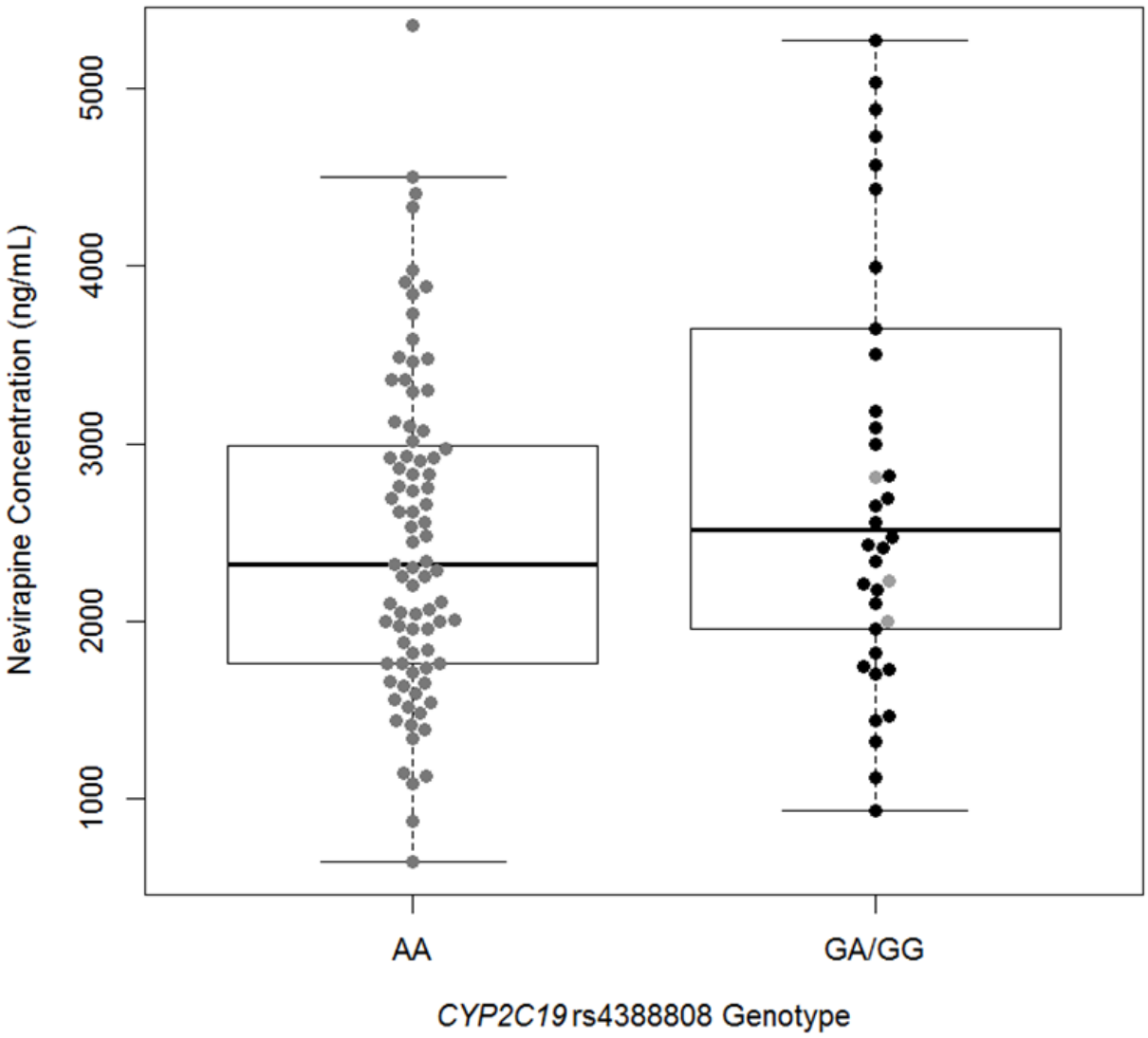

Figure 3.7. Nevirapine C_{min} as a function of *CYP2C19* rs4388808 genotype. Concentrations (mean ± SEM) are stratified by *CYP2C19* rs4388808 genotype (adjusted p = 0.05). In the GA/GG genotype group black circles = GA, grey circles = GG.

In the multivariate analysis, *CYP2B6* 983T>C (p = 0.007), *ABCC10* rs2125739 (p = 0.008) and age (p = 0.045) are significantly associated with NVP C_{min} values (Table 3.4).

Table 3.4. Multivariate analysis of the association of NVP C_{min} with Genotypes and Demographic Covariates

Independent Variable[1]	Beta	SE	Upper 95% CI	Lower 95% CI	*P*[2]
Age	0.002	0.002	0.006	-0.002	**0.045**
Gender (M)	-0.03	0.04	0.049	-0.109	0.116
CYP2B6 983T>C (CT/CC)	0.09	0.04	0.169	0.011	**0.007**
ABCC10 rs2125739 (CT/CC)	0.09	0.03	0.149	0.031	**0.008**
CYP2C19 rs4917623 (CT/CC)	0.05	0.06	0.169	-0.069	0.178
CYP2C19 rs4388808 (GA/GG)	-0.0009	0.06	0.118	-0.120	0.998

[1]Beta and SE values are reported for each factor tested against the control level. Level in parentheses is the level being tested against the control level.
[2]Significant values are highlighted bold

3.4.4 CYP2B6 and ABCC10 Composite Genotypes have Significant Effects on NVP C_{min}:

In order to investigate the combined effects of *CYP2B6* 516G>T and 983T>C, composite genotypes were constructed. There was a significant effect with subjects carrying more variant alleles having higher NVP C_{min} values (Figure 3.8, p = 0.0002). Due to the significant effects seen for SNPs in *CYP2B6* and *ABCC10*, a composite genotype that included SNPs from both genes was constructed. This *CYP2B6/ABCC10* composite genotype was highly correlated with NVP C_{min} levels (p = 2.5×10^{-6}; Figure 3.9).

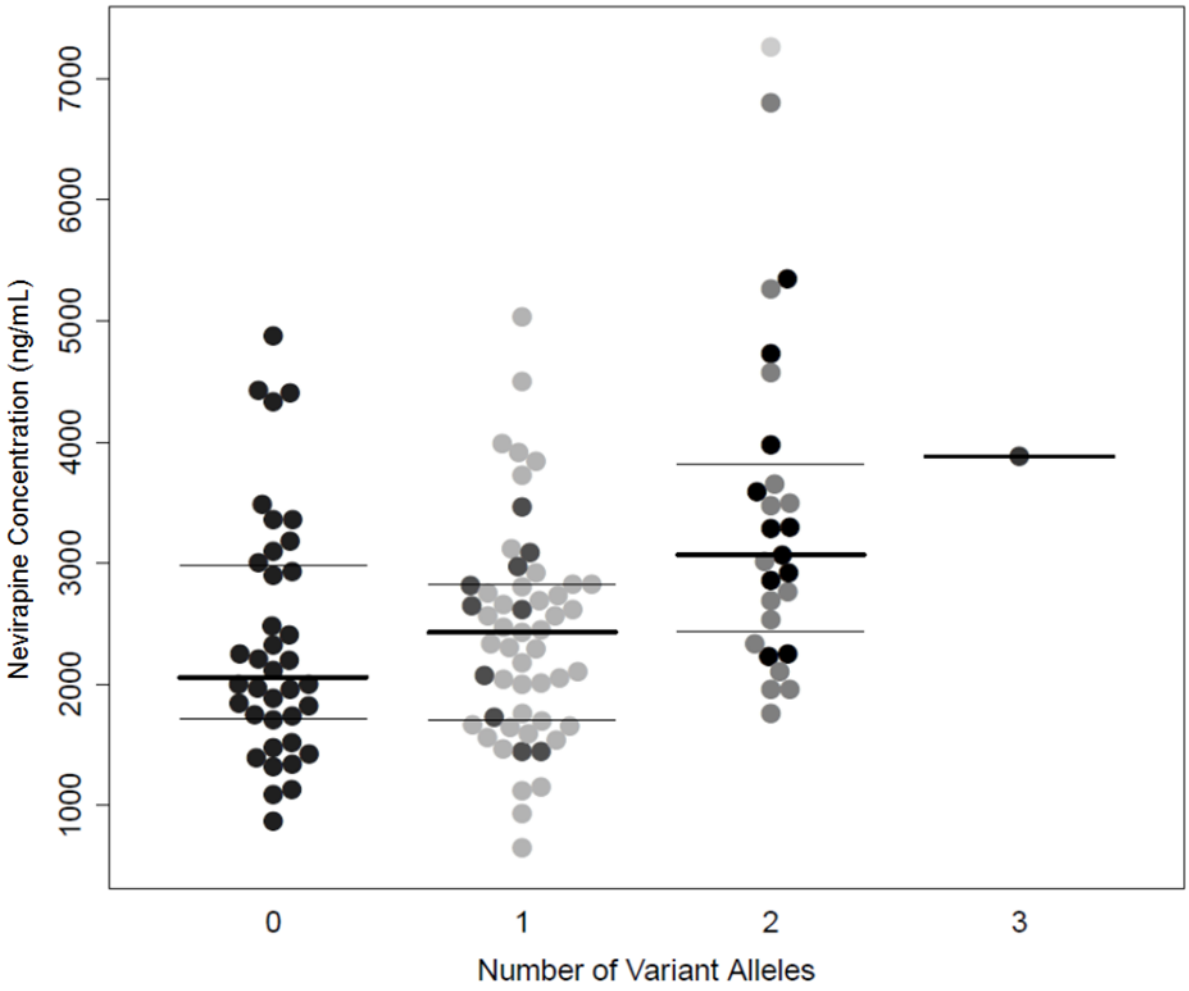

Figure 3.8. Nevirapine C_{min} as a function of *CYP2B6* composite genotype.
Nevirapine C_{min} stratified by the number of variant alleles present in *CYP2B6 516G>T* and *CYP2B6 983T>C* genotypes (p = 0.0002). Dark bars indicate the mean and light bars indicate standard deviations. All subjects in the "0" group (n = 38) have GG/TT genotypes. In the "1" group (n = 53) light grey subjects are GT/TT, dark grey are GG/TC. In the "2" group (n = 27) light grey subjects are GG/CC, medium grey are TT/TT and black are GT/TC. The "3" group (n = 1) is TT/TC.

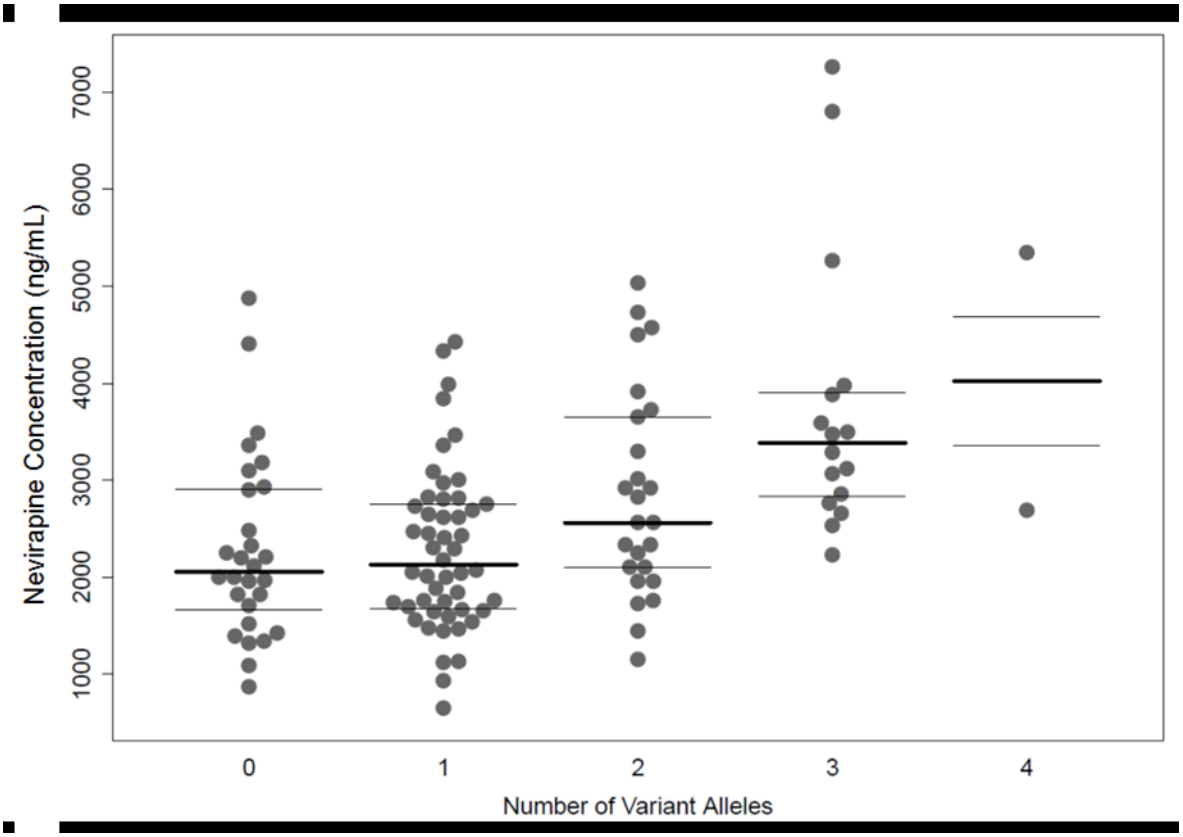

Figure 3.9. Nevirapine C_{min} as a function of *CYP2B6/ABCC10* composite genotype. Nevirapine C_{min} stratified by the number of variant alleles present in *CYP2B6 516G>T*, *CYP2B6 983T>C* and *ABCC10* rs2125739 genotypes ($p = 2.5x10^{-6}$). Dark bars indicate the mean and light bars indicate standard deviations. 0 alleles, n = 27; 1 allele, n = 49; 2 alleles, n = 25; 3 alleles, n = 16; 4 alleles, n = 2.

3.5 Discussion:

There have been many studies that show that *CYP2B6* polymorphisms are an important predictor of NNRTI pharmacokinetics. However, the majority of studies have focused on EFV, are conducted in Caucasian populations and polymorphisms are studied for their independent effects. This study explored the association of SNPs in established candidate genes and identified several for further analysis. Consistent with other studies, the importance of *CYP2B6* in NVP pharmacokinetics was confirmed[9,10,14,21,28]. The importance of *ABCC10* polymorphisms on NVP pharmacokinetics in African populations, which has only recently been demonstrated in

Caucasians, was also described[16]. Interestingly, the importance of the combined effects of polymorphisms in *CYP2B6* and *ABCC10* on NVP pharmacokinetics was also evident; composite phenotypes have previously only been explored for *CYP2B6*[21,29].

Soon after the approval of NVP, unexplained variation in its pharmacokinetic parameters not attributable to environmental factors was observed[30]. Much of this variation has been explained by *CYP2B6* polymorphisms, notably *CYP2B6* 516G>T and *CYP2B6* 983T>C[5,8,10,14,29,31]. This is only the second study to demonstrate the effect of a composite *CYP2B6* 516/983 genotype on NVP pharmacokinetics, which underlines the importance of the combined effects of both alleles for NVP pharmacokinetics[21]. While other studies have investigated the effect of *CYP2B6* 516/983 genotypes on NVP pharmacokinetics and did not observe an effect, this may have been due to the lack of *CYP2B6* autoinduction caused by extended NVP treatment, as the populations they were studying had only received a single dose of NVP[13,29].

This study is the first to demonstrate the effect of *ABCC10* rs2125739 on NVP plasma concentrations in an African population. A previous study showed that NVP is a substrate for MRP7 and found an association between rs2125739 and NVP pharmacokinetics in Caucasians, but not in Africans[16]. It is possible that the current study was better powered to observe an effect since the sample was larger than the previous study. ABCC10 is expressed higher in the kidney than in the liver and MRP7 may efflux NVP from the kidney into the urine. In such a case, reducing function of this transporter could result in reduced renal clearance and higher plasma concentrations of NVP[32]. This study confirms the importance of MRP7 in controlling NVP plasma

concentrations and suggests that it is worthwhile to further investigate its influence on NVP pharmacokinetics and efficacy.

The most interesting finding in this study is the substantial combined effect of *CYP2B6/ABCC10* genotype on NVP plasma concentrations. The number of variant alleles in both genes contributed significantly to a rise in NVP plasma concentrations. This could be due to a combination of reduced metabolism and decreased efflux of NVP into the urine. This finding could lead to more precise dosing guidelines for NVP which would increase efficacy, decrease viral resistance and decrease toxicity. The effect of multiple genotypes in several genes along with demographic parameters has successfully been employed to design the dosing regimen for other drugs, most notably warfarin[33].

This study did not find any associations between NVP C_{min} and *CYP2C19*, *CYP2C9*, *NR1L2*, *CYP2D6*, *CYP3A4* and *CYP3A5*[12,13,22]. These genes were selected on the basis of their role in NVP pharmacokinetics. Only the *CYP3A5**3 variant has previously been associated with a reduction in NVP AUC[34]. No effect of this allele was observed in this study, however, only NVP C_{min} was evaluated.

3.6 Conclusions

In summary, this study found that the combined *CYP2B6/ABCC10* genotype has a significant effect on NVP C_{min} concentrations in Ugandan patients receiving antiretroviral treatment. Due to the high rates of HIV infection in sub-Saharan Africa and the widespread use of NVP in that region, this information could be used to tailor dosing in patients which would lead to increases in efficacy and decreases in viral resistance and toxicity.

3.7 References

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Chapter 4

Genetic Predictors of HIV-1 Induced Peripheral Neuropathy in Ugandan HIV-1+ Subjects

4.1 Abstract

Sensory peripheral neuropathy is one of the most common neurological complications associated with HIV infection. HIV related sensory neuropathy (HIV-SN) is common in the developing world due to complications in the delivery of timely antiretroviral therapy. The objective of our study is to identify genetic predictors of HIV-SN in a treatment naive Ugandan HIV+ population. DNA and symptom data were collected from 638 patients enrolled in a cohort study in Uganda to examine treatment outcomes in HIV+ subjects. Patients were treatment naïve at enrollment and an extensive symptom questionnaire was completed to capture HIV-SN symptoms. Genotyping was performed using the Illumina OmniExpress® platform with 681,315 SNPs and 580 samples passing quality control. Imputation with IMPUTE2 added >16 million SNPs. HIV-SN was scored on a Likert scale and recorded prior to the initiation of antiretroviral therapy. An additive genetic model and logistic regression were used to identify genes associated with HIV-SN. Replication was performed in a new set of Ugandan subjects for selected SNPs using Taqman genotyping assays. The genome-wide analyses identified multiple SNPs associated with HIV-induced PN, including several SNPs proximal to *FOLH1* (top SNP: rs2007068, $p = 2.63 \times 10^{-7}$), a protein associated with folate metabolism. Replication studies and meta-analyses were performed on three SNPs that had putative biological or functional effects on *FOLH1*. One SNP (rs7925419) showed a statistical trend ($p = 0.12$) in the replication study for

association with HIV-SN development. This study suggests that genetic variation in *FOLH1*, a gene important to folate metabolism, may influence an individual patient's risk of developing HIV-SN. Further studies are warranted to determine the precise role that *FOLH1* may play in the development of HIV-SN.

4.2 Introduction

Over 35 million people worldwide are currently living with HIV infections and 69% of these people live in sub-Saharan Africa[1]. The advent of Highly Active Antiretroviral Therapy (HAART) has greatly improved patient mortality but the developing world still struggles to treat HIV patients in a timely and effective manner[1,2]. As a consequence, many HIV+ patients in sub-Saharan Africa experience greater morbidity and mortality than HIV+ patients in the developed world[3].

HIV related sensory neuropathies (HIV-SN) are a common neurological complication of HIV infection[4]. Reports from the pre-HAART era indicate that up to 35% of HIV infected patients will develop HIV-SN[5-7]. There are several proposed mechanisms for the development of HIV-SN, the primary being macrophage invasion of the peripheral nerve and viral protein toxicity[4]. Although the mechanism of HIV-SN is still not fully understood, risk factors including nutritional deficiencies, alcoholism, age, disease stage, low CD4+ T-cell counts and high viral load have been extensively documented[8,9]. Because antiretroviral drugs, specifically nucleoside reverse transcriptase inhibitors (NRTIs), may also cause sensory neuropathies that are clinically indistinguishable from HIV-SN, it is difficult to ascertain whether patients receiving HAART develop sensory neuropathies due to HIV infection or drug toxicity[9]. There have been several candidate gene studies to characterize the effect of specific genes on the development of peripheral neuropathy due to NRTI toxicity, however to date no genetic studies have been conducted to characterize the role of host genetics on HIV-SN[10-13].

Genome-wide association studies (GWAS) have identified the role of patient genetics for many complex diseases, including HIV infection, and have emphasized the

importance of host genetics on HIV infection and progression[14,15]. However, the role of host genetics in the development of HIV-SN is still unknown.

The goal of this GWAS study was to identify genetic predictors of HIV-SN in a treatment naïve HIV+ Ugandan population. Replication and bioinformatics analyses were used to further define genomic regions likely to influence the HIV-SN phenotype.

4.3 Materials and Methods:

4.3.1 Participants

Patients were recruited from the Uganda AIDS Rural Treatment Outcomes (UARTO) and Antiretrovirals in Kaposi Sarcoma (ARKS) cohorts. Study participants were treatment naïve HIV+ patients living in Mbarra, Uganda and Kampala, Uganda, respectively. Patients enrolled in the study received a treatment regimen consisting of two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) or one protease inhibitor (PI). Whole blood or saliva samples were obtained at enrollment and shipped to the University of California San Francisco for DNA isolation. Study visits were conducted upon enrollment and every three months thereafter and consist of an extensive symptom interview, CD4+ T-cell counts and viral load measurements. Genotype and phenotype data were collected on 638 patients for the initial study and 209 patients for the replication study.

4.3.2 Genotyping

In the discovery study, genomic DNA from 638 subjects was extracted from either saliva or whole blood samples using standard DNA extraction techniques. DNA concentrations were determined by Quant-iT™ PicoGreen® dsDNA Assays (Life Technologies, Grand Island, NY) performed according to the manufacturer's instructions and samples were normalized in Tris-EDTA buffer to a concentration of 50 ng/µl and stored at -80°C. Genotyping was performed on the HumanOmniExpress BeadChip (Illumina, San Diego, CA), which interrogated 733,202 SNPs. Subjects with genotyping call rates < 95% (n = 19) were excluded from further analysis and the remaining genotypes were reclustered using BeadStudio data analysis software (Illumina, San Diego, CA). Gender was determined from the genotype data using PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/) and compared to the database recorded sex to ensure concordance[16]. Eleven samples did not have concordant genders and were excluded from further analysis. As closely related individuals may confound downstream statistical analyses, Identify by Descent (IBD) was determined using PLINK [16]. Twenty-five individuals were found to be greater than 12.5% related and were investigated to determine the source of the relatedness. In the instances where it was possible to establish legitimate relatedness, the subject with better quality genotyping was retained for further analysis, otherwise the samples were excluded from further analysis. In total, 15 subjects were excluded due to relatedness issues. To further ensure sample quality, genomic heterozygosity was evaluated using PLINK and two subjects were excluded due to heterozygosity issues[16]. Six subjects were excluded from downstream analyses due to study related issues such as

withdrawal from the study. A total of 585 subjects passed genotyping and subject quality control procedures. A summary of quality control procedures for samples and single nucleotide polymorphisms (SNPs) can be viewed in Table 4.1.

Table 4.1. Quality Control of Genotype and Subject Data

Parameter	Potential Cause	# Excluded	# Remaining
Pre QC Dataset	-	0	645
Genotyping Call Rate (>95%)	Poor quality DNA	19	626
Sex Concordance	Database error/sample mix-up	11	615
Relatedness (IBD) (<12.5% related)	Subjects are related/Poor quality DNA	15	600
Heterozygosity	Poor quality DNA	2	598
Population Stratification	Defines ethnicity/Used to correct for differences in ethnicities	0	598
Study Related Issues	*e.g.,* Subject withdrew from study, inadequate longitudinal data	6	592
Genotyping Controls/Duplicate Sample	HAPMAP trios included to verify accuracy and precision of genotyping	7	585
Final Dataset	-		585

To ensure SNP quality, filters for SNPs with call rates of greater than 95% and greater than 1% minor allele frequency were applied using PLINK software[16]. Out of the 733,202 SNPs genotyped, 681,315 passed quality control procedures (Table 4.2).

Table 4.2. Quality Control of Genotype Data

Parameter	# SNPs Excluded	# SNPs Remaining
Pre-QC	0	733,202
Call rate > 95%	12,356	720,846
MAF ≥ 1%	39,531	681,315

To ensure that population stratification due to ethnicity did not introduce bias into downstream analyses, a principal components analysis comparing the study cohort with HAPMAP world populations was performed using Eigenstrat software[17,18]. All subjects clustered near the HAPMAP African population samples (Figure 4.1).

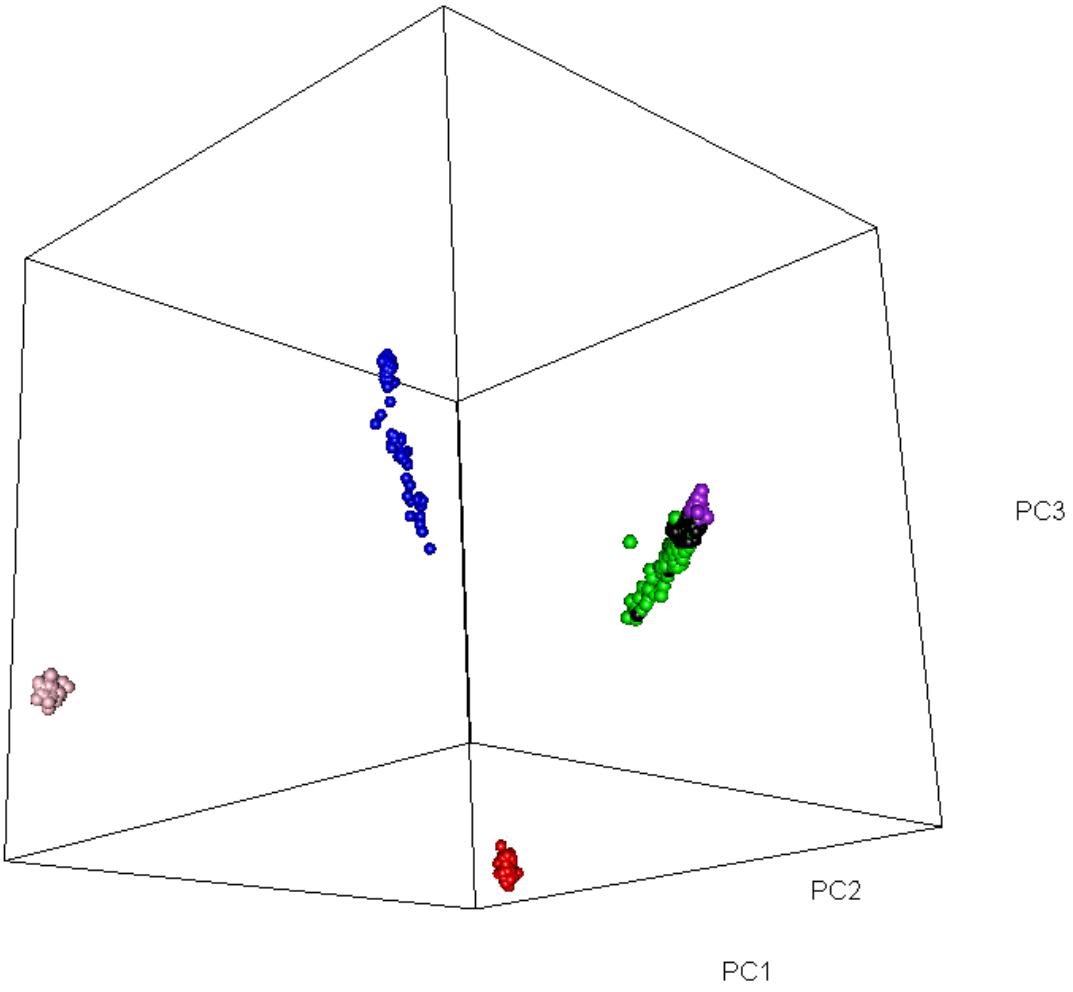

Figure 4.1. Principal component analysis of study samples compared to world HAPMAP populations.
Red circles are Utah residents (CEPH) with Northern and Western European ancestry (CEU), pink circles are Han Chinese in Bejing, China (CHB), blue circles are Gujarati Indian from Houston, Texas (GIH), purple circles are Yoruba in Ibadan, Nigera (YRI), black circles are ARKS and green are UARTO populations.

Genotyping for the replication study was performed according to the manufacturer's instructions using the SNP specific Taqman Genotyping assays (Life Technologies, Grand Island, NY) outlined in Table 4.3.

Table 4.3. SNPs Selected for Replication

SNP	Assay ID	Tag SNPs (r^2 >0.8)
rs7925419	Custom: AHLJXQZ	
rs2007068	Custom: AHI12Y6	rs9332434, rs7937386, rs2007090
rs11245616	Custom: AHKAZKR	rs11245609, rs12361625

Taqman assay IDs are listed. SNPs that are in LD (r^2 >0.8) in the LWK 1000 Genomes database are also listed.

Imputation was performed using SHAPEIT (http://www.shapeit.fr/) and IMPUTE2 (http://mathgen.stats.ox.ac.uk/impute/impute_v2.html) software. Prephasing of haplotypes was performed according to the developer's instructions for prephasing for imputation using SHAPEIT version 1[19]. Imputation was performed on samples and SNPs passing quality control steps in IMPUTE2 using as a reference panel the 1000 Genomes Phase I integrated variant set which includes 1,092 individuals from Africa, Asia, Europe, and the Americas[20]. Approximately 40 million SNPs were imputed which were subsequently filtered for quality (info score > 0.8), leaving 16.9 million SNPs for analysis[21]. Because this dataset is intended for further use in additional studies, filtering SNPs for MAF > 1% was performed after statistical analyses were performed.

4.3.3 Phenotype

Peripheral neuropathy data were gathered during the study visits as a component of the symptom questionnaire. Peripheral neuropathy is graded on a Likert-type scale with subjects asked if they are experiencing "pain, numbness or tingling in the hands or feet." A score of "0" denotes no symptoms, "1" means "bothers me not at all", "2" means

"bothers me a little", "3" denotes "bothers me a moderate amount" and "4" means "bothers me a lot". Subjects who reported no symptoms were assigned as "controls" (n = 342). Subjects who reported symptoms in the 3 and 4 categories were assigned as "cases" (n = 129).

4.3.4 Statistical Analyses

Statistical analyses to determine the effect of demographic covariates were performed in R using ANOVA for continuous variables and Chi-squared tests for categorical variables[22]. Standard case/control analyses using logistic regression were performed for the primary and replication analyses using PLINK to test the association between each SNP and the phenotype[16]. Odds ratios (OR), 95% confidence intervals (95% CI) and *p*-values were generated for each SNP. To account for the multiple testing burden genome-wide significance was considered $p \leq 5 \times 10^{-8}$ and genome-wide suggestive was considered $p \leq 5 \times 10^{-7}$; 'promising' SNPs at $p \leq 5 \times 10^{-6}$ were considered for further bioinformatic analysis[23]. Allele frequencies in the control group for SNPs of interest were examined to ensure they were in Hardy-Weinberg Equilibrium using a Chi-squared test. Linkage disequilibrium calculations were performed in PLINK[16]. Plots were produced in R and Microsoft Excel 2010[22]. Meta analyses to combine *p*-values from the discovery and replication studies were performed in R using the meta package[24].

4.3.5 Bioinformatic Analyses

To explore the putative biological significance of SNPs that had 'promising' *p*-values ($p \leq 5 \times 10^{-6}$), these SNPs were selected for further bioinformatic analysis. SNPs were

annotated to genes using SNPnexus and the UCSC genome browser[25,26]. GTEx (http://www.broadinstitute.org/gtex/) and GeneVar (http://www.sanger.ac.uk/resources/software/genevar/) databases were employed to examine the effect of a SNP on gene expression[27]. To determine regulatory functions of SNPs of interest, Haploreg and ENCODE databases were employed[28,29]. SNPs with 'promising' *p*-values that also had putative biological function or regulatory consequences were chosen for replication.

4.4 Results

4.4.1 Demographic Data

The demographic characteristics of the discovery and replication cohorts are described in Table 4.4. The two cohorts were similar in all demographic variables assessed. None of the demographic variables tested had an effect on case/control status with the exception of gender in the discovery cohort ($p < 0.001$). However, this effect was not seen in the replication cohort ($p = 0.8$). The case percentages in the initial and replication cohorts were 27% and 23%, respectively.

Table 4.4. Patient Demographic Data in the Discovery and Replication Cohorts

	Discovery	**Replication**
Sample size, n	471	157
Age, years, median ± SD	34 ± 8.53	33 ± 9.25
Gender, n (%M)	177 (38%)	66 (42%)
Baseline CD4+ T-cell counts, cells/mm^3, median ± SD	133 ± 117	200 ± 204
Baseline Viral Load, log(copies/mL), median ± SD	5.20 ± 0.68	5.07 ± 0.67
Case, n (%)	129 (27%)	36 (23%)
Cohort, n (%ARKS)	139 (30%)	45 (35%)

4.4.2 Loci in Chromosome 11 are associated with HIV induced peripheral neuropathy

After quality control procedures, 16.9 million imputed and genotyped SNPs were tested for association with HIV-SN using a standard case/control testing methodology. Figure depicts the *p*-value for each SNP plotted at their position in the genome. The Q-Q plot and genomic inflation factor, λ (1.04, Figure 4.3), indicate that there is no significant population stratification.

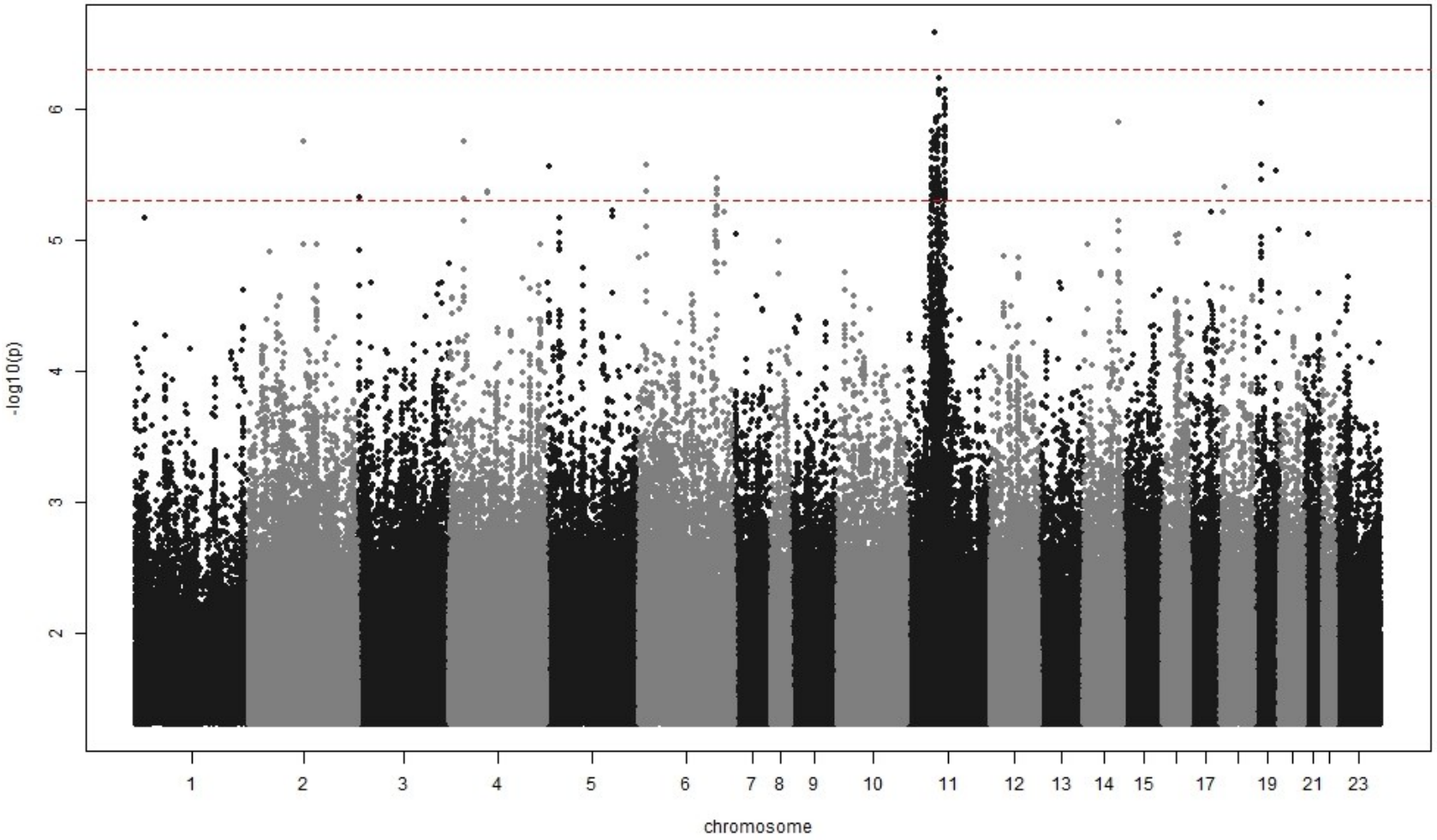

Figure 4.2. Manhattan plot showing the distribution along the human autosomes of -log10 (*p* values) obtained for SNP association with HIV-SN cases versus control subjects. The lower red line ($p < 5 \times 10^{-6}$) marks the cutoff for 'promising' SNPs considered for further bioinformatics analysis. The upper red line marks the genome-wide suggestive cutoff of $p < 5 \times 10^{-7}$.

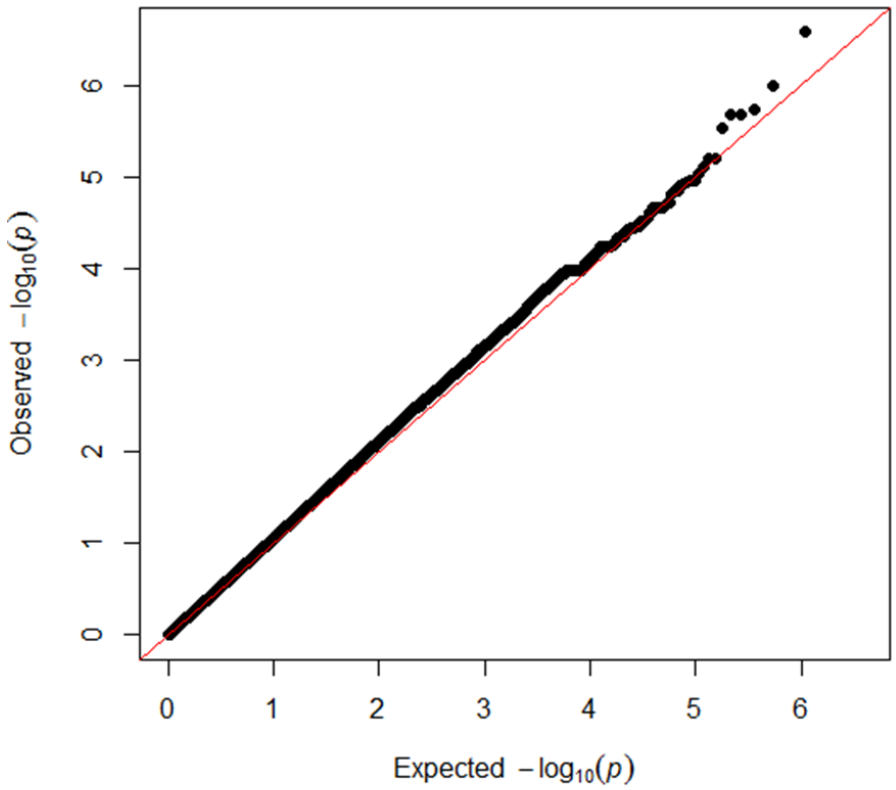

Figure 4.3. Observed versus expected *p*-values (-log base 10 scale) for SNP association with HIV-SN cases versus control subjects. Red line indicates the null distribution. The genomic inflation factor λ was 1.04 and was calculated using R statistical computing software.

Only 142 SNPs had *p*-values < 5 x 10^{-6}. SNPs with the greatest association to HIV-SN were filtered for linkage disequilibruim (r^2 > 0.8) with the SNP with the lowest *p*-value for each LD block being retained (Bold SNPs, Table 4.5). The SNP with the most significant *p*-value was on chromosome 11 (rs2007068, *p* = 2.63 x 10^{-7}, OR = 2.69, 95% CI 3.90 - 1.85). A robust peak can be seen at chromosome 11 with sporadic signals from other regions of the genome.

Table 4.5. Top Variants Associated with HIV Induced Peripheral Neuropathy

Chr	SNP	Frq	OR	U95CI	L95CI	*P*	Gene	Feature	Left Gene	Right Gene
11	**rs2007068**	**0.15**	**2.69**	**3.90**	**1.85**	**$2.63x10^{-7}$**	**-**	**Intergenic**	***OR4C12***	***OR4A5***
11	**rs12288743**	**0.23**	**2.41**	**3.43**	**1.69**	**$5.88x10^{-7}$**	**-**	**Intergenic**	***OR4C12***	***OR4A5***
11	rs8189012	0.43	2.20	3.01	1.61	$7.21x10^{-7}$	-	Intergenic	*OR4C12*	*OR4A5*
11	**rs2512730**	**0.30**	**2.13**	**2.86**	**1.59**	**$7.24x10^{-7}$**	***OR5W2***	**5' upstream**	***OR5W2***	***OR5I1***
11	rs146970082	0.28	2.29	3.20	1.64	$7.62x10^{-7}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs11246460	0.27	2.29	3.20	1.64	$7.72x10^{-7}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4939007	0.33	2.15	2.94	1.57	$8.42x10^{-7}$	-	Intergenic	*OR5D16*	*TRIM51*
19	**rs10403857**	**0.33**	**0.39**	**0.57**	**0.27**	**$9.24x10^{-7}$**	***OR1I1***	**5' upstream**	***CASP14***	***OR1I1***
11	rs2457232	0.30	2.11	2.83	1.57	$9.40x10^{-7}$	-	Intergenic	*OR5W2*	*OR5I1*
11	rs10895994	0.19	2.41	3.43	1.69	$9.53x10^{-7}$	-	Intergenic	*OR5BE1P*	*OR8I2*
11	rs4939008	0.30	2.11	2.83	1.57	$1.01x10^{-6}$	-	Intergenic	*OR5W2*	*OR5I1*
11	rs2449144	0.19	2.36	3.36	1.66	$1.11x10^{-6}$	-	Intergenic	*OR5F1*	*OR5F2P*
11	rs4261267	0.37	2.10	2.82	1.57	$1.14x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4963111	0.11	3.06	4.80	1.95	$1.19x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs11512987	0.11	3.05	4.79	1.94	$1.20x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
14	**rs75721606**	**0.16**	**2.70**	**4.07**	**1.79**	**$1.27x10^{-6}$**	***TC2N***	**Intronic**	***CATSPERB***	***FBLN5***
11	rs7294146	0.11	3.05	4.79	1.94	$1.28x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2512928	0.20	2.31	3.22	1.66	$1.39x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs2512925	0.20	2.30	3.21	1.65	$1.42x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs4598652	0.38	2.07	2.78	1.54	$1.47x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4423169	0.38	2.07	2.78	1.54	$1.47x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4268491	0.38	2.07	2.78	1.54	$1.47x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2512759	0.20	2.30	3.21	1.65	$1.47x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	11-55777327	0.20	2.33	3.32	1.64	$1.47x10^{-6}$	-	Intergenic	*OR5F1*	*OR5S1*
11	rs6485948	0.25	2.32	3.24	1.66	$1.48x10^{-6}$	-	Intergenic	*OR4A47*	*TRIM49B*
11	rs4363603	0.25	2.28	3.18	1.63	$1.50x10^{-6}$	-	Intergenic	*OR4A47*	*TRIM49B*
11	rs7118155	0.20	2.34	3.33	1.64	$1.50x10^{-6}$	-	Intergenic	*OR5J1P*	*OR8I2*
11	rs148900318	0.22	2.51	3.64	1.73	$1.59x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs8189038	0.38	2.05	2.75	1.53	$1.61x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2460196	0.20	2.30	3.21	1.65	$1.62x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs4451712	0.38	2.05	2.75	1.53	$1.63x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4256959	0.38	2.05	2.75	1.53	$1.63x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2512966	0.20	2.30	3.21	1.65	$1.63x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs8189236	0.37	2.04	2.74	1.52	$1.66x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	**rs11245616**	**0.12**	**2.92**	**4.49**	**1.90**	**$1.67x10^{-6}$**	**-**	**Intergenic**	***OR4C12***	***OR4A5***
11	rs2512740	0.20	2.29	3.20	1.64	$1.67x10^{-6}$	-	Intergenic	*OR5F2P*	*OR5AS1*
11	rs11518847	0.12	2.84	4.37	1.85	$1.71x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
4	**rs13148227**	**0.05**	**5.45**	**11.04**	**2.69**	**$1.78x10^{-6}$**	**-**	**Intergenic**	***SMIM20***	***RBPJ***
2	**rs4848126**	**0.31**	**2.35**	**3.34**	**1.65**	**$1.81x10^{-6}$**	***GLI2***	**Intronic**	***FLJ14816***	***TFCP2L1***

Chr	SNP	Frq	OR	U95Cl	L95Cl	*P*	Gene	Feature	Left Gene	Right Gene
11	**rs7925419**	**0.14**	**2.67**	**4.03**	**1.77**	**$1.82x10^{-6}$**	***FOLH1***	**5' upstream**	***FOLH1***	***OR4C13***
11	rs10902288	0.37	2.07	2.78	1.54	$1.84x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2851533	0.26	2.23	3.11	1.60	$1.84x10^{-6}$	-	Intergenic	*TRIM64C*	*FOLH1*
11	rs1600823	0.20	2.30	3.21	1.65	$1.89x10^{-6}$	-	Intergenic	*OR5F1*	*OR5F2P*
11	rs4080494	0.12	2.79	4.29	1.81	$1.91x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs11246421	0.37	2.05	2.75	1.53	$1.91x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs8189029	0.37	2.04	2.74	1.52	$1.92x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4515955	0.37	2.04	2.74	1.52	$1.92x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4350349	0.37	2.04	2.74	1.52	$1.92x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2449126	0.20	2.30	3.27	1.62	$1.92x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs12361625	0.12	2.87	4.42	1.86	$1.94x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs11245609	0.12	2.87	4.42	1.86	$1.94x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs7952311	0.28	2.12	2.90	1.55	$1.96x10^{-6}$	-	Intergenic	*OR5I1*	*OR10AF1P*
11	rs12360596	0.37	2.04	2.74	1.52	$1.99x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs10902283	0.37	2.04	2.74	1.52	$1.99x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4881692	0.12	2.87	4.42	1.86	$2.02x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2512941	0.20	2.25	3.14	1.61	$2.04x10^{-6}$	*OR5F1*	3' downstream	*OR7E5P*	*OR5F1*
11	rs2460207	0.20	2.29	3.20	1.64	$2.09x10^{-6}$	*OR5F1*	3' downstream	*OR7E5P*	*OR5F1*
11	rs2512942	0.20	2.27	3.17	1.63	$2.10x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs2460195	0.20	2.28	3.18	1.63	$2.10x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs1164685	0.20	2.41	3.50	1.66	$2.12x10^{-6}$	-	Intergenic	*FOLH1*	*OR4C13*
11	rs2449124	0.20	2.27	3.17	1.63	$2.13x10^{-6}$	-	Intergenic	*OR5AS1*	*OR5AQ1P*
11	rs6591816	0.20	2.28	3.18	1.63	$2.14x10^{-6}$	-	Intergenic	*OR10AG1*	*OR7E5P*
11	rs6591812	0.20	2.28	3.18	1.63	$2.14x10^{-6}$	-	Intergenic	*OR10AG1*	*OR7E5P*
11	11-50652459	0.30	2.18	2.98	1.59	$2.14x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs7939844	0.28	2.11	2.89	1.54	$2.17x10^{-6}$	-	Intergenic	*OR5I1*	*OR10AF1P*
11	rs4103567	0.13	2.66	4.01	1.76	$2.40x10^{-6}$	-	Intergenic	*FOLH1*	*OR4C13*
11	rs2460204	0.20	2.27	3.17	1.63	$2.43x10^{-6}$	-	Intergenic	*OR7E5P*	*OR5F1*
11	rs7294221	0.42	2.09	2.86	1.53	$2.44x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs10902013	0.07	4.22	7.75	2.30	$2.46x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs7927383	0.34	2.02	2.71	1.51	$2.58x10^{-6}$	-	Intergenic	*OR5I1*	*OR10AF1P*
11	rs10895998	0.20	2.26	3.15	1.62	$2.59x10^{-6}$	*OR8I2*	3' downstream	*OR8I2*	*OR8I4P*
19	rs7249208	0.33	0.41	0.59	0.28	$2.67x10^{-6}$	-	Intergenic	*CASP14*	*OR1I1*
6	**rs686070**	**0.11**	**2.84**	**4.37**	**1.85**	**$2.69x10^{-6}$**	**-**	**Intergenic**	***OFCC1***	***TFAP2A***
11	rs59591431	0.30	2.05	2.75	1.53	$2.69x10^{-6}$	-	Intergenic	*OR5D16*	*OR9M1P*
11	**rs11231253**	**0.30**	**2.05**	**2.75**	**1.53**	**$2.72x10^{-6}$**	***OR5D16***	**Synonymous**	***OR5L2***	***OR9M1P***
5	**rs11134288**	**0.47**	**2.16**	**2.96**	**1.58**	**$2.80x10^{-6}$**	**-**	**Intergenic**	***MTRR***	***SEMA5A***
11	rs1164666	0.20	2.37	3.37	1.67	$2.87x10^{-6}$	-	Intergenic	*FOLH1*	*OR4C13*
11	rs8188994	0.33	2.08	2.85	1.52	$2.88x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
19	**rs6966**	**0.34**	**2.08**	**2.85**	**1.52**	**$2.97x10^{-6}$**	***PPP1R13L***	**3' UTR**	***ERCC2***	***CD3EAP***
11	rs8189086	0.44	2.13	2.91	1.56	$2.97x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*

Chr	SNP	Frq	OR	U95CI	L95CI	*P*	Gene	Feature	Left Gene	Right Gene
11	rs10839243	0.30	2.31	3.29	1.62	$3.01x10^{-6}$	-	Intergenic	*FOLH1*	*OR4C13*
11	rs4556555	0.20	2.29	3.26	1.61	$3.11x10^{-6}$	-	Intergenic	*OR5D16*	*TRIM51*
11	rs7294283	0.40	2.02	2.71	1.51	$3.16x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs76392625	0.40	2.03	2.72	1.51	$3.21x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs10902312	0.45	2.10	2.87	1.53	$3.30x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs10839211	0.21	2.41	3.50	1.66	$3.31x10^{-6}$	-	Intergenic	*OR4A47*	*TRIM49B*
11	rs7942630	0.21	2.35	3.34	1.65	$3.32x10^{-6}$	-	Intergenic	*FOLH1*	*OR4C13*
11	11-50207195	0.23	2.26	3.22	1.59	$3.41x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
6	**rs74609646**	**0.14**	**2.80**	**4.31**	**1.82**	**$3.43x10^{-6}$**	***PDE7B***	**Intronic**	***GAPDHL19***	***MTFR2***
19	rs7245995	0.22	0.32	0.52	0.20	$3.50x10^{-6}$	-	Intergenic	*CASP14*	*OR1I1*
11	rs2512734	0.29	2.03	2.72	1.51	$3.50x10^{-6}$	-	Intergenic	*TRIM51*	*OR5W2*
11	rs28582835	0.07	4.07	7.33	2.26	$3.58x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs28456013	0.07	4.04	7.27	2.24	$3.60x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs950413	0.07	4.00	7.20	2.22	$3.64x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs76892297	0.29	2.11	2.89	1.54	$3.68x10^{-6}$	-	Intergenic	*TRIM51*	*OR5W2*
11	11-50760329	0.46	2.11	2.89	1.54	$3.69x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	11-51241844	0.39	2.00	2.68	1.49	$3.70x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs11827319	0.40	2.01	2.70	1.50	$3.73x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs8188846	0.33	2.05	2.81	1.50	$3.76x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	11-49034349	0.21	2.40	3.48	1.65	$3.78x10^{-6}$	-	Intergenic	*OR4A47*	*TRIM49B*
11	rs10901992	0.07	3.89	6.87	2.20	$3.78x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs2193303	0.33	2.07	2.83	1.51	$3.85x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs115617184	0.40	2.01	2.70	1.50	$3.89x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs56080733	0.21	2.40	3.48	1.65	$3.98x10^{-6}$	-	Intergenic	*OR4A47*	*TRIM49B*
11	rs8189163	0.40	2.01	2.70	1.50	$4.00x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
18	**rs1701503**	**0.13**	**2.62**	**3.95**	**1.74**	**$4.01x10^{-6}$**	**-**	**Intergenic**	***DLGAP1***	***PPIAP14***
11	rs7294222	0.40	2.01	2.70	1.50	$4.04x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs12274709	0.40	2.01	2.70	1.50	$4.04x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs7294240	0.40	2.01	2.70	1.50	$4.07x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs7294226	0.40	2.00	2.68	1.49	$4.08x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs142781985	0.43	2.07	2.83	1.51	$4.08x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
6	rs188640115	0.14	2.70	4.16	1.75	$4.10x10^{-6}$	*PDE7B*	Intronic	*GAPDHL19*	*MTFR2*
11	rs2851564	0.26	2.20	3.07	1.58	$4.12x10^{-6}$	-	Intergenic	*TRIM64C*	*FOLH1*
11	rs2727015	0.26	2.20	3.07	1.58	$4.12x10^{-6}$	-	Intergenic	*TRIM64C*	*FOLH1*
11	rs12224686	0.29	2.02	2.71	1.51	$4.16x10^{-6}$	*TRIM51*	Intronic	*OR5D6*	*OR5W1P*
11	rs4553353	0.40	2.00	2.68	1.49	$4.19x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs4393301	0.40	2.00	2.68	1.49	$4.20x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs8189043	0.40	2.01	2.70	1.50	$4.21x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
6	rs9376172	0.14	2.69	4.14	1.75	$4.23x10^{-6}$	*PDE7B*	Intronic	*GAPDHL19*	*MTFR2*
11	rs4362131	0.40	2.01	2.70	1.50	$4.24x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs7294255	0.40	2.01	2.70	1.50	$4.26x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*
11	rs58036605	0.07	3.86	6.81	2.19	$4.30x10^{-6}$	-	Intergenic	*OR4C12*	*OR4A5*

Chr	SNP	Frq	OR	U95CI	L95CI	*P*	Gene	Feature	Left Gene	Right Gene
11	rs11245788	0.07	3.86	6.81	2.19	4.30×10^{-6}	-	Intergenic	*OR4C12*	*OR4A5*
11	rs8188898	0.35	2.08	2.85	1.52	4.34×10^{-6}	-	Intergenic	*OR4C12*	*OR4A5*
4	**rs28633232**	**0.23**	**2.21**	**3.08**	**1.58**	**4.35×10^{-6}**	**-**	**Intergenic**	***TMPRSS11 B***	***YTHDC1***
6	rs672962	0.14	2.60	3.92	1.72	4.36×10^{-6}	-	Intergenic	*OFCC1*	*TFAP2A*
4	rs11940780	0.23	2.21	3.08	1.58	4.37×10^{-6}	-	Intergenic	*TMPRSS11B*	*YTHDC1*
11	rs2512736	0.20	2.23	3.17	1.57	4.47×10^{-6}	-	Intergenic	*OR5W1P*	*OR5W2*
6	rs4896193	0.14	2.68	4.04	1.78	4.50×10^{-6}	*PDE7B*	Intronic	*GAPDHL19*	*MTFR2*
11	rs1396625	0.20	2.23	3.17	1.57	4.50×10^{-6}	-	Intergenic	*OR5W1P*	*OR5W2*
11	11-50464664	0.26	2.16	3.01	1.55	4.51×10^{-6}	-	Intergenic	*OR4C12*	*OR4A5*
11	rs17492683	0.19	2.35	3.41	1.62	4.62×10^{-6}	-	Intergenic	*OR4A16*	*OR4A15*
11	11-48945608	0.30	2.24	3.19	1.57	4.68×10^{-6}	-	Intergenic	*OR4A47*	*TRIM49B*
3	**rs6779831**	**0.35**	**0.45**	**0.64**	**0.32**	**4.72×10^{-6}**	**-**	**Intergenic**	***GRM7***	***LMCD1***
11	rs61897488	0.20	2.27	3.23	1.60	4.72×10^{-6}	-	Intergenic	*OR5D16*	*TRIM51*
11	rs61897487	0.20	2.27	3.23	1.60	4.72×10^{-6}	-	Intergenic	*OR5D16*	*TRIM51*
11	11-55686442	0.32	2.08	2.85	1.52	4.73×10^{-6}	-	Intergenic	*OR5W2*	*OR5I1*
11	rs8188856	0.45	2.06	2.82	1.51	4.81×10^{-6}	-	Intergenic	*OR4C12*	*OR4A5*
11	rs11245888	0.42	2.03	2.72	1.51	4.81×10^{-6}	-	Intergenic	*OR4C12*	*OR4A5*
11	rs10444243	0.07	3.96	7.13	2.20	4.82×10^{-6}	-	Intergenic	*OR4C13*	*OR4C12*
4	4-25982104	0.11	3.00	4.80	1.87	4.92×10^{-6}	-	Intergenic	*SMIM20*	*RBPJ*
11	rs2512726	0.20	2.21	3.08	1.58	4.99×10^{-6}	-	Intergenic	*OR5W2*	*OR5I1*

The lowest p-value SNP for each LD block is highlighted in bold. Upper 95% Confidence interval, U95CI; Lower 95% Confidence interval, L95CI.

4.4.3 Polymorphisms on chromosome 11 may have an effect on FOLH1 regulation and expression

The region around the most significant SNPs associated with HIV-SN on chromosome 11 has a multitude of genes, with the majority being olfactory receptors and uncharacterized genes (**Error! Reference source not found.** 4.4). Of biological interest in the region was folate hydrolase 1 (*FOLH1*), which catalyzes the step to transform dietary folate to folic acid prior to the entry of folic acid into the folate cycle. All of the SNPs on chromosome 11 that had *p*-values < 5×10^{-6} clustered within four independent loci near *FOLH1* (Figure 4.5). Three of the loci were at the 5' end of the gene and one was at the 3' end of the gene. Initially the SNP closest to *FOLH1*

(rs7925419, 1.6 kb from the 5'-end of *FOLH1*) was analyzed for putative functional or regulatory effects.

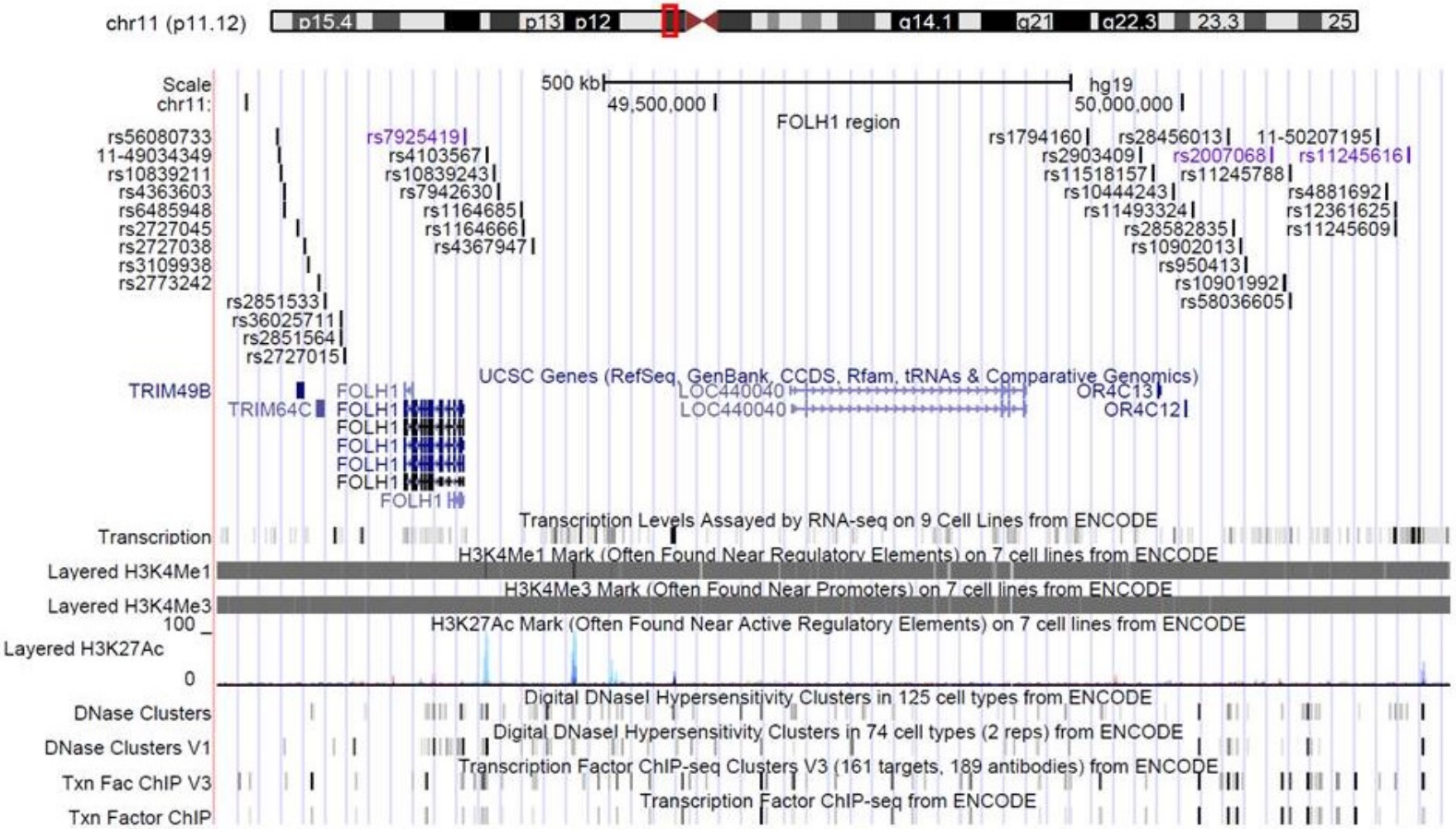

Figure 4.4. UCSC genome browser image showing position of SNPs associated with HIV-SN susceptibility in the vicinity of the *FOLH1* gene. SNPs in the *FOLH1* region track were implicated with HIV-SN case vs. control status ($p < 1 \times 10^{-5}$). SNPs in purple ($p < 5 \times 10^{-6}$) were considered 'promising' and selected for replication. Rs12288743 is further upstream and is not visible in this figure.

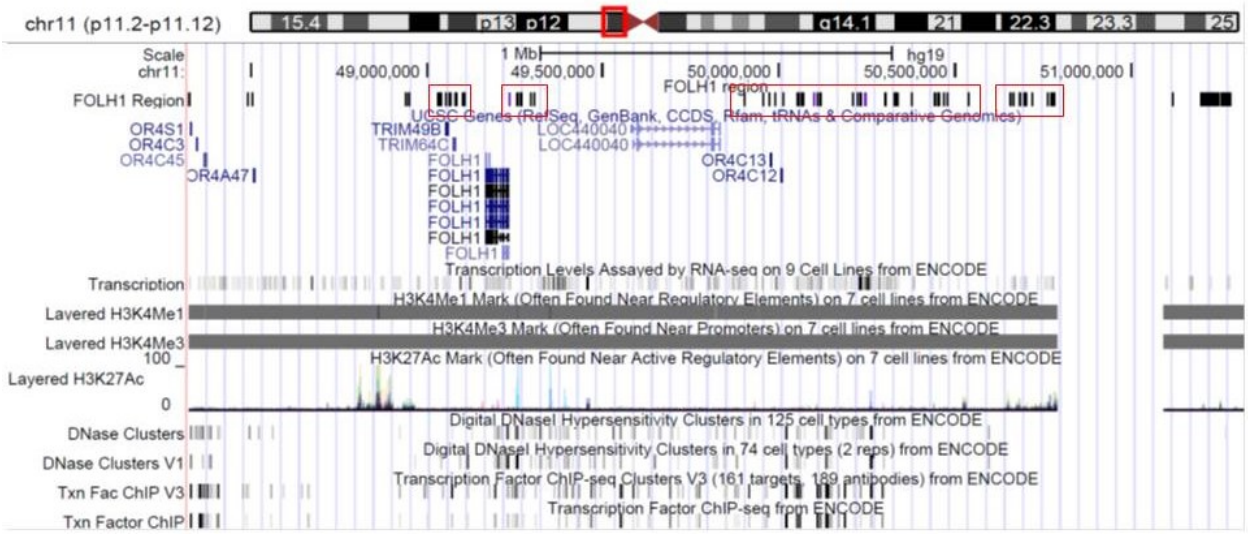

Figure 4.5. Loci proximal to *FOLH1* associated with HIV-SN. UCSC browser detail of SNPs associated with HIN-SN ($p < 10^{-5}$) proximal to *FOLH1*. The four independent loci are outlined in red.

The *FOLH1* SNP rs7925419 had a minor allele frequency of 11% and 23% in control and case groups, respectively (Figure 4.6). The control group minor allele frequency of 11% was comparable to the 1000 Genomes project's African population minor allele frequency for this SNP of 12%[30].

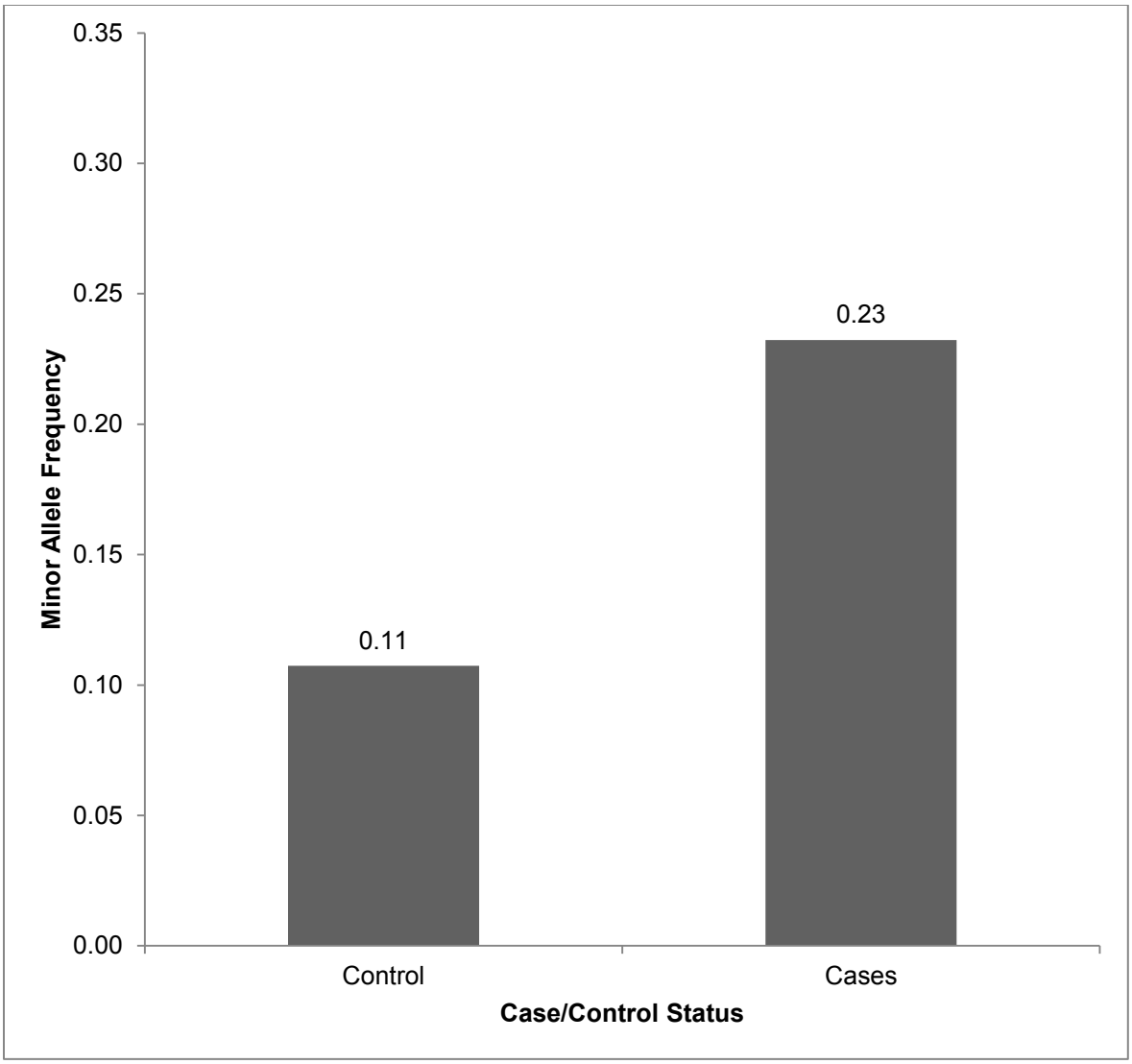

Figure 4.6. Plot of the association of *FOLH1* SNP rs7925419 with HIV-SN case and control status in HIV+ Ugandan subjects.

Minor allele frequencies of rs7925419 in the discovery cohort are significantly higher (p = 1.82 x 10^{-6}) in case subjects (n=129, 23%) versus control subjects (n=342, 11%).

Analysis for regulatory function using the Haploreg database indicated that rs7925419 is a weak enhancer in several neuronal tissues (brain inferior temporal lobe, cingulate gyrus, hippocampus and substantia nigra) and alters the binding affinity for the transcription factor NF-I[28]. The association between rs7925419 and FOLH1 RNA expression was explored in the GTEx database and the variant allele was associated with decreased FOLH1 RNA expression in brain cervical cord C-1 tissue (Figure 4.7, $p = 0.04$).

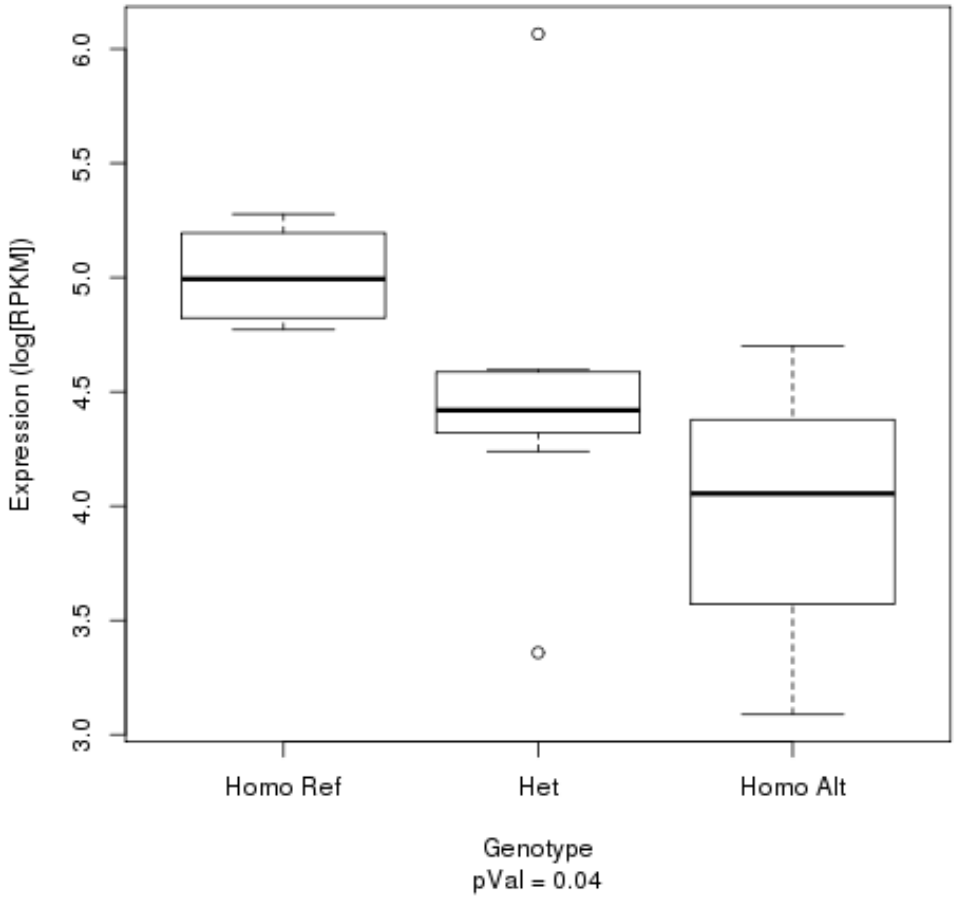

Figure 4.7. The effect of rs7925419 genotype on RNA expression of FOLH1 in brain spinal cord cervical c1 tissue. FOLH1 levels decrease significantly with the number of variant alleles present in the genotype ($p < 0.04$). Data are from RNA-seq (log[RPKM]; n = 17) and was provided by the GTEx bioinformatic database. RPKM = reads per kilobase per million.

Additional analyses were conducted on the lowest *p*-value SNPs from each of the remaining three loci proximal to *FOLH1*. The top SNP associated with HIV-SN is rs2007068 which is 865 kb from the 5' region of *FOLH1*. Regulatory functional analyses performed in Haploreg indicate that this SNP is an expression quantitative loci (eQTL) in the Gibbs Frontal cortex[28]. GTEx data indicates that the variant allele is associated with a reduction in RNA expression of FOLH1 in brain cervical cord C-1 tissue ($p = 3 \times 10^{-4}$).

The second most associated SNP with HIV-SN was rs12288743, which is in the loci most distal to *FOLH1*, being 1180 kb from the 5' end of *FOLH1*. While regulatory analyses in the Haploreg database did not reveal any effects due to the SNP, GTEx expression analyses found that the variant allele is significantly associated with FOLH1 expression in the brain cervical cord C-1 tissue ($p = 3 \times 10^{-4}$).

4.4.4 Replication Results

An additional 157 subjects from the original UARTO and ARKS cohorts were available for the replication study. Three SNPs that had 'promising' *p*-values and also showed the highest potential for regulatory and functional effects were chosen for replication and genotyped using Taqman genotyping assays. A meta-analysis was also performed to see the overall effect of these SNPs in both the discovery and replication cohorts. The results of the replication study and the meta-analysis are shown in Table 4.6. Of these SNPs, rs7925419 showed a trend toward significance in the replication study ($p = 0.12$) and had an improved *p*-value in the meta-analysis (7.60×10^{-7}). The two other SNPs chosen for replication showed no trend towards significance and did not have improved *p*-values in the meta-analysis.

Table 4.6. Results from replication and meta-analyses

				Discovery				Replication				Meta-analysis			
SNP	**Chr**	**Gene**	**Minor Allele**	**OR**	**U95 %CI**	**L95 %CI**	**P**	**OR**	**U95% CI**	**L95 %CI**	**P**	**OR**	**U95 %CI**	**L95 %CI**	**P***
rs7925419	11	*FOLH1*	T	2.67	4.03	1.77	1.82×10^{-6}	1.74	3.52	0.86	0.12	2.39	3.42	1.68	7.60×10^{-7}
rs20087068	11		T	2.69	3.90	1.85	2.63×10^{-7}	1.11	2.31	0.56	0.77	2.23	3.10	1.61	9.21×10^{-7}
rs11245616	11		A	2.92	4.49	1.90	1.67×10^{-6}	0.72	2.21	0.24	0.79	2.40	3.65	1.61	1.07×10^{-5}

* Meta-analysis *p*-values reflect the one-sided *p*-value because the effect direction was assumed *a priori* to be the same direction in the replication cohort as the discovery cohort.

4.5 Discussion

HIV related sensory neuropathies are a common complication to HIV infection, particularly in the developing world[3,4]. Because of the rarity of HIV-SN in the developed world, little research has been conducted into the role host genetics play in its development. This study used a genome-wide association study to determine the genetic predictors of HIV-SN in a Ugandan HIV+ treatment naïve population. While no SNPs reached genome-wide significance, multiple loci of suggestive or 'promising' significance were identified on chromosome 11 proximal to the *FOLH1* gene. Bioinformatic analyses indicated that three SNPs had the potential to modulate FOLH1 expression or function and these SNPs were chosen for independent replication in a subset of patients from the same cohorts as the discovery study. Replication identified one SNP (rs7925419) that showed a trend towards significance (p = 0.12) and meta-analyses of the discovery and replication cohorts showed improved statistical significance (7.60×10^{-7}). The lack of statistical significance may be due to the small sample sizes of the discovery and replication cohorts; however the results for rs7925419 are intriguing. While this SNP is upstream from the 5' end of *FOLH1* it has been shown *in vitro* to be in the promoter region of *FOLH1* and therefore may affect FOLH1 expression as supported by data obtained from the GTEx database[31].

FOLH1 is a folate hydrolase that converts dietary folate to folic acid before folic acid enters the one-carbon cycle. Polymorphisms in *FOLH1* have been shown to influence plasma levels of folate and have been implicated in neural tube defects in developing fetuses[32,33]. Sensory peripheral neuropathies may be caused by many diseases and drug toxicities and folate-responsive peripheral neuropathies have been extensively

documented in the scientific literature[34-37]. Generally, these folate-responsive neuropathies are due to dietary deficiency or impaired folate metabolism, as is seen in peripheral neuropathy in alcoholic patients[36]. It has also been reported that HIV patients commonly are folate deficient[38,39].

Several lines of evidence support a potential role for folate in HIV-induced SN. First, folate levels have been found to impact the development of sensory neuropathies in other diseases and HIV infection may lead to folate deficiency[35-39]. In addition, *FOLH1* polymorphisms have been shown to effect folate plasma levels[33,40,41]. The rs7925419 *FOLH1* SNP identified in this study has been associated with decreased expression of FOLH1 in brain tissue and has the potential to reduce the amount of folate that is converted to dietary folate. This is the same mechanism of folate deficiency that is observed in some alcoholism related neuropathies where the absorption of folate is impaired prior to entry into the one-carbon metabolic cycle[42,43].

The primary limitation of this study is the small sample sizes of the discovery and replication cohorts which decreases the power to detect variants with small effect sizes. However, this study does provide an interesting potential mechanism of HIV-SN that could be verified with additional clinical and functional studies. Another potential limitation of this study is the use of a symptom questionnaire in lieu of a clinical test to define the HIV-SN phenotype. It has been previously demonstrated though that single question neuropathy screens (SQNS) are remarkably specific (80.0%) and sensitive (95.7%) for the diagnosis of HIV related neuropathies[44].

4.6 Conclusion

In conclusion, this study identified a potential genetic predictor for the development of HIV-SN, *FOLH1*. Since the exact role that *FOLH1* and *FOLH1* polymorphisms play in the development of HIV-SN is not known, further *in vitro* and clinical studies are warranted. These findings may lead to a potentially simple and cost effective treatment for HIV-SN, folate supplementation in HIV+ patients.

4.7 References

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Chapter 5

Genetic Predictors of NRTI Induced Peripheral Neuropathy in Ugandan HIV-1+ Subjects

5.1 Abstract

Sensory peripheral neuropathy is one of the most common toxicities associated with the use of nucleoside reverse transcriptase inhibitors (NRTI-SN), a primary component in antiretroviral therapy. While the role that host genetics plays in the development of NTRI-SN has been investigated in candidate gene studies, a genome-wide association study to identify genetic predictors of NRTI-SN has not been reported. The objective of our study is to identify genetic predictors of NRTI-SN in a treatment naive Ugandan HIV+ population. Genotype and phenotype data were collected from 580 Ugandan HIV+ patients enrolled in a treatment outcome cohort study. Patients received a treatment regimen consisting of zidovudine/lamivudine or stavudine/lamivudine combinations along with either nevirapine or efavirenz. Whole genome genotyping was performed using the Illumina OmniExpress® platform with 681,315 SNPs and imputation with IMPUTE2 resulted in >16 million SNPs available for analysis. Sensory PN was scored on a Likert scale from symptom recording at baseline and each quarterly clinic visit. Associations between genetic markers and NRTI-SN were performed using an additive genetic model and logistic regression. Analyses identified several SNPs associated with NRTI-SN at a genome-wide suggestive significance level ($p < 5 \times 10^{-7}$), including an intergenic SNP, rs188298690 ($p = 1.47 \times 10^{-7}$, OR = 8.61, 95% confidence interval = 20.80 - 3.56), that may influence VAMP4 expression, a protein that regulates asynchronous neurotransmitter release. A candidate gene subanalysis also identified

SNPs in two genes associated with NRTI-SN: SNPs in *ABCC4* (rs7317112: unadjusted $p = 2.8 \times 10^{-3}$, OR = 0.54, 95% confidence interval = 0.80 - 0.36) and *SLC28A1* (rs2242046: unadjusted $p = 3.1 \times 10^{-3}$, OR = 0.19, 95% confidence interval =0.57 – 0.06). These genes are drug transporters and are important for NRTI disposition. These studies suggest that genetic variation in novel genes involved in nerve function and drug transport may influence an individual patient's risk of developing NRTI-SN. Further studies are warranted to investigate the roles these genes play in the development of NRTI-SN.

5.2 Introduction

Great progress has been made in the treatment of HIV since the beginning of the HIV epidemic in the 1980s. HIV infections are treated with Highly Active Antiretroviral Therapy (HAART) which consists of a combination of drugs that target different stages in the HIV life cycle[1,2]. One of the most commonly used drug combinations consists of a backbone of two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI)[1,2]. While HAART is extremely effective at controlling HIV replication, there are drug toxicities associated with all the classes of drugs used to treat HIV[1-3]. NRTIs have several toxicities associated with their use including anemia, sensory peripheral neuropathy and renal toxicity[4-9]. Specifically, the first and second generation NRTIs, including azidothymidine (AZT) and stavudine (d4T) are associated with the development of NRTI induced sensory neuropathies (NRTI-SN). While AZT and d4T are not first line treatments in the developing world, they are still critical to the treatment of HIV in the developing world, particularly in sub-Saharan Africa.

Since the earliest days of HAART, cases of NRTI-SN have been observed, especially when used in combination therapies[10,11]. NRTI-SN is characterized by the development of numbness, tingling or pain in a distinctive “glove and sock” pattern[12]. NRTI-SN is clinically indistinguishable from neuropathies caused by other factors such as HIV infection, diabetes or alcoholism[10,11]. Unlike other drug toxicities that are dose dependent, the risk of developing NRTI toxicity is highest during the first year of HAART and is reduced after this point[13,14]. NRTI-SN has been attributed to increases in mitochondrial toxicity mediated by the inhibition of the mitochondrial polymerase,

polymerase γ, which is encoded by *POLG*[6,15,16]. The inhibition of polymerase γ causes increases in oxidative stress that eventually leads to cellular apoptosis and Wallerian degeneration of peripheral nerves[10,17,18]. Like other neurodegenerative conditions, NRTI-SN has also been associated with dysregulation of iron metabolism[19].

Multiple genetic studies focusing on genes involved in mitochondrial function or iron metabolism have been conducted, some with positive results. Specifically, genetic mutations in the mitochondrial haplogroup T and the genes *HFE* and *POLG* have both been associated with the development of NRTI-SN[19-22]. While these studies have helped to characterize the role that host genetics play in the development of NRTI-SN, no study to date has examined the whole genome for potential genetic predictors of the development of NRTI-SN.

5.3 Materials and Methods:

5.3.1 Participants

The participants of this study are fully described in Chapter 4.3.1. Genotype and phenotype data from Ugandan HIV+ individuals were collected for the discovery study (n = 638) and the replication study (n = 209).

5.3.2 Genotyping

The full details of the sample collection and processing, genotyping and quality control procedures for samples and single nucleotide polymorphisms (SNPs) are provided in Chapter 4.3.2. In the discovery study, genomic DNA from 638 subjects was extracted from either saliva or blood samples using standard DNA extraction or

normalization techniques. Out of 645 samples (638 patient samples with seven control samples) a total of 585 subjects passed genotyping quality control procedures. After genotyping and imputation quality control procedures 16.9 million SNPs were available for analysis[23].

For the replication studies, a total of 169 patients had complete genotype and phenotype data. Genotyping for the replication study was performed according to the manufacturer's instructions using the Taqman Genotyping assay (Life Technologies, Grand Island, NY) outlined in Table 5.1.

Table 5.1. SNPs Selected for Replication

SNP	Assay ID	Tag SNPs (r^2 >0.8)
rs188298690	Custom: AHHS28B	rs144134647, rs139815631, rs144690537, rs138815589, rs141776039

Taqman assay IDs are listed. SNPs that are in LD (r^2 >0.8) in the LWK 1000 Genomes database are also listed.

5.3.3 Phenotype

Peripheral neuropathy data was gathered quarterly during the study visits as a component of the symptom questionnaire. Peripheral neuropathy is graded on a Likert-type scale with subjects asked if they are experiencing "pain, numbness or tingling in the hands or feet." A score of "0" denotes no symptoms, "1" means "bothers me not at all", "2" corresponds to "bothers me a little", "3" corresponds to "bothers me a moderate amount" and "4" denotes "bothers me a lot". Case and control status were evaluated from baseline to 12 months from the date of treatment initiation. Subjects that reported "0" (n = 148) or "1" (n = 31) during the study period or had a decrease in symptoms from baseline (n = 91) were classified as "controls" (n = 270). Subjects that reported "0" or "1" at baseline and had at least 1 value greater than or equal to "2" during the study

period (n = 83), reported “2” at baseline and had at least one value greater than or equal to “3” during the study period (n = 11) or reported “3” at baseline and had at least one value greater than or equal to “4” during the study period (n = 9) were classified as “cases” (n = 103). A graphical representation of this can be seen in Figure 5.1.

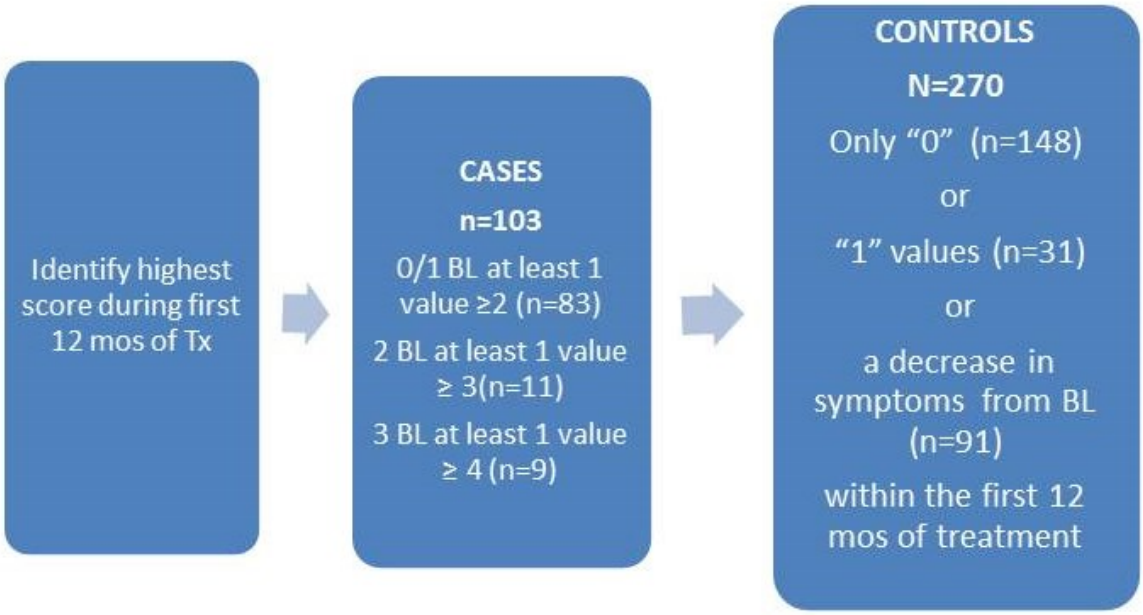

Figure 5.1. NRTI-SN case/control definition method.

5.3.4 Statistical Analyses

Demographic variables were tested for statistical significance using linear regression for continuous variables and ANOVA tests for categorical variables in R[24]. Standard case/control analyses using logistic regression were performed for the primary and replication analyses using PLINK to test the association between each SNP and the phenotype[25]. Odds ratios (OR), 95% confidence intervals (95% CI) and *p*-values were reported for each SNP. To account for the multiple testing burden genome-wide significance was considered $p \leq 5x10^{-8}$ and genome-wide suggestive was considered $p \leq 5x10^{-7}$; ‘promising’ SNPs at $p \leq 5x10^{-6}$ were considered for further bioinformatic

analysis[26]. The control group minor allele frequency was examined for Hardy-Weinberg Equilibrium for SNPs of interest (X^2 test, $\alpha = 0.05$). Linkage disequilibrium calculations were performed in PLINK[25]. Plots were produced in R and Microsoft Excel 2010[24]. Meta analyses to combine *p*-values from the discovery and replication studies were performed in R using the meta package[27].

5.3.5 Candidate Gene Analysis

Candidate genes (n = 16) were selected based on literature documenting their function in the metabolic, pharmacokinetic and pharmacodynamic pathways of NRTIs[7,14,19,28-30]. Table 5.2 describes the candidate genes selected for this study and their role in NRTI disposition, pharmacology or toxicity. Statistical analyses were carried out in the same fashion as the genome-wide analysis with the exception that the *p*-value cutoff for significance was determined by performing a Bonferroni correction for the number of haplotype blocks tested (n = 121). SNPs were considered significant if the adjusted $p < 0.05$. The number of linkage disequilibrium blocks present in each gene was calculated in Haploview using the LWK population[31]. A gene based cutoff was also considered at $p \leq 0.003$ using the number of genes (n = 16) for the multiple testing comparison.

Table 5.2. Candidate Genes with NRTI Transport or Functional Evidence

Gene	Protein	Function/Evidence
ABCC4	MRP4	Nucleoside transport[32]
ABCG2	MXR	Drug transport[33]
HFE	HFE	Iron metabolism[19]
POLG	POLG	Mitochondrial polymerase[22]
SLC22A6	OAT1	Organic anion transport[34-36]
SLC22A7	OAT2	Organic anion transport[34-36]
SLC22A8	OAT3	Organic anion transport[34-36]
SLC25A19	DNC	Nucleoside transport, mitochondrial
SLC28A1	CNT1	Nucleoside transport[34-36]
SLC28A2	CNT2	Nucleoside transport[34-36]
SLC28A3	CNT3	Nucleoside transport[34-36]
SLC29A1	ENT1	Nucleoside transport[37]
SLC29A2	ENT2	Nucleoside transport[37]
SLC29A3	ENT3	Nucleoside transport[37,38]
TK1	TK1	Intracellular nucleoside phosphorylation[38]
TK2	TK2	Intracellular nucleoside phosphorylation[38]

5.3.6 Bioinformatic Analyses

To further explore the putative biological significance of SNPs that had 'promising' *p*-values (genome-wide $p \leq 5x10^{-6}$, candidate gene $p \leq 0.003$) bioinformatic analyses were performed. SNPs were annotated to genes using SNPnexus, SCANdb (http://www.scandb.org/) and the UCSC genome browser[39,40]. GTEx (http://www.broadinstitute.org/gtex/) and GeneVar (http://www.sanger.ac.uk/resources/software/genevar/) databases were employed to examine the effect of a SNP on gene expression[41]. To determine any regulatory functions of SNPs of interest, Haploreg and ENCODE databases were employed[42,43].

5.4 Results

5.4.1 Demographic Data

The demographic characteristics of the discovery and replication cohorts are described in Table 5.3. The two cohorts were similar in the majority of demographic variables assessed with the exception of gender percentages, percent of ARKS participants and case rate. The discrepancy of gender is most likely due to the higher percentage of ARKS participants, as this cohort has a larger male participation rate. The higher rate of cases may also be due to the number of ARKS participants since this cohort takes place in a hospital setting and patients may have better access to care. None of the demographic variables tested had an effect on case/control status with the exception of gender in the initial cohort (p = 0.013). This effect was not seen in the replication cohort (p = 0.99). The case percentages in the initial and replication cohorts were 23% and 44%, respectively.

Table 5.3. Patient Demographic Data in the Discovery and Replication Cohorts

	Discovery	**Replication**
Sample size, n	373	169
Age, years, median ± SD	34 ± 8.3	34 ± 9.3
Gender, n (%M)	127 (34%)	73 (43%)
Baseline CD4+ T-cell counts, cells/mm^3, median ± SD	137 ± 118	200.0 ± 213
Baseline Viral Load, log(copies/mL), median ± SD	5.13 ± 0.69	5.07 ± 0.67
Case, n (%)	103 (28%)	74 (44%)
Cohort, n (%ARKS)	117 (23%)	58 (34%)

5.4.2 A loci on chromosome 1 is associated with NRTI-SN

After quality control procedures, 16.9 million imputed and genotyped SNPs were tested for association with NRTI-SN using a standard case/control testing methodology. Figure 5.2 shows the Manhattan plot where the y-axis is the *p*-value for each SNP and the x-axis is the genomic position. A Q-Q plot showing the observed versus expected *p*-values and the genomic inflation factor, λ (1.01, Figure 5.3), indicate that there is no significant population stratification.

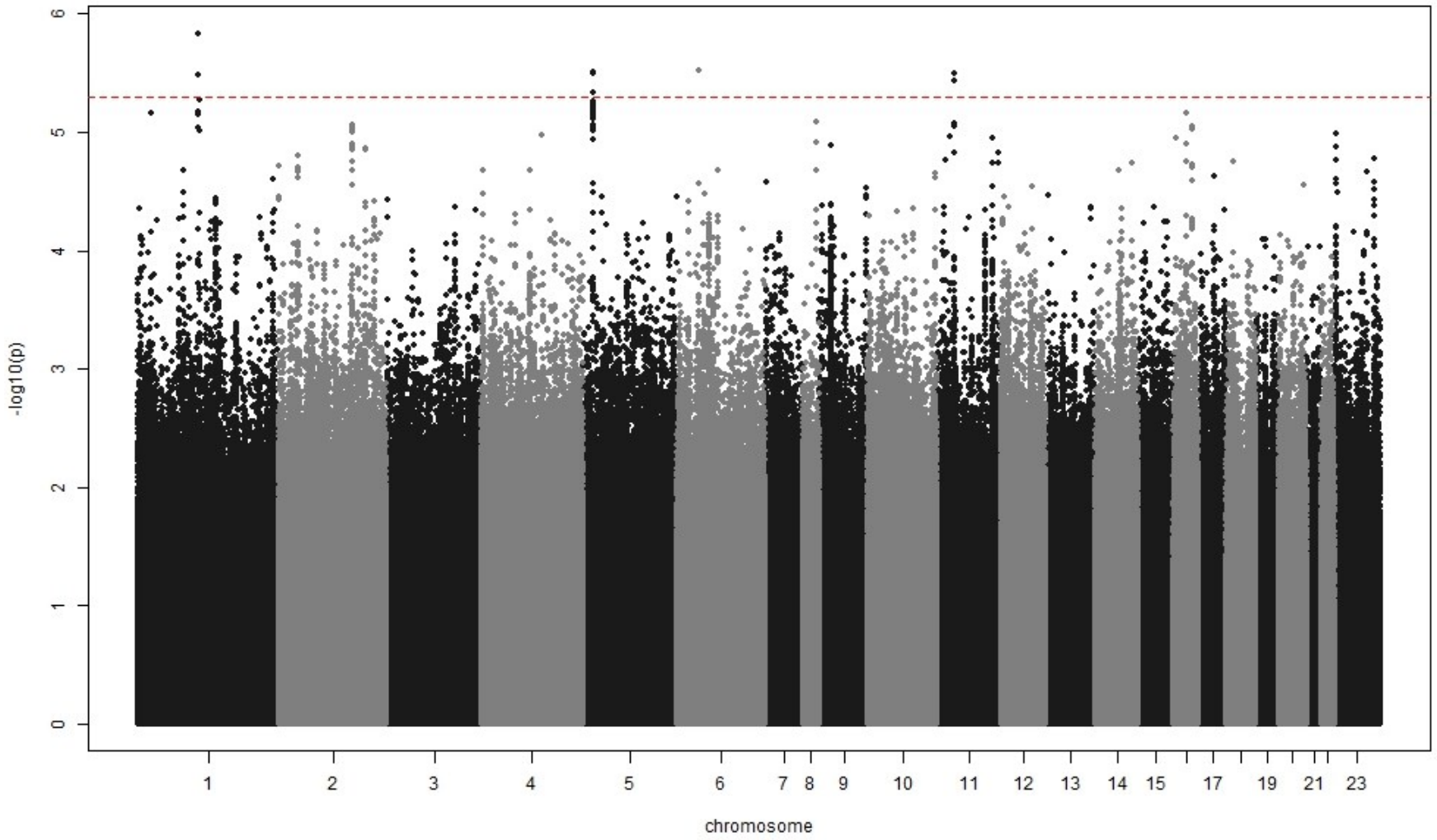

Figure 5.2. Manhattan plot showing the distribution along the human autosomes of -log10 (P values) obtained for SNP association with NRTI-SN cases versus control subjects. The red line ($p < 5 \times 10^{-6}$) marks the cutoff for 'promising' SNPs considered for further bioinformatics analysis.

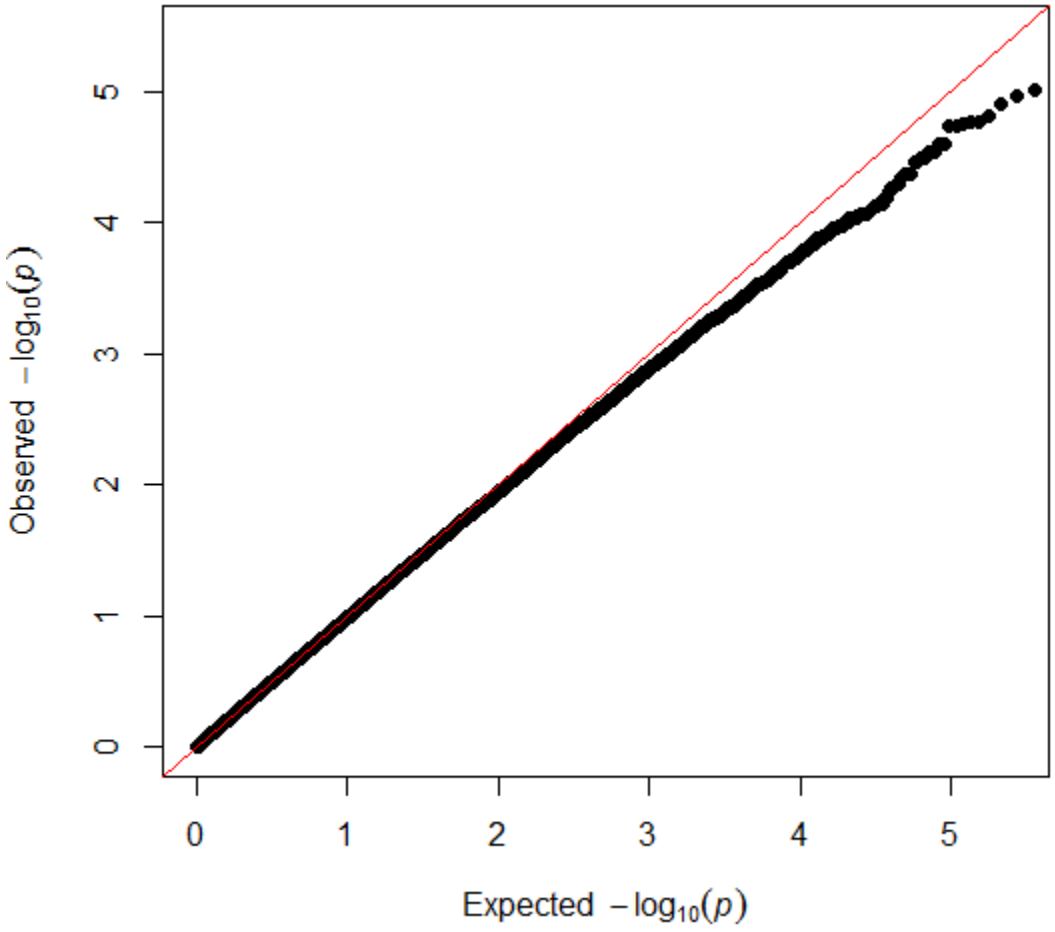

Figure 5.3. Observed versus expected *p*-values for SNP association with NRTI-SN cases versus control subjects.

P-values are plotted on a -log base 10 scale. The red line indicates the null distribution. The genomic inflation factor λ was 1.01 and was calculated using R statistical computing software.

Only 13 SNPs had *p*-values < 5 x 10^{-6}. SNPs with the greatest association to HIV-SN were filtered for linkage disequilibrium (r^2 >0.8) with the SNP with the lowest *p*-value for each LD block being retained highlighted in bold in Table 5.4. The SNP with the most significant *p*-value was on chromosome 1 (rs188298690, $p = 1.47 \times 10^{-6}$, OR = 8.61, 95% CI = 20.80 - 3.56). This SNP is located in an intergenic region between *VAMP4 and METTL13* with *DMN3* closely downstream of *METTL13* (Figure5.4). Because this was the top SNP in the GWAS and no SNPs reached genome-wide significance, this locus was chosen for further bioinformatic analysis.

Table 5.4. Top Variants Associated with NRTI-Induced Peripheral Neuropathy

Chr	SNP	MAF	OR	U95CI	L95CI	P	Gene	Feature	Left Gene	Right Gene
1	**rs188298690**	**0.04**	**8.61**	**20.80**	**3.56**	**1.47 x 10^{-6}**	**-**	**Intergenic**	***VAMP4***	***DNM3***
6	**rs2234245**	**0.11**	**3.39**	**5.64**	**2.04**	**3.00 x 10^{-6}**	***TREM1***	**missense**	***NCR2***	***TREM4***
5	**rs116426216**	**0.04**	**8.01**	**19.35**	**3.32**	**3.05 x 10^{-6}**	**-**	**Intergenic**	***BASP1***	***CDH18***
11	**rs4755601**	**0.22**	**2.57**	**3.80**	**1.74**	**3.16 x 10^{-6}**	**-**	**Intergenic**	***LRRC4C***	***API5***
5	rs114363753	0.04	7.96	19.23	3.30	3.17 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
5	rs115212730	0.04	7.96	19.23	3.30	3.19 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
5	rs141776039	0.04	8.58	21.14	3.48	3.25 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
5	rs138815589	0.04	8.58	21.14	3.48	3.25 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
5	rs144134647	0.04	8.56	21.09	3.47	3.31 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
5	rs139815631	0.04	8.56	21.09	3.47	3.31 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
5	rs144690537	0.04	8.56	21.09	3.47	3.31 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*
11	rs7949101	0.23	2.47	3.66	1.67	3.69 x 10^{-6}	-	Intergenic	*LRRC4C*	*API5*
5	rs75759888	0.04	7.37	17.46	3.11	4.67 x 10^{-6}	-	Intergenic	*BASP1*	*CDH18*

The lowest p-value SNP for each LD block is highlighted in bold. Upper 95% Confidence interval, U95CI; Lower 95% Confidence interval, L95CI.

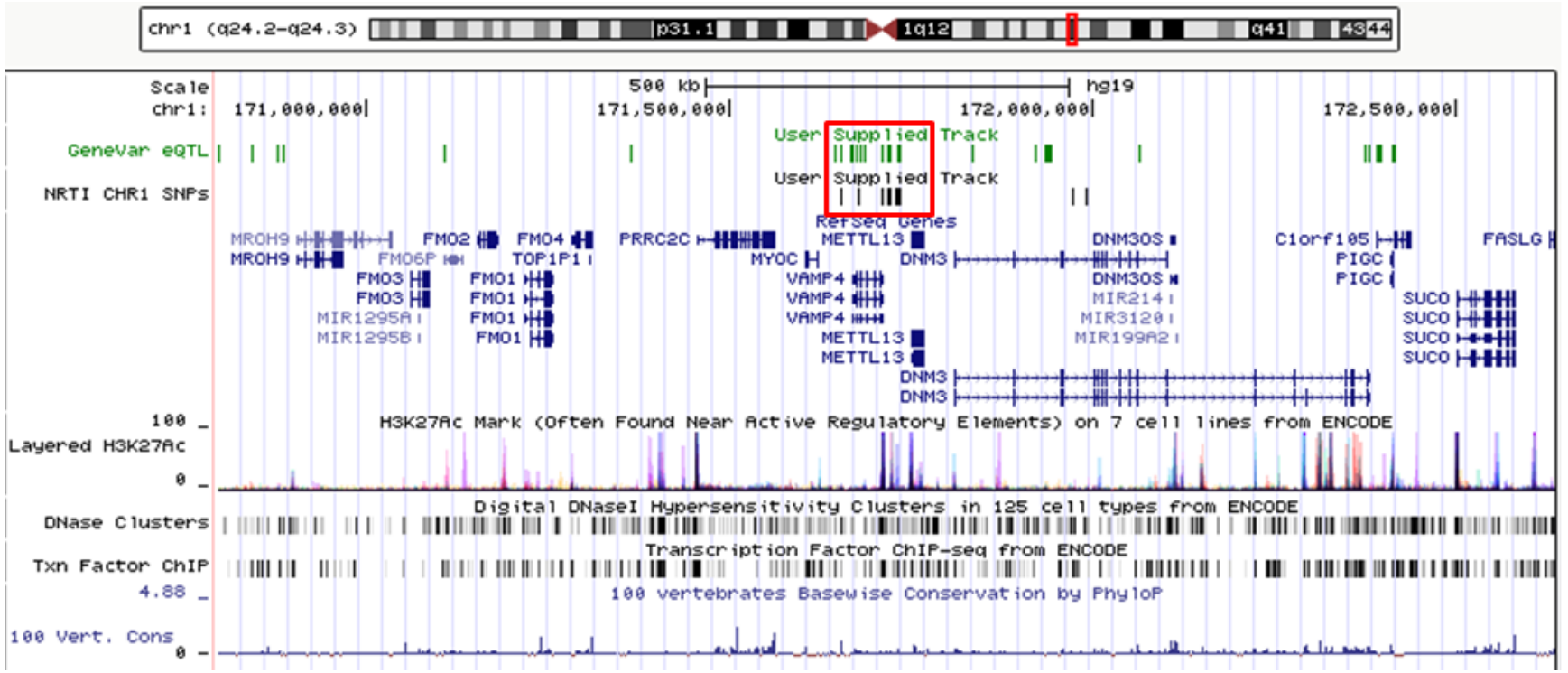

Figure 5.4. UCSC genome browser image showing position of SNPs associated with NRTI-SN susceptibility in the vicinity of the VAMP4 gene. SNPs in the NRTI CHR1 region track were marginally associated with HIV-SN case vs. control status ($p < 1 \times 10^{-5}$). SNPs in the GeneVar eQTL track are SNPs significantly associated ($p < 1 \times 10^{-3}$) with VAMP4 expression in the LWK Hapmap population. The red box highlights the eQTL region that is unique to the LWK population.

5.4.3 The rs188298690 polymorphism is in an active regulatory region and is located within a VAMP4 eQTL

All of the genes proximal to rs188298690 were investigated to determine if this SNP could regulate their expression. According to Haploreg, rs188298690 is in a region that has active regulatory elements; specifically, it is a weak enhancer in a leukemia cell line, K562. SNPs that are in LD (r^2 >0.8) also change several transcription factor binding motifs. Figure 5.5 shows the genomic position of SNPs in LD with rs188298690 and where they overlap transcription factor binding sites in K562 cells.

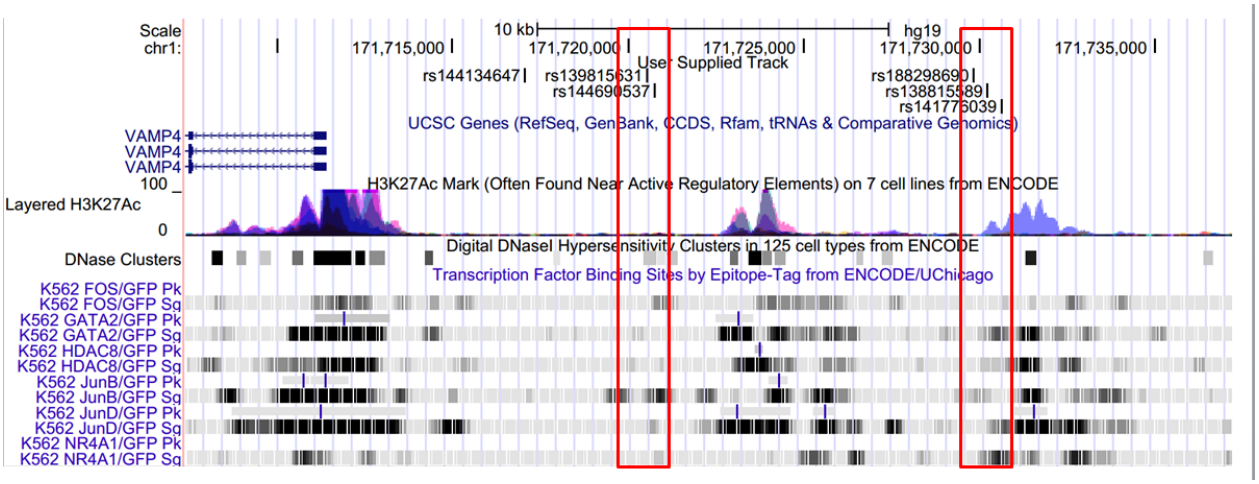

Figure 5.5. SNPs that are in LD with rs188298690 are in regions that have active regulatory elements. The "User Supplied Track" plots SNPs that are in LD (r^2 > 0.8) with rs188298690. The red boxes highlight the alignment of SNPs with H3K27Ac marks, DNase1 hypersensitivity clusters and transcription factor binding sites in the ENCODE data.

Unfortunately, rs188298690 and the SNPs in LD with it are not present in the GTEx database, so tissue specific expression analyses could not be performed. However, eQTL data in the GeneVar database was available for VAMP4, which allowed an

examination of the eQTL expression pattern of VAMP4 around the rs188298690 locus. The eQTL pattern in LCL cells was examined for three African Hapmap populations: Luhya in Webuye, Kenya (LWK), Maasai in Kinyawa, Kenya (MKK) and Yoruba in Ibadan, Nigeria (YRI). The study population is most genetically similar to the LWK population. This examination revealed that the LWK population has a unique additional eQTL locus for VAMP4 which is located farther upstream from the eQTL loci that is not seen in the other populations (Figure 5.6).

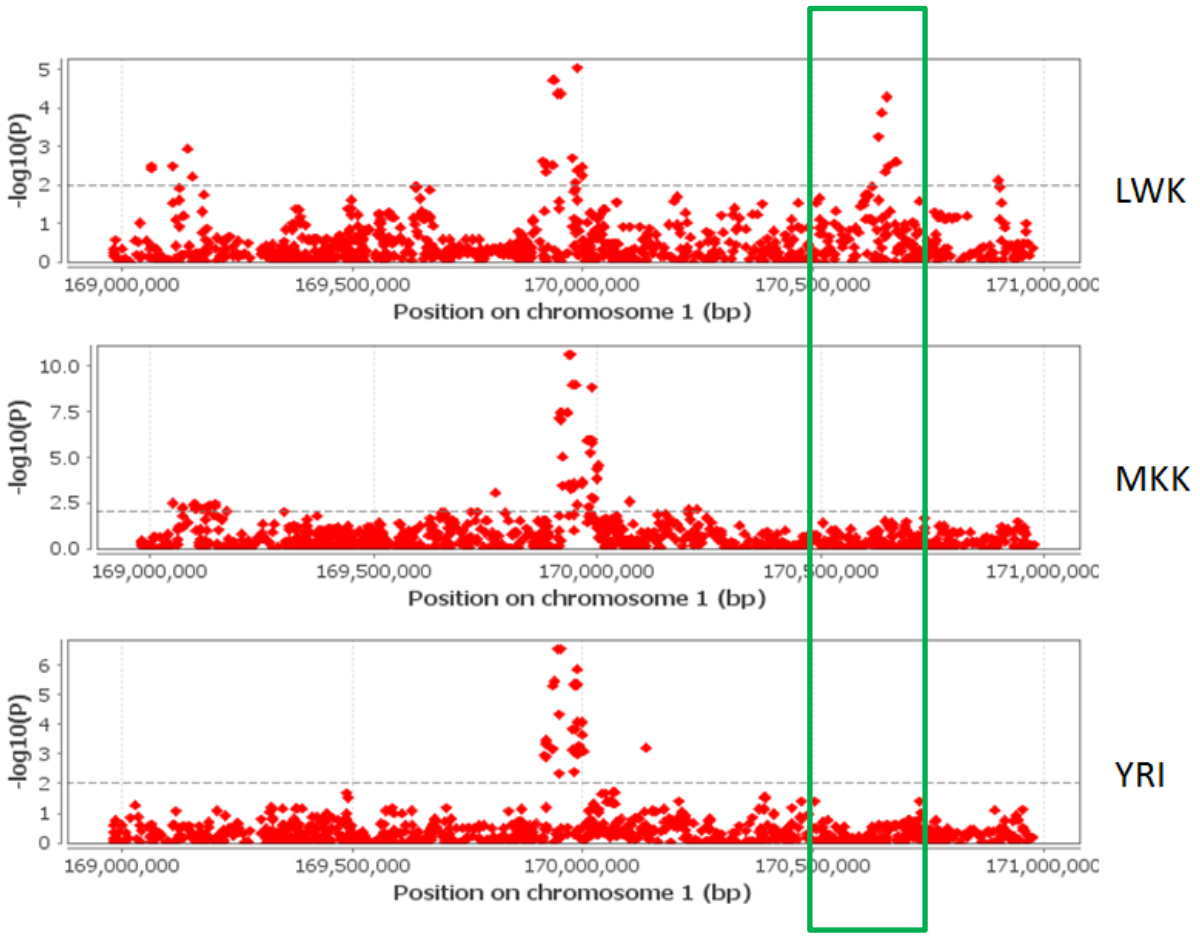

Figure 5.6. Genevar VAMP4 eQTL LCL data for three African populations.
The LWK population possesses a unique eQTL locus (highlighted in the green box) that is associated with VAMP4 expression. LWK = Luhya in Webuye, Kenya, MKK = Maasai in Kinyawa, Kenya, YRI = Yoruba in Ibadan, Nigeria

rs188298690 had a minor allele frequency of 10% and 1% in case and control groups, respectively (Figure 5.7). The control group minor allele frequency of 1% was comparable to the 1000 Genomes African population minor allele frequency for this SNP of 1%[44]. Expression of METTL13 and DNM2 was not associated with the rs188298690 locus.

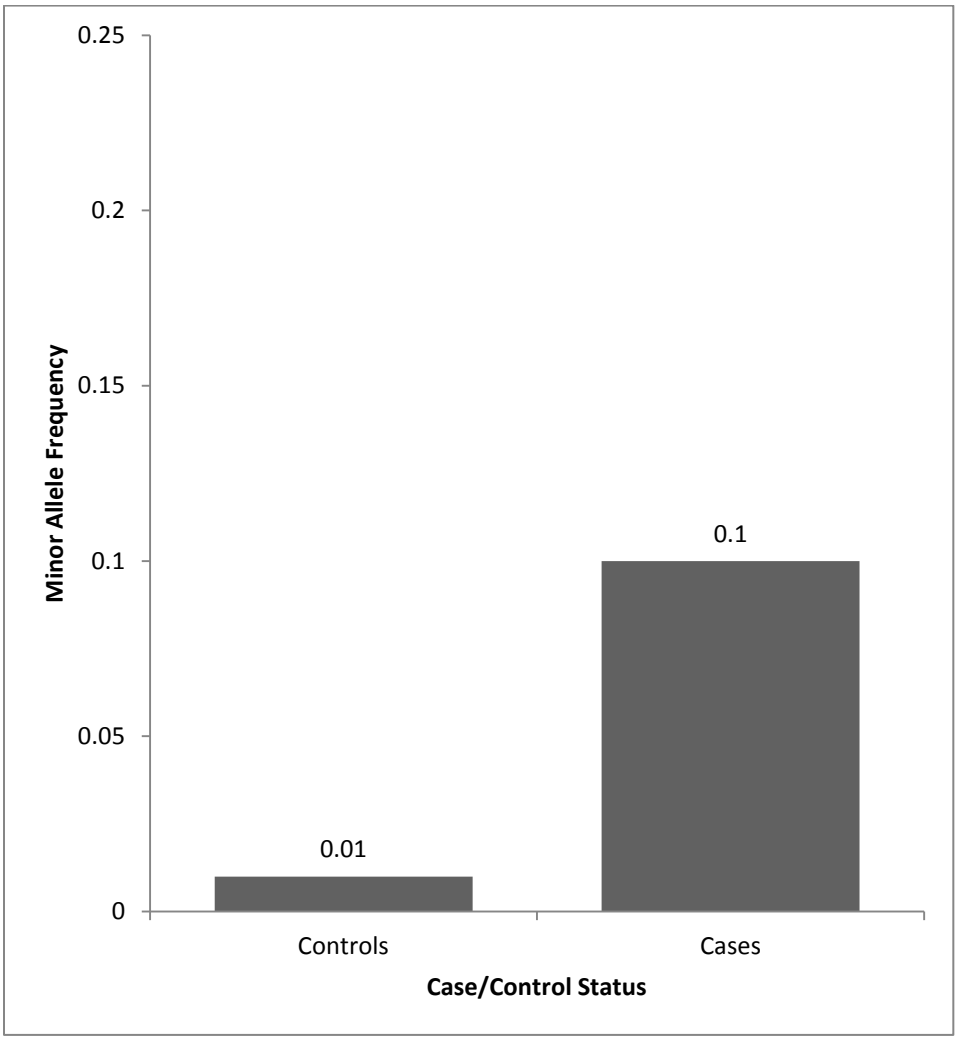

Figure 5.7. The association of intergenic SNP rs188298690 with NRTI-SN case and control status in HIV+ Ugandan subjects.

Minor allele frequencies of rs188298690 in the discovery cohort are significantly higher (p = 1.47 x 10^{-6}) in case subjects (n=103) versus control subjects (n=270).

5.4.4 Replication Results

A cohort of 169 new subjects from the original UARTO and ARKS cohorts were available for the replication study (Table 5.5). The top SNP from the GWAS study was chosen for replication and genotyped using Taqman genotyping assays. A meta-analysis was also performed to see the overall effect of this SNP in both the discovery and replication cohorts. While the SNP did not replicate it did maintain odds ratio directionality.

Table 5.5. Results from Replication and Meta-Analysis

			Discovery				Replication				Meta-analysis			
SNP	**Chr**	**Minor Allele**	**OR**	**U96 CI**	**L96 CI**	**P**	**OR**	**U96 CI**	**L96 CI**	**P**	**OR**	**U96 CI**	**L96 CI**	**P***
rs188298690	1	A	8.61	20.8	3.56	1.47×10^{-6}	1.59	3.78	0.67	0.29	3.67	6.80	1.98	1.8×10^{-5}

* Meta-analysis *p*-values reflect the one-sided *p*-value because the effect direction was assumed *a priori* to be the same direction in the replication cohort as the discovery cohort.
Upper 95% Confidence interval, U95CI; Lower 95% Confidence interval, L95CI.

5.4.5 Candidate gene study reveals an association of ABCC4 polymorphisms and NRTI-SN

In a candidate gene subanalysis of 16 genes, only three SNPs had *p*-values reaching the gene-based significance cutoff of $p < 3 \times 10^{-3}$ (Table 5.6) and no SNPs met the haplotype-based significance cutoff of adjusted $p < 0.05$. The top two unadjusted *p*-value SNPs are in strong LD ($r^2 = 1$) in the LWK Hapmap population and are located in the first intron of the *ABCC4* gene, which encodes the MRP4 drug transporter (rs7317112: unadjusted $p = 2.8 \times 10^{-3}$, OR = 0.54, 95% CI = 0.80 - 0.36; rs8001475: unadjusted $p = 2.9 \times 10^{-3}$, OR = 0.55, 95% CI = 0.81 - 0.37) (Figure 5.8). The third most associated SNP is a missense mutation in *SLC28A1*, which encodes the CNT1 drug transporter (rs2242046: unadjusted $p = 3.1 \times 10^{-3}$, OR = 0.19, 95% CI = 0.57 - 0.06). These SNPs were chosen for further bioinformatic analysis.

Table 5.6. Top Candidate Gene Variants Associated with NRTI-Induced Peripheral Neuropathy

SNP	MAF	OR	U95 CI	L95 CI	*P*	*P* adjusted*	Gene	Feature
rs7317112	**0.46**	**0.54**	0.80	0.36	**0.0028**	**0.34**	***ABCC4***	**Intronic**
rs8001475	0.46	0.55	0.81	0.37	0.0029	0.35	*ABCC4*	Intronic
rs2242046	0.08	0.19	0.57	0.06	0.0031	0.38	*SLC28A1*	Missense

**P* values adjusted for the number of haplotype blocks tested across genes.
The lowest p-value SNP for each LD block is highlighted in bold. Upper 95% Confidence interval, U95CI; Lower 95% Confidence interval, L95CI.

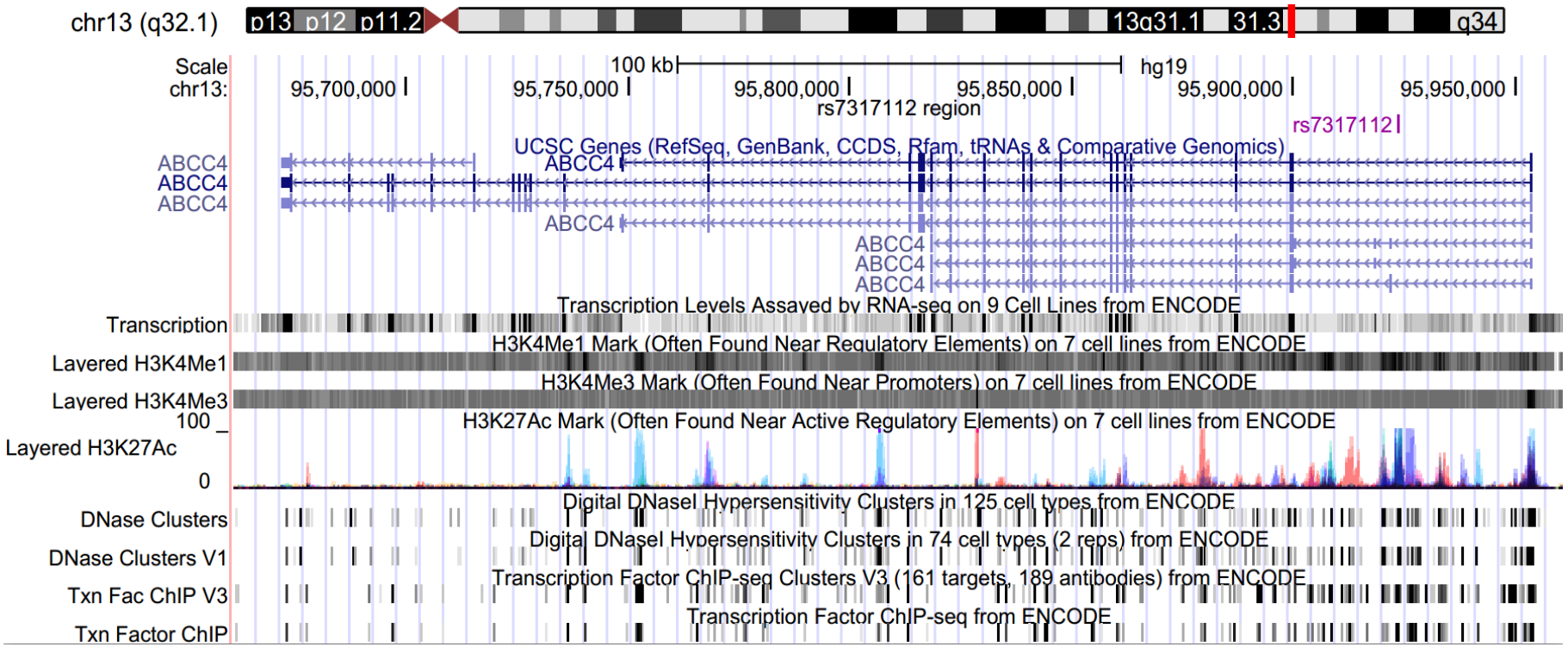

Figure 5.8. UCSC genome browser image showing position of the top candidate SNP (rs7317112, in purple) associated with NRTI-SN susceptibility in the vicinity of *ABCC4* ($p = 2.8 \times 10^{-3}$).

Haploreg indicates that rs7317112 is highly evolutionarily conserved and located within the first intron of *ABCC4* with multiple active regulatory elements that function as a weak enhancer in multiple tissues. In a GTEx analysis, rs7317112 is associated with increased RNA expression (p = 0.02; Figure 5.9). According to Haploreg, rs2242046 is an enhancer; however GTEx bioinformatic analyses did not reveal any association with the SNP and SLC28A1 RNA expression.

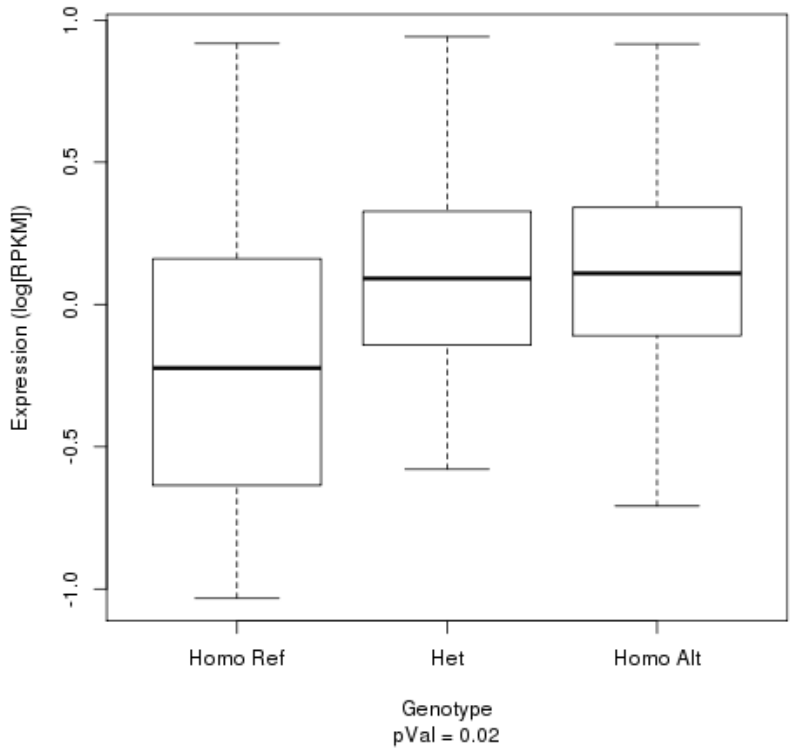

Figure 5.9. The effect of *ABCC4* SNP rs7317112 genotype on RNA expression in nerve tissue.

ABCC4 levels increase significantly with the number of variant alleles present in the genotype (n = 97, p < 0.02). Expression is from RNA-seq (log[RPKM]) and was provided by the GTEx bioinformatic database. RPKM = reads per kilobase per million.

5.5 Discussion

NRTI-SN is a commonly observed side effect of HAART therapy[10]. While some risk factors and genetic predictors of NRTI-SN are known, there still is uncertainty about what genes have the most influence on the development of NRTI-SN. This study used a whole genome and candidate gene approach to discover novel genetic predictors of NRTI-SN. While no SNPs reached genome-wide significance, the top hit (rs188829890) had a 'promising' *p*-value ($p = 1.47 \times 10^{-6}$). This SNP was found to be in a LWK population specific eQTL locus for *VAMP4*. The candidate gene study revealed three SNPs in the *ABCC4* (rs731112, unadjusted $p = 2.8 \times 10^{-3}$; rs8001475, unadjusted $p = 2.9 \times 10^{-3}$) and *SLC28A1* (rs2242046, unadjusted $p = 3.1 \times 10^{-3}$) genes that reached gene-based significance ($p < 3 \times 10^{-3}$). No SNPs reached haplotype-corrected significance. Publically available data shows that rs731112 is associated with increased ABCC4 expression in nerve tissue and is in an active regulatory region. Neither of these SNPs reached statistical significance in the replication study; however this may be due to the small sample size of the replication cohort. rs731112 had a slightly improved *p*-value in the meta-analysis. Despite the limited power of these studies, the results for these genome-wide and candidate gene studies are worthy of follow-up studies.

VAMP4 is a largely uncharacterized gene that is in the vesicle-associated membrane protein (VAMP)/synaptobrevin family. These proteins generally are involved in the docking and/or fusion of synaptic vesicles with the presynaptic membrane. VAMP4, unlike other VAMPs, has been shown to selectively maintain bulk Ca^{2+}-dependent asynchronous release in neuronal cells[45]. When the function of VAMP4 is

reduced, nerves continue conducting without a pause between neurotransmitter releases[45]. The role that VAMP4 may play in the development of NRTI-SN is still unclear, however it can be surmised that a reduction of VAMP4 would lead to increases in presynaptic neurotransmitter release, which will lead to increases in stimulation of the post-synaptic neuron. An increase of stimulation of the postsynaptic neuron leads to increases in the expression of several molecular signaling molecules associated with neuropathic pain including cytokines, COX2 enzymes and ion channels[46]. NRTIs are known to cause neuronal toxicity by causing mitochondrial damage[7,28,47]. This is the same mechanism that is seen in some inherited neuropathies such as Charcot-Marie Tooth disease[48]. Additionally, changes in neurotransmitter release have been documented to be one mechanism of neuropathic pain[49]. Therefore it is possible that after a neuron has been damaged by NRTI exposure, a decrease in VAMP4 expression could lead to increases in neuropathic pain due to a lack of regulation of neurotransmitter release.

CNT1 (*SLC28A1*) and MRP4 (*ABCC4*) can transport NRTIs[50,51,52]. CNT1 is an influx transporter and MRP4 is an efflux transporter; both of these transporters should influence NRTI systemic exposure and possibly the levels of NRTI in the dorsal root ganglion. rs7317112 is associated with increased expression of ABCC4 in nerve tissue. As MRP4 is an efflux transporter, increases in expression of this protein would lead to a reduced amount of drug accumulating in the nerve cell, which would decrease the neuronal damage caused by NRTI exposure, consistent with the protective effect observed in the current study. This SNP has also been shown to predict methotrexate plasma levels in pediatric acute lymphoblastic leukemia patients[53]. SLC28A1 is

primarily apically expressed in the liver and kidney and importantly is expressed in the dorsal root ganglion [54,55]. rs2242046 causes a missense mutation in the *SLC28A1* gene and would be expected to result in less systemic drug exposure and less drug entering the nerve cell and therefore less exposure to NRTIs. While this SNP has not been functionally characterized, it has been shown to predict clinical outcomes in breast cancer patients receiving gemcitabine which is also a nucleoside analog[56]. While other studies have seen an association with HFE and POLG and NRTI-SN, these findings have not been replicated and this study was also not able to replicate these findings[19,22]. Other studies have also identified mutations in mitochondrial DNA (mtDNA) that are associated with NRTI-SN, however, mtDNA was not available in this study[20,21,57].

The largest limitation to this study is a lack of power to detect small effect sizes due to a small sample size. This is evident by the lack of SNPs that met genome-wide significance. However, this study is meant to be hypothesis generating and provides novel genes for further investigation. Another potentially confounding factor is neuropathy caused by HIV infection. Although an effort was made to account for peripheral neuropathy due to HIV infection, there is a possibility that case status was assigned incorrectly. Additionally, the definition of the phenotype may be skewed by the patients' perception of pain due to advanced HIV infection. To attenuate this effect a clinician that specializes in HIV patients was consulted to ensure proper phenotype definition.

5.6 Conclusion

This study identified several potential genetic predictors for the development of NRTI-SN in genes with known roles in nerve function or intracellular NRTI exposure. Further *in vitro* and clinical studies are warranted to define a role for VAMP4, MRP4, CNT and OAT1 in NRTI-induced peripheral neuropathy. A long term goal is to define genetic markers that could be used to identify patients with modified risk for NRTI-SN prior to drug exposure. Genetic studies such as these may also identify novel targets for treating peripheral neuropathies including, diabetic neuropathy, chemotherapeutic neuropathy and congenital neuropathies.

5.7 References

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Chapter 6

Conclusions

Since its emergence in the 1980's, HIV has been one of the most important epidemics worldwide[1]. HIV is a lentivirus that affects the immune system by depleting the T-cell population of the host and if left untreated results in death due to opportunistic infections[2]. In addition to immune system dysfunction, HIV infection also results in numerous complications which may result in wasting, neurologic and other complications[3,4]. HIV is a genetically diverse virus that includes groups and subgroups that have differing regional prevalences[5].

Since the approval of azidothymidine, 28 drugs have been approved for the treatment of HIV and more are currently in the drug development pipeline. HIV treatment generally consists of inhibiting viral replication through multiple mechanisms using highly active antiretroviral therapy (HAART)[6-8]. In the developing world, HAART consists of drug regimens containing two nucleoside reverse transcriptase inhibitors (NRTI) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI)[7]. These drug regimens are efficacious and consistently reduce viral load and increase $CD4^+$ T-cell counts, however, the first and second generation of antiretrovirals (ARV) that are used in the developing world have greater toxicity than their newer counterparts[9,10].

There have been numerous pharmacogenomic associations impacting the pharmacokinetics, pharmacodynamics and toxicity of ARVs, many of them in genes important in metabolism and disposition of ARVs[11-14]. The focus of these studies has

been primarily on drug metabolizing enzymes (CYP450s and UGTs) and in membrane transporters in the SLC and ABC superfamilies.

This dissertation describes research to further characterize the role that host genetics play in the development of HIV infection complications, ARV pharmacology and ARV toxicity. Specifically, it is focused on the role of host genetics in HIV-induced peripheral neuropathy (HIV-SN), the pharmacogenetics of nevirapine (NVP) pharmacokinetics and the pharmacogenetics of NRTI-induced peripheral neuropathy (NRTI-SN). The overall goal of this dissertation is to increase the understanding of the role of host genetics on HIV infection complications and ARV pharmacogenetics and to provide data to direct further research into these fields of study.

In chapters 2 and 3, research was performed to assess and characterize the heritability of NVP pharmacokinetics. It has been shown previously that NVP is metabolized by CYP2B6 and associations between polymorphisms in this gene have been shown to impact NVP pharmacokinetics[15-19]. The role is unclear for *ABCB1*, which has been associated with NVP toxicity, but associations between *ABCB1* and NVP pharmacokinetics have been controversial[13,17,20]. While numerous candidate gene studies investigating the effect of genetics on NVP pharmacokinetics have been performed, the heritability of NVP pharmacokinetics is unknown. To address this, in chapter 2, a study to determine the overall relative genetic contribution to the variance in NVP $AUC_{0\text{-}6hr}$ was performed and a significant relative genetic contribution was found in European and African American subjects. To investigate the role that polymorphisms in the *CYP2B6* and *ABCB1* genes play in NVP pharmacokinetics, a study investigating the association between candidate polymorphisms and NVP $AUC_{0\text{-}6hr}$ was performed.

While no statistically significant associations were found, likely due to the limited sample size, a trend towards association was observed for the *CYP2B6 516G>T* polymorphism. To further investigate the results in chapter 2, an additional candidate gene study investigating the genetics of NVP pharmacokinetics was performed in HIV+ Ugandans. Significant associations between NVP C_{min} and previously known polymorphisms in *CYP2B6* and a novel polymorphism in *ABCC10* were observed. These results reiterate the importance of *CYP2B6* in NVP pharmacokinetics and also provide novel evidence that *ABCC10* is also important in NVP pharmacokinetics. Additionally, a composite genotype consisting of these polymorphisms was was a predictor of NVP C_{min}. The effect of composite genotypes has been reported for *CYP2B6* and efavirenz pharmacokinetics, but not for NVP[21]. The combined effect of *CYP2B6* and *ABCC10* suggests that variation in NVP pharmacokinetics is polygenic.

One of the main complications of HIV infection is peripheral neuropathy[22]. While the development of HIV-SN is rare in the developed world, it occurs with a higher frequency in the developing world because of difficulties in access to healthcare[23]. The mechanism of HIV-SN is poorly understood and no studies investigating the role that host genetics play in the development of HIV-SN have been performed. In chapter 4, a genome-wide association study with a case vs. control design was used to investigate the role that host genetics play in the development of HIV-SN. A SNP proximal to the 5' end of *FOLH1*, a gene important in folate metabolism, was associated with the development of HIV-SN, with a higher incidence of the variant allele in HIV-SN cases. This SNP is in a region that has active regulatory features and is associated with a decrease in *FOLH1* expression in neuronal tissue. A trend towards an association between the *FOLH1*

SNP and HIV-SN was found in a replication cohort and statistical significance was improved when a meta analysis of the discovery and replication studies was performed. Folate responsive peripheral neuropathies have been extensively documented, particularly in alcoholic patients[24]. Folate deficiencies are also commonly observed in African HIV+ patient populations[25]. The results of the present study suggest that deficiencies in folate metabolism may play a role in the development of HIV-SN. This study also may be informative for other peripheral neuropathies, *e.g.* Type 2 diabetic neuropathy, and warrants additional clinical and experimental study.

A common NRTI toxicity is peripheral neuropathy, thought to be caused by mitochondrial toxicity[26]. NRTIs compete with endogenous nucleotides during mitochondrial DNA (mtDNA) replication, which leads to errors in mtDNA replication and depletion of mtDNA. This effect is seen more with older NRTIs, such as AZT and d4T, due to their higher affinity for the mitochondrial polymerase, polγ[27]. Several candidate gene studies have been performed to investigate the effect of host genetics on the development of NRTI-SN, however, few associations have been observed[28-30]. A GWAS was performed in this dissertation to characterize unknown genetic predictors of NRTI-SN. The SNP with the lowest *p*-value in this study was an intergenic SNP nearest to the *VAMP4* gene, however, it did not reach genome-wide significance, likely due to a small study population. *VAMP4* is largely uncharacterized, but has been implicated in the regulation of asynchronous synaptic transmission. This *VAMP4* SNP is in an active regulatory region and is associated with a decrease in VAMP4 expression but replication was not successful in the small sample set available. A candidate gene study was also performed and SNPs in SLC22A1 and SLC28A1 were associated with

the development of NRTI-SN. These genes are known to transport NRTIs and may influence the exposure of a patient to NRTIs. The findings in the GWAS and candidate gene studies are interesting, although the sample size was limited, and suggest that further research into these genes is necessary.

The research in this dissertation highlights the importance of host genetics in HIV infection, ARV pharmacokinetics and toxicity. It also is important to study the effects of host genetics in multiple ethnic populations due to the differences in genetic variation observed in different ethnicities. By better understanding the role of host genetics, advances can be made in the prevention of disease complications and drug toxicities. The results presented in this dissertation provide novel targets, but require additional experimental and clinical study.

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