HIV DRUG RESISTANCE, TROPISM, AND GENETIC DIVERSITY IN BLACK MEN WHO HAVE SEX WITH MEN

by
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Abstract

Black men who have sex with men (MSM) are disproportionately affected by HIV in the United States (US). Despite representing less than 1% of the US population, Black MSM accounted for the highest number of new HIV diagnoses in 2014. Culturally-tailored interventions are needed to control the HIV epidemic in this population. The HIV Prevention Trials Network (HPTN) 061 study was designed to assess the feasibility of a multi-component intervention for reducing HIV incidence among Black MSM. The HPTN 061 study enrolled 348 HIV-infected men and 1,205 HIV-uninfected men; 28 men seroconverted during the study for an annual incidence rate of 3.0% overall and 5.9% among younger men (aged 18-30 years). Men in the HPTN 061 study completed detailed demographic and behavioral assessments at each study visit, which included an evaluation of risk behaviors associated with HIV transmission and acquisition. Demographic and behavioral factors that influence HIV transmission and acquisition may also impact the characteristics of infecting viral populations and their subsequent evolution. The work in this dissertation analyzed factors associated with HIV drug resistance, tropism, and genetic diversity in this cohort of Black MSM in the US. These findings provided new insights relevant to HIV treatment and prevention in this high-risk population.

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List of Abbreviations

3TC	lamivudine
ACASI	audio computer-assisted self-interview
AIDS	acquired immune deficiency syndrome
APV	amprenavir
ART	antiretroviral treatment
ARV	antiretroviral
ATV	atazanavir
BED-CEIA	BED capture enzyme immunoassay
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CTL	cytotoxic T lymphocyte
DM	dual/mixed
DNA	deoxyribonucleic acid
DRM	drug resistance mutation
DRV	darunavir
EFV	efavirenz
FDA	Food and Drug Administration
FTC	emtricitabine
HIV	human immunodeficiency virus
HPTN	HIV Prevention Trials Network
HRM	high resolution melting
IDV	indinavir
INSTI	integrase strand transfer inhibitor
LPV	lopinavir
MAA	multi-assay algorithm

MCR	multi-class drug resistance
MSM	men who have sex with men
NFV	nelfinavir
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
NVP	nevirapine
PCR	polymerase chain reaction
PEP	post-exposure chemoprophylaxis
PI	protease inhibitor
PIC	pre-integration complex
PrEP	pre-exposure chemoprophylaxis
R5	CCR5-using
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
RTV	ritonavir
STI	sexually transmitted infection
TDR	transmitted drug resistance
TFV	tenofovir
US	United States
X4	CXCR4-using
ZDV	zidovudine

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Chapter 1: Introduction

Disparities in the US HIV epidemic among Black MSM

In the United States (US), the incidence of human immunodeficiency virus (HIV) infection has remained relatively stable since the mid-1990's [1-3]. HIV infects approximately 50,000 individuals each year, over half of whom are men who have sex with men (MSM) [1-3]. MSM comprise just 2% of the US population. Yet, the US Centers for Disease and Control and Prevention (CDC) estimate that MSM represent over half of all diagnosed individuals living with HIV in 2011 and half of all individuals who have died from acquired immune deficiency syndrome (AIDS) since the start of the HIV epidemic [4]. Young MSM (aged 13-29 years) are also the only risk group in the US with a rising HIV incidence rate [2]. These numbers highlight the urgency of identifying effective interventions for HIV prevention in MSM.

Among MSM, Black MSM are most affected by HIV. Black MSM represent 36% of new HIV infections among MSM, and approximately half of all new infections among young MSM occur in young Black MSM [2, 3]. Despite their elevated HIV incidence, Black MSM typically engage in fewer risk behaviors than other MSM. Black MSM report less unprotected anal intercourse, less substance use during sex, and lower numbers of male sexual partners [5]. Black MSM also participate in more HIV prevention behaviors, such as HIV testing, condom use, and disclosure of HIV status to partners [5].

Recent studies suggest that the high rates of HIV among Black MSM may be the result of structural barriers to HIV testing and care [5, 6]. Compared to White MSM with HIV infection, HIV-infected Black MSM are less likely to have health insurance and more likely to be diagnosed at a lower CD4 cell count [5, 6], which suggests late HIV diagnosis. More than half of HIV-infected Black MSM are not aware of their seropositive status [7]; therefore, they are not receiving antiretroviral treatment (ART) and are at higher risk for transmitting HIV to others. Several studies also indicate that Black MSM tend to have same-race sexual partners [5, 8, 9], which may contribute to the high HIV

incidence rates among Black MSM when combined with the high prevalence of undiagnosed HIV infection.

HPTN 061: The Brothers Study

Because of the racial disparity of HIV infection in MSM, the HIV Prevention Trials Network (HPTN) evaluated the feasibility of a multi-component intervention for reducing HIV incidence in Black MSM [10, 11]. The HPTN 061 study is the largest longitudinal cohort of Black MSM in the US to date. The study enrolled both HIV-infected men at a high risk for HIV transmission and HIV-uninfected men at a high risk for HIV acquisition in six US cities between 2009 and 2010. Men were followed for one year, provided samples for laboratory testing, and completed detailed behavioral assessments and social and sexual network questionnaires. Many of the men enrolled in HPTN 061 were poor (annual household income <$10,000) and did not have a college education [11]. Nearly a quarter of the men were HIV infected at study enrollment [11]. The majority of those men were classified as previously diagnosed by either self-report or antiretroviral (ARV) drug testing [12]. Compared to previously-diagnosed men, HIV-infected men who were classified as newly diagnosed were more likely to be younger, have sexual partners of unknown HIV status, and have multiple sexually transmitted infections (STIs) [11]. Compared to HIV-uninfected men, newly-diagnosed men were more likely to be unemployed, have unprotected receptive anal intercourse, and have STIs [11].

The annual HIV incidence rate in HPTN 061 was 3.0% overall (95% confidence interval [CI]: 2.0-4.4%) with the highest HIV incidence among younger men (aged 18-30 years, 5.9%, 95% CI: 3.6-9.1%) [10]. In addition to younger age, HIV incidence was higher among men who reported unprotected receptive anal intercourse with HIV-positive partners or partners of unknown HIV status. Younger men in HPTN 061 were more likely to have STIs during follow-up and have unmet healthcare needs, such as not

having a usual place for health care [10]. These findings from HPTN 061 further highlight the need for culturally-tailored interventions to increase Black MSM engagement in health care, including HIV treatment and prevention, to reduce HIV transmission and acquisition.

The HIV life cycle

HIV infection is initiated when the virus infects and replicates within CD4+ T cells to form infectious viral particles. Cellular HIV replication occurs in stages [13]. First, the viral envelope binds to the host cell CD4 receptor. This facilitates conformational changes in the viral envelope that allow the virus to bind either the CCR5 or CXCR4 coreceptor and fuse to the cellular membrane. HIV ribonucleic acid (RNA) and proteins are then released into the cytoplasm. The HIV RNA is reverse transcribed into deoxyribonucleic acid (DNA) by HIV reverse transcriptase, and a pre-integration complex (PIC) forms that contains HIV DNA and viral proteins. The PIC is translocated into the nucleus where the HIV DNA is integrated into the host cell DNA by HIV integrase. New HIV RNA and viral proteins are transcribed and translated from the integrated HIV DNA using host cell machinery. These components are assembled at the cellular membrane, and new immature virions form via budding. After budding, HIV protease cleaves the structural polyprotein to form mature, infectious viral particles.

HIV treatment and prevention

Over 20 ARV drugs have been approved by the US Food and Drug Administration (FDA) for HIV treatment to date [14]. These drugs comprise five ARV drug classes: entry inhibitors (i.e., coreceptor antagonists and fusion inhibitors), nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), integrase strand transfer inhibitors (INSTIs), and

protease inhibitors (PIs). Each ARV drug class targets a specific step in the HIV life cycle. The goal of ART for HIV infection is to drastically reduce cellular HIV replication, which is reflected by a decrease in plasma HIV viral load to below the limit of detection for clinical assays (<20-400 HIV RNA copies/mL). Individuals who achieve viral suppression have better clinical outcomes due to immune restoration (i.e., an increase in the number of circulating CD4 cells) [15].

Since the mid-1990's, treatment guidelines have recommended triple ARV drug combinations for ART. Triple drug regimens comprised of at least two ARV drug classes help combat the development of HIV drug resistance. HIV variants with resistance to individual ARV drugs naturally occur before ART initiation [16]. This is due to the high genetic diversity of HIV resulting from its large viral population, short viral half-life, error-prone reverse transcription, and frequent viral recombination [17]. Treatment with a single ARV drug rapidly selects for these drug-resistant HIV variants. However, the probability that a HIV variant with resistance to three ARV drugs from multiple drug classes will naturally occur is much lower [16, 18].

ARV drugs can also be used for pre- or post-exposure chemoprophylaxis (PrEP or PEP) to prevent HIV infection. The efficacy of chemoprophylaxis for HIV prevention was first demonstrated in adults through PEP. In a case-control study of healthcare workers after needlestick injury; healthcare workers who took zidovudine (ZDV) alone within 72 hours of injury had an 80% lower chance of acquiring HIV [19]. Additional studies showed that dual regimens for non-occupational PEP (i.e., after possible HIV exposure from sex or injection drug use) were also effective [20, 21]. Current US guidelines recommend initiating a triple drug regimen for PEP (similar to ART) within 72 hours of exposure to bodily fluids from an individual who may be HIV infected [22, 23]. PEP studies validated the use of ARV drugs to prevent HIV infection, which led to the development of PrEP as an alternative approach for HIV prevention. In 2012, the US

FDA approved the use of Truvada® (co-formulated emtricitabine [FTC] and tenofovir [TFV] disoproxil fumarate) for PrEP in HIV-uninfected individuals at a high risk for HIV acquisition [24]. Taken daily, Truvada can decrease the risk for HIV acquisition among HIV-uninfected individuals by more than 90% [25-27].

HIV drug resistance

As mentioned above, drug-resistant HIV variants can naturally arise due to the extensive viral diversity of HIV. Prolonged exposure to non-suppressive levels of ARV drugs can select for drug-resistant viruses [28], which can paradoxically lower HIV diversity if genetic bottlenecking occurs [29]. Individuals can be exposed to non-suppressive levels of ARV drugs in a variety of settings. In the context of ART, individuals may be taking a suboptimal ART regimen or a regimen that includes one or more drugs to which they already have resistance. Certain medications and other substances can also interfere with ARV drug absorption or activation, or enhance the metabolism or clearance of ARV drugs [30]. Non-suppressive levels of ARV drugs may also occur in individuals who are non-adherent to ART and in individuals taking ARV drugs for PrEP, PEP, or recreational use before knowing they are HIV infected [31-33].

HIV drug resistance can lead to treatment failure and limit future treatment options. Resistance mutations to one ARV drug also often confer resistance to other ARV drugs in the same drug class [28]. Drug-resistant HIV strains can also be transmitted to others, which can affect treatment options for newly-infected individuals. Transmitted drug resistance (TDR) has been associated with an increased risk for virologic failure in patients on ART [34]. Determining the prevalence of HIV drug resistance and TDR is important for understanding the current state of the HIV epidemic and guiding treatment recommendations.

HIV coreceptor tropism can also affect HIV susceptibility to certain ARV drug classes. Cellular HIV entry is mediated by the viral envelope, CD4 receptor, and CCR5 or CXCR4 coreceptors. CCR5 coreceptor antagonists bind to the CCR5 coreceptor to prevent cellular HIV entry; currently, the only CCR5 antagonist approved for use in the US is maraviroc [14]. HIV tropism is determined by coreceptor usage; viruses can use CCR5 exclusively (R5), CXCR4 exclusively (X4), or both coreceptors (dual-tropic). Viral populations can be comprised of solely R5 or X4 viruses or include dual/mixed (DM) viruses. R5 viruses are susceptible to CCR5 antagonists while X4 viruses are not. Consequently, maraviroc use in individuals who have DM viruses may select for X4 and dual-tropic viruses [35].

The work in this dissertation characterized HIV drug resistance, tropism, and genetic diversity in samples from HIV-infected Black MSM enrolled in the HPTN 061 study. In particular, this work explored the association of demographic factors and risk behaviors with the characteristics of viral populations in these men. These studies included men who were HIV infected at study enrollment and men who acquired HIV infection (i.e., seroconverted) during the study. Understanding HIV virology in these two groups provides new insights into the general HIV epidemic in this population, as well as HIV-related issues affecting newly-infected Black MSM. These findings will help guide interventions to improve the quality of HIV treatment and prevention among Black MSM in the US.

Chapter 2: Methods

Study cohort

The samples used in this research were obtained from the HPTN 061 study. HPTN 061 (NCT 00951249) was a multi-site study that enrolled 1,553 self-identified Black MSM between July 2009 and October 2010 in the US [10, 11]. The study enrolled men at eight sites in six cities: Atlanta, GA; Boston, MA; Los Angeles and San Francisco, CA; New York City, NY; and Washington, DC. Men were recruited from the community (index participants) or were sexual partners referred by index participants; men were eligible for the study if they reported having unprotected anal intercourse with a man in the six months prior to study enrollment. Index participants were comprised of three groups: (1) HIV-uninfected men; (2) HIV-infected men who reported that they were unaware of their HIV status (newly diagnosed); and (3) HIV-infected men who reported that they were aware of their HIV status (previously diagnosed) [11]. Index participants who reported that they were previously diagnosed included men who were at increased risk for HIV transmission (defined as not receiving HIV care and having unprotected sex with HIV-uninfected partners or partners of unknown HIV status) or decreased risk for HIV transmission (defined as being in HIV care or only having unprotected sex with HIV-infected partners; capped at a maximum of 10 per study site). HPTN 061 study participants were followed for one year. Men provided demographic data to an interviewer at the enrollment visit and completed a behavioral assessment and social and sexual network questionnaire using audio computer-assisted self-interview (ACASI) technology at each visit [10, 11]. Plasma samples were stored at each visit for laboratory assessments.

HIV and STI testing were performed at the study sites at enrollment and at the 6- and 12-month follow-up visits [10]. HIV screening was performed at the study sites using a single HIV rapid test; if the rapid test was reactive, a Western blot was performed at a local laboratory. CD4 cell count and HIV viral load were measured for men with HIV

infection. Stored plasma samples were tested retrospectively at the HPTN Laboratory Center (Johns Hopkins University, Baltimore, MD) for quality assurance, to identify men who had acute HIV infection at enrollment, and to confirm cases of HIV seroconversion [10, 11]. Enrollment samples from participants with low or undetectable HIV RNA (<1,000 copies/mL) who reported they were newly diagnosed were also tested for the presence of ARV drugs; men who had ARV drugs detected that were consistent with ART were considered previously diagnosed [10-12].

All study participants in HPTN 061 provided written informed consent for participation. Each study was approved by institutional review boards at each participating institution.

Identification of recent HIV infection

Multi-assay algorithms (MAAs) that use a combination of serologic and non-serologic biomarkers can discriminate between new (recent) HIV infections and non-recent infections [36-38]. The MAA used in this dissertation identifies recent infections in clade B HIV populations using two serologic assays (the BED-capture enzyme immunoassay [BED-CEIA] and an avidity assay) and two non-serologic biomarkers (CD4 cell count and HIV viral load) [36, 38]. Clade B is the predominant HIV subtype in the US [39]. Men in HPTN 061 were classified as recently infected if they had a CD4 cell count >50 cells/mm, a BED-CEIA <1.6 normalized optical density units, an avidity index <85%, and a HIV viral load >400 copies/mL. The window period for recent infection using this MAA is 159 days (95% CI: 134-186 days) [37].

Detection of ARV drugs

A rapid, high-throughput ARV drug assay was used to test samples for the presence of 15 ARV drugs: four NRTIs (FTC, TFV, ZDV, and lamivudine [3TC]); two

NNRTIs (efavirenz [EFV] and nevirapine [NVP]), and nine PIs (atazanavir [ATV], amprenavir [APV], darunavir [DRV], indinavir [IDV], lopinavir [LPV], nelfinavir [NFV], saquinavir, tipranavir, and ritonavir [RTV]). Plasma samples were prepared by combining 100 µL plasma with 300 µL acetonitrile containing 125 ng/mL deuterated morphine-d3. The resulting supernatant was dried and reconstituted with 150 µL water. ARV drugs were detected using high-performance liquid chromatography coupled with high-resolution accurate mass-mass spectrometry (Exactive-Orbitrap; Thermo Scientific, Pittsburgh, PA). The mobile phase system consisted of 10 mM ammonium acetate and 0.1% ammonium hydroxide in methanol. Samples were introduced onto a 1.9-µm Hypersil Gold perfluorinated phenyl column at 100% aqueous composition; elution occurred during a 3.25 minute step-and-hold isocratic step to 100% methanol. Positive full-ion MS and all-ion fragmentation were conducted at 100,000 and 25,000 resolution, respectively; negative full-ion MS was conducted at 100,000 resolution. ZDV and EFV were detected using parent-ion scanning in negative ion mode and fragment identification in positive ion mode. All other ARV drugs were analyzed in positive ion mode for both the parent and fragment ions. The limit of identification for all 15 ARV drugs in this assay was 10 ng/mL.

Detection of HIV drug resistance mutations

The ViroSeq HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA) is a population (bulk) sequencing method that generates a consensus sequence that can be used to analyze HIV drug resistance mutations (DRMs) associated with three ARV drug classes: NRTIs, NNRTIs, and PIs. The ViroSeq System reliably detects mutations present in more than 20% of the viral population [40, 41], but can detect some mutations at much lower levels [42]. ViroSeq is approved by the US FDA for diagnostic use to guide ART.

ViroSeq HIV-1 Genotyping System

HIV RNA was isolated from 500 µL of plasma, purified, and resuspended in 50 µL RNA diluent. Moloney murine leukemia virus reverse transcriptase and a single primer were used to reverse transcribe 10 µL HIV RNA into complementary DNA (cDNA), and a 1.8 kb region of HIV *pol* was amplified from the cDNA by polymerase chain reaction (PCR). A nested PCR was performed for samples with low HIV viral loads that failed to amplify using the standard procedure [43]. The resulting PCR product was quantified, purified, and diluted to an optimized concentration for sequencing. Samples were sequenced using seven primers (four forward and three reverse) and standard dideoxy chain termination using an Applied Biosystems 3130*xl* Genetic Analyzer (Life Technologies Corporation, Grand Island, NY). Sequences from each sample were assembled into a 1.3 kb consensus sequence and compared to a reference sequence to identify mutations using the ViroSeq HIV-1 Genotyping Software, v2.8. The 1.3 kb consensus sequence includes the coding regions for all of HIV *protease* and approximately two-thirds of HIV *reverse transcriptase* (codons 1-335). HIV drug resistance to NRTIs, NNRTIs, and PIs was predicted using the ViroSeq Algorithm Advisor included in the software package.

ViroSeq HIV-1 Integrase Genotyping Kit

The ViroSeq HIV-1 Integrase Genotyping Kit (Celera Diagnostics, Alameda, CA) uses HIV RNA prepared by the ViroSeq HIV-1 Genotyping System; the kit is for research use only. A 1.1 kb amplicon of HIV *integrase* was amplified using a one-step reverse transcription-PCR (RT-PCR) with Omniscript and Sensiscript reverse transcriptases and 10 µL HIV RNA. The resulting PCR product was quantified, purified, and diluted to an optimized concentration for sequencing. Samples were sequenced using four primers

(two forward and two reverse) and standard dideoxy chain termination using an Applied Biosystems 3130*xl* Genetic Analyzer. Sequence data from each sample were assembled into a 0.9 kb consensus sequence and compared to a reference sequence to identify mutations using the ViroSeq Integrase Software, v1.0. The 0.9 kb consensus sequence includes all of HIV *integrase*. HIV drug resistance to INSTIs was predicted using the ViroSeq Algorithm Advisor included in the software package.

Prediction of rilpivirine resistance

HIV drug resistance to rilpivirine, a NNRTI, was predicted using the HIV *reverse transcriptase* consensus sequence (codons 1-335) generated by the ViroSeq HIV-1 Genotyping System. Consensus sequences were manually analyzed for rilpivirine-associated DRMs included in the ViroSeq Algorithm Advisor for the ViroSeq HIV-1 Genotyping Software, v3.0 (Celera Diagnostics, Alameda, CA). Rilpivirine resistance was predicted according to the ViroSeq Algorithm Advisor criteria.

Determination of HIV coreceptor tropism

The Trofile assay (Monogram BioSciences, San Francisco, CA) is a clinically-validated, phenotypic tropism assay, which can distinguish HIV coreceptor usage for HIV variants present in 0.3% of the viral population [44]. Full-length *env* sequences from each sample were amplified and cloned into expression vectors [44, 45]. Pseudoviruses were prepared by cotransfecting human embryonic kidney cell cultures with the *env* expression vectors and a replication-defective HIV genomic vector containing a luciferase reporter gene. HIV coreceptor tropism was determined by infecting $CCR5^+$ and $CXCR4^+$ cells in the presence and absence of CCR5 and CXCR4 inhibitors. Viral replication was quantified by measuring luciferase activity (reported as relative light units, which reflect the level of luciferase activity). Analysis of individual viral variants

(clonal analysis) was also performed at Monogram BioSciences using samples from seroconverters who had DM viruses. HIV tropism was determined for 16-20 *env* clones from the viral populations of each individual. In addition, the V3 loop of each clone was sequenced using standard dideoxy-chain termination [46].

Measurement of HIV diversity

Our research group developed a high resolution melting (HRM) diversity assay that can quantify HIV diversity as a single numeric HRM score without sequencing [47, 48]. The HRM scores generated by the assay are highly correlated with sequence-based diversity measures [47, 49].

Preparation of DNA templates for the HRM diversity assay

HIV DNA used to analyze HIV *gag* and *pol* was generated using the ViroSeq HIV-1 Genotyping System. The diluted PCR products used for sequencing were diluted 1:10 for HRM analysis [47]. HIV DNA used to analyze HIV *env* was generated using the QIAGEN OneStep RT-PCR Kit (QIAGEN, Valencia, CA) and HIV RNA prepared by the ViroSeq HIV-1 Genotyping System. A 0.7 kb amplicon of HIV *env* was amplified using Omniscript and Sensiscript reverse transcriptases, 10 µL HIV RNA, and a forward (JH35F: 5'-TGARGGACAATTGGAGAARTGA-3') and reverse (JH38R: 5'-GGTGARTATCCCTKCCTAAC-3') primer [48]. The resulting PCR product was quantified and diluted to an optimized concentration for additional analysis. ExoSap-IT (United States Biochemical Corporations, Cleveland, OH) was then used to purify 5 µL of the diluted PCR product. The purified PCR product was added to 45 µL diethylpyrocarbonate-treated water and then diluted 1:10 for HRM analysis.

HRM diversity assay and DivMelt analysis

The HRM diversity assay was performed for six regions of the HIV genome: two in *gag* (GAG1 and GAG2), one in *pol* (POL), and three in *env* (ENV1, ENV2, and ENV3, Table 2.1) [47, 48]. Amplicons for each region were generated using 1 µL 1:10 diluted and purified PCR product, forward and reverse primers, and 1x LightScanner Master Mix amplification buffer (Idaho Technology, Salt Lake City, UT). The LightScanner Master Mix includes Taq polymerase and LCGreen Plus dye, which is incorporated into the amplicons. The resulting amplicons were melted using the LightScanner Instrument (Model HR 96, Idaho Technology, Salt Lake City, UT) at a melting range of 68-98°C with a 65°C hold (for GAG1, GAG2, and POL amplicons) or 60-98°C with a 57°C hold (for ENV1, ENV2, and ENV3 amplicons). Melting data for each amplicon were processed using the LightScanner software (Idaho Technology, Salt Lake City, UT) to generate melting curves that represent the negative derivative of the fluorescence vs. temperature (-dF/dT).

Melting curves were analyzed using the DivMelt software package, version 1.0.2 [50] in R, version 2.14.1 (The R Foundation for Statistical Computing, Vienna, Austria). The width of the melting peak corresponds to the HRM score (i.e., the output of the HRM diversity assay). Region-specific DivMelt analysis protocols were used.

Statistical methods

All statistical analyses were performed by the HPTN Statistical and Data Management Center (Fred Hutchinson Cancer Research Center, Seattle, WA). Details of these analyses are described in Chapters 3, 4, and 5.

Tables

Table 2.1. HRM regions analyzed using the HRM diversity assay.

Region	HXB2 coordinates[a]	HIV genomic region[a]	Primer Sequences
GAG1	1998-2097	*gag* p7 and p1	Forward: 5'-AAATTGCAGGGCCCCTAGGAA-3' Reverse: 5'-TTTCCCTAAAAAATTAGCCTGTCT-3'
GAG2	2068-2278	*gag* p1 and p6; *pol* transframe	Forward: 5'-ACTGAGAGACAGGCTAATTTTTTAG-3' Reverse: 5'-GGTCGTTGCCAAAGAGTGATTTG-3'
POL	2373-2597	*pol* protease and reverse transcriptase	Forward: 5'-AAATGGAAACCAAAAATGATAG-3' Reverse: 5'-CATTCCTGGCTTTAATTTTACTG-3'
ENV1	7798-8036	*env* gp41 heptad repeat 1	Forward: 5'-CAGCAGGWAGCACKATGGG-3' Reverse: 5'-GCARATGWGYTTTCCAGAGCADCC-3'
ENV2	7950-8119	*env* gp41 immunodominant region	Forward: 5'-CTYCAGRCAAGARTCYTGGC-3' Reverse: 5'-TCCCAYTSCAKCCARGTC-3'
ENV3	8016-8299	*env* gp41 heptad repeat 2	Forward: 5'-TGCTCTGGAAARCWCATYTGC-3' Reverse: 5'-AARCCTCCTACTATCATTATRA-3'

Legend for Table 2.1.

Abbreviations: HRM: high resolution melting.

[a] Each HRM amplicon corresponds with the genomic coordinates listed for HXB2 and portions of the genomic regions that encode for the HIV proteins listed.

Chapter 3: Analysis of ARV drug use and HIV drug resistance

ARV drug use and HIV drug resistance to NRTIs, NNRTIs, and PIs

This work has been published and is reprinted here. At the journal's request, no modifications of the material in this dissertation are permitted.

Chen I, Connor MB, Clarke W, Marzinke MA, Cummings V, Breaud A, Fogel JM, Laeyendecker O, Fields SD, Donnell D, Griffith S, Scott HM, Shoptaw S, del Rio C, Magnus M, Mannheimer S, Wheeler DP, Mayer KH, Koblin BA and Eshleman SH. Antiretroviral drug use and HIV drug resistance among HIV-infected Black men who have sex with men: HIV Prevention Trials Network 061. 2015. *J Acquir Immune Defic Syndr*. 69(4):446-452. Wolters Kluwer Health, Lippincott Williams & Wilkins©.

Introduction

HPTN 061 provided important information about the HIV epidemic in Black MSM. By design, most of the HIV-infected men enrolled in HPTN 061 reported that they were unaware of their HIV status (newly diagnosed) or that they were aware of their status (previously diagnosed) but were not in care [11]. However, 54% of the HIV-infected men who reported that they were newly diagnosed had low or undetectable HIV viral loads at study enrollment. Although none of these men reported prior or current ARV drug use, ARV drug testing revealed that 78% of these men were taking ARV drugs consistent with ART, but chose not to disclose this to study staff [12]. Some men had unusual patterns of ARV drugs detected (e.g., two NNRTIs, a NNRTI with a PI, or multiple PIs). A few men had one or two NRTIs detected in the absence of an NNRTI or PI, which suggested that they may have been using ARV drugs for PrEP or PEP [12]. ARV drugs were also detected in some men with low but detectable HIV viral loads (400-1,000 copies/mL). This was concerning, since exposure to non-suppressive levels of ARV drugs promotes selection of HIV with DRMs [28], which can be transmitted to others.

These findings indicated the need for further evaluation of ARV drug use and HIV drug resistance among Black MSM in the HPTN 061 cohort.

This chapter presents a comprehensive analysis of ARV drug use and HIV drug resistance among HIV-infected men in the HPTN 061 cohort who were not virally suppressed. By combining ARV drug testing with HIV genotyping, it was possible to assess levels of drug resistance, the risk for increasing drug resistance, and patterns of ARV drug use in this cohort. Inclusion of ARV drug testing in these assessments, rather than relying solely on self-reported ARV drug use, also provided a more accurate estimate of the frequency of TDR.

Methods

Study cohort

HIV genotyping was performed retrospectively using plasma samples from men who had viral loads >400 copies/mL, including men who were HIV infected at study enrollment and men who seroconverted during the study. All men were asked about ARV drug use for PrEP or PEP at their study visits; men were also asked about prior or current ART.

GenBank accession numbers: KM357930-KM358121.

Statistical methods

Fisher's exact and chi-square tests were used for univariate analyses. Correlates of HIV drug resistance, multi-class drug resistance (MCR), and ARV drug detection were also compared among men who were included in this sub-study (those with HIV genotyping results) vs. men who were not included in this sub-study (those with viral suppression). Multivariate analyses were attempted via generalized linear mixed models

but had inadequate power for modeling the site-to-site variability among covariates. All statistical analyses were performed using SAS, version 9.2 (SAS Institute, Cary, NC).

Results

Analysis of HIV-infected men at study enrollment

Study cohort

In HPTN 061, 348 men were HIV infected at enrollment; 163 were virally suppressed (viral load ≤400 copies/mL) and 14 men refused further testing or had no sample available. The remaining 171 men had HIV viral loads >400 copies/mL; genotyping was successful for 169 of those men (men included in this sub-study). Eighty-one (48%) of the 169 men reported that they were newly diagnosed and 88 (52%) reported that they were previously diagnosed; 31 (35%) of the 88 men reported that they were in HIV care. Demographic and behavioral characteristics of the 169 men are shown in Table 3.1. Compared to the 163 HIV-infected men who were virally suppressed and not included in this sub-study, the 169 men included in this sub-study were more likely to be younger than 30 years old (P<0.001) and were less likely to have reported prior or current ART at study enrollment (P=0.004 and P<0.001, respectively).

HIV drug resistance

At least one DRM was detected in 48 (28%) of the 169 men (Table 3.1). Nineteen (11%) of the men had MCR, including two (1%) who had HIV that was resistant to all three ARV drug classes (NRTIs, NNRTIs, and PIs). NNRTI-associated DRMs were most common, followed by NRTI- and PI-associated DRMs; the most prevalent DRMs were K103N/S and M184V/I. In univariate analyses, older age (>30 years), reporting being in HIV care, and reporting prior or current ART use were significantly associated

with any HIV drug resistance and with MCR; MCR was also more common among men who reported having no or one sexual partner in the previous six months (Table 3.1).

ARV drug detection

None of the men reported ARV drug use for PrEP, and only one reported ARV drug use for PEP. Twenty-one of the 139 men who were asked about ART reported both prior and current ART (30 of the newly-diagnosed men were not asked about ART); 11 men reported prior ART only. At least one ARV drug was detected in samples from 60 (36%) of the 169 men (Table 3.2); 42 (70%) of the 60 men reported no prior or current ARV drug use. ARV drug detection was associated with reporting prior or current ART (P=0.02 and P=0.03, respectively), but was not associated with any of the other demographic or behavioral factors shown in Table 3.1 (data not shown).

NRTIs and PIs were the most frequently detected ARV drug classes, and FTC was the most frequently detected ARV drug (Figure 3.1). Thirty-one (52%) of the 60 men had ARV drugs detected that were consistent with treatment regimens that are currently recommended or were recommended at the time of study enrollment (2009-2010) by the US Department of Health and Human Services (Table 3.2) [51-53]. Because the half-life of NRTIs is shorter than the half-life of NNRTIs and PIs, detection of a PI or NNRTI alone was considered to be consistent with recommended ART if the PI or NNRTI was included in one of the recommended treatment regimens. Detection of ARV drugs that were consistent with a recommended treatment regimen was more frequent among men who reported that they were previously diagnosed (regardless of whether they reported that they were in care) than among men who reported that they were newly diagnosed (68% vs. 31%, P=0.009).

The remaining 29 men had ARV drugs or combinations of drugs detected that are not consistent with recommended treatment regimens (Table 3.2). NFV was

detected in 13 men (7 had NFV alone) and IDV was detected in 2 men (one had IDV alone); these drugs were no longer recommended for treatment at the time the HPTN 061 study was performed. Thirteen men had a single NRTI detected (nine had ZDV, three had FTC, and one had TFV). Detection of a single NRTI or other ARV drugs and drug combinations that were not consistent with recommended ART was more common among men who reported no prior HIV diagnosis than among men who reported that they were previously diagnosed (69% vs. 32%, P=0.009).

Relationship between HIV drug resistance and ARV drug detection

We next evaluated the relationship between HIV drug resistance and ARV drug use in the 48 men who had drug-resistant HIV at enrollment (Figure 3.1). ARV drugs were more frequently detected in these 48 men than in the 121 men without drug resistance (56% vs. 27%, P<0.001). Men with drug-resistant HIV were more likely to have NRTIs and PIs detected (P=0.001 and P<0.001, respectively), including FTC (P<0.001), TFV (P=0.01), 3TC (P=0.002), RTV (P<0.001), ATV (P=0.01), and LPV (P=0.02). Among the 48 men who had drug-resistant HIV, 18 (38%) had at least one ARV drug detected that was not consistent with the DRMs detected, indicating that they were at risk of acquiring additional DRMs. The data obtained from ARV drug testing was also used to estimate the rate of TDR. When the rate of TDR was based solely on the frequency of DRMs among the 137 men who reported no prior or current ARV drug use (i.e., based solely on self-report of ARV drug use), the estimated rate of TDR was 23%. When 14 men with DRMs who also had ARV drugs detected (i.e., when ARV drug testing was used in addition to self-report of ARV drug use) were excluded, the estimated rate of TDR was only 12%. Five (29%) of the 17 men who were classified as having TDR in this assessment had MCR.

Analysis of newly-infected men

Study cohort

Thirty-nine men in this sub-study were classified as newly infected: three had acute HIV infection at study enrollment, 13 had recent HIV infection at study enrollment (identified using a MAA [37]), and 23 seroconverted during the HPTN 061 study [10]. The 23 seroconverters had a median viral load of 36,078 copies/mL (interquartile range: 5,933 to 85,140 copies/mL) at the first HIV-positive visit. Five additional seroconverters in HPTN 061 were not included in this sub-study (one did not have sufficient sample available from the first HIV-positive visit, and four had viral loads ≤400 copies/mL [54]).

HIV drug resistance

DRMs were detected in nine (23%) of the 39 newly-infected men. No DRMs were detected in any of the acutely-infected men. The NNRTI-associated K103N/S mutation was detected in four (31%) of the 13 recently-infected men and four (17%) of the 23 seroconverters (one of whom also had the NNRTI-associated P225H mutation); one additional seroconverter had the PI-associated L90M mutation.

ARV drug detection

Ten (26%) of the 39 newly-infected men had at least one ARV drug detected. ARV drugs were detected in one (33%) of the three acutely-infected men (NFV and ZDV) and nine (69%) of the 13 recently-infected men (three with NFV, two of whom had the K103N mutation; five with ZDV and one with NVP, none of whom had DRMs). No ARV drugs were detected in samples from the seroconverters at the first HIV-positive visit. None of the 39 newly-infected men reported PrEP or PEP use.

Discussion

In HPTN 061, 48 (28%) of the HIV-infected men who were not virally suppressed had drug-resistant HIV at study enrollment and 11% had MCR. HIV drug resistance and MCR were associated with older age, reporting being in HIV care, and reporting prior or current ART. A novel, high-throughput, multi-drug assay was used to assess ARV drug use in this cohort. ARV drug testing identified many men who were at risk for acquiring additional DRMs. At least one ARV drug was detected in 27% of the men who did not have drug-resistant HIV, and 38% of the men who had drug-resistant HIV had at least one ARV drug detected that was not associated with their pattern of drug resistance. ARV drug testing also revealed that some men were using ARV drugs that were no longer recommended for HIV treatment (e.g., NFV and IDV) and that some men had unusual patterns of ARV drug use. ARV drug testing also provided a more accurate estimate of the rate of TDR. The estimated rate of TDR in this cohort was 23% when calculated based solely on the rate of drug resistance among men who reported no prior or current ARV drug use. When the results of ARV drug testing were taken into account, the estimated rate of TDR was 12%, which is similar to that reported in other recent, multi-city US studies [55-58]. This should be considered a maximum estimate of TDR, since some men may have taken ARV drugs in the past that were not detected in their samples. It is notable that 29% of the men classified as having TDR had MCR.

ARV drugs were also detected in 10 (26%) of 39 newly-infected men. This group included acutely-infected men, recently-infected men (identified with a MAA), and seroconverters [10, 11, 37]. Most of these men had NFV or ZDV alone detected. Neither of these drugs is currently recommended as part of first-line regimens for PrEP, PEP, or ART [22-24, 51]. HIV drug resistance was detected in nine (23%) of the newly-infected men; eight had the K103N/S mutation. The prevalence of drug resistance among these

men is higher than the prevalence of drug resistance in other studies of newly-diagnosed or recently-infected MSM in the US [56, 58].

None of the men in this sub-study reported PrEP use, and only one reported PEP use. The first reports of successful use of co-formulated FTC/TFV for PrEP appeared in 2010 [25], after enrollment in HPTN 061 ended. In 2012, Truvada® was approved by the US FDA for PrEP in high-risk populations [24]. While this report only included HIV-infected men, some men may have been using PrEP or PEP if they were not aware of their HIV status. In this sub-study, FTC was the most frequent ARV drug detected; TFV was also detected in some men. M184V/I, which confers resistance to FTC and other NRTIs, was the second most common DRM detected in this cohort; this mutation increases susceptibility to TFV [28]. K65R, which is associated with resistance to FTC and TFV [28], was detected in only one man. These results are relevant to expanded use of Truvada for PrEP among MSM at high risk for HIV acquisition.

Most studies of HIV drug resistance among MSM in the US have focused on men who were failing ART [59, 60], reported they were newly diagnosed [55-57], or were recently infected [57, 58, 61-63]. HPTN 061 enrolled both HIV-uninfected and HIV-infected self-identified Black MSM, including men who reported that they were in care [10, 11]. This sub-study (which included men with viral loads >400 copies/mL) and our previous report (which included men with low or undetectable HIV viral loads [12]) demonstrate that many men in HPTN 061 chose not to disclose that they were using ARV drugs. Although other studies from both clinical and research settings have also reported undisclosed ARV drug use [64-66], the frequency of undisclosed ARV drug use was considerably higher in the HPTN 061 cohort than in other studies. Men may have not disclosed past or current ARV drug use because of concerns that it would impact their eligibility for the HPTN 061 study. The study also included monetary incentives and medical services that may have also contributed to nondisclosure due to the high

poverty levels in this cohort [10, 11]. These findings emphasize the benefit of using objective, biomedical measures to supplement self-reported data on ARV drug use.

This study had the following limitations. First, HIV drug resistance testing was performed using a method based on population sequencing; DRMs present at low levels may not have been detected. Previous studies have shown that minority HIV variants with DRMs can impact ART outcomes [67] and that resistance rates may be underestimated if these minority variants are not taken into account [68]. Second, only resistance and ARV drug use for NRTIs, NNRTIs, and PIs was assessed. Resistance to and use of other ARV drug classes, such as entry inhibitors and INSTIs, were not assessed. Entry inhibitors and INSTIs are used for ART [51], and INSTIs are also recommended for PEP [22]. Therefore, the prevalence of HIV drug resistance and ARV drug use in the HPTN 061 cohort may be even higher than reported here.

The results in this report should be considered in the context of the evolving landscape of ARV drug use. Unusual patterns of ARV drug use were detected in this sub-study and in our previous report [12]. ARV drugs were also detected in 26% of the newly-infected men. In many cases, the reason for ARV drug use could not be determined. In addition to ARV drug use for PrEP, PEP, and ART, some men in the HPTN 061 cohort may have been using ARV drugs for other reasons. For example, 3TC and TFV are recommended by the US FDA for treating chronic hepatitis B virus infection [69], and recent reports suggest that ARV drugs may be useful for treating other conditions [70]. ARV drugs, such as EFV and RTV, are also used for recreational purposes [32, 71, 72]. Increasing evidence also suggests that ARV drugs are shared or channeled into illicit markets in some settings, which could compromise ART adherence and expanded use of PrEP [71, 73, 74]. More research is needed to understand how Black MSM acquire and use ARV drugs. The high rate of undisclosed ARV drug use among men in HPTN 061 and other recent reports of undisclosed ARV drug use should

also raise awareness of the limitations of self-reported ARV drug use in research and clinical settings.

HIV drug resistance to rilpivirine and INSTIs

Introduction

In the previous study, HIV drug resistance to NRTIs, NNRTIs, and PIs was frequently detected in the HPTN 061 cohort using the ViroSeq HIV-1 Genotyping System, v2.8. This version of the ViroSeq System does not predict resistance to newer ARV drugs, such as the NNRTI rilpivirine, and is not designed to analyze HIV drug resistance to additional ARV drug classes, such as INSTIs. In the US, rilpivirine and INSTIs are now recommended for ART [51] and are also being evaluated for PrEP [75, 76]. Rilpivirine was not approved by the US FDA for ART until 2011 [14], after enrollment in HPTN 061 ended. However, men in HPTN 061 may have DRMs selected by other NNRTIs that also confer resistance to rilpivirine. In contrast, raltegravir, the first INSTI, was approved by the US FDA for ART in 2007 [14], before enrollment in HPTN 061 began. The prevalence of rilpivirine and INSTI resistance, particularly among MSM, is not well characterized. In this study, rilpivirine and INSTI resistance were analyzed among HIV-infected men in HPTN 061, including men who were HIV infected at study enrollment and men who seroconverted during the study.

Methods

Samples from men who had viral loads >400 copies/mL at their enrollment or seroconversion visit were analyzed. The analysis of rilpivirine resistance included all 169 men who were HIV infected at enrollment and 23 seroconverters who were analyzed in the previous study. Testing for INSTI resistance was performed using HIV RNA samples leftover from the previous study. The analysis of INSTI resistance included 141 men who were HIV infected at enrollment and 23 seroconverters.

Results

Rilpivirine resistance

Two (1%) of the 169 men who were HIV infected at enrollment had HIV genotypes that predicted resistance to rilpivirine. Both men also had resistance to NRTIs, NNRTIs, and PIs. None of the seroconverters had rilpivirine resistance.

INSTI resistance

Genotyping results for HIV integrase were obtained for 134 (95%) of the 141 men who were HIV infected at enrollment and for 23 seroconverters. None of the men had HIV genotypes that predicted resistance to raltegravir, dolutegravir, or elvitegravir. Ten (8%) of the men who were HIV infected at enrollment and two (9%) of the seroconverters had one or two accessory or polymorphic mutations detected that are associated with INSTI resistance. The most frequently detected mutation was E157Q, which was detected in seven men who were HIV infected at enrollment and one seroconverter.

Discussion

Although NRTI, NNRTI and PI resistance were frequently observed in this cohort in the previous study, rilpivirine resistance was only detected in two men and INSTI resistance was not detected. The INSTI-associated DRMs that were detected in this study were not sufficient to cause INSTI resistance and are most likely viral polymorphisms. These results are promising, since INSTIs are now recommended in first-line ART regimens in the US [51] and both rilpivirine and INSTIs are under evaluation for use as PrEP agents [75, 76]. New INSTIs are also under development for HIV treatment and prevention.

Tables

Table 3.1. Association between HIV drug resistance and demographic and behavioral factors.

			Any resistance			Multi-class resistance		
		Total	**Yes**	**No**		**Yes**	**No**	
Characteristic		169	48 (28%)	121 (72%)	**P value**	19 (11%)	150 (89%)	**P value**
Age	≤ 30 years	47 (28%)	7 (15%)	40 (85%)	**0.02**	0 (0%)	47 (100%)	**0.002**
	> 30 years	122 (72%)	41 (34%)	81 (66%)		19 (16%)	103 (84%)	
Household income	≤ $30,000/year	131 (78%)	35 (27%)	96 (73%)	0.37	15 (11%)	116 (89%)	1.00
	> $30,000/year	38 (22%)	13 (34%)	25 (66%)		4 (11%)	34 (89%)	
Employment status	Employed	32 (19%)	9 (28%)	23 (72%)	0.97	4 (13%)	28 (88%)	0.76
	Unemployed	137 (81%)	39 (28%)	98 (72%)		15 (11%)	122 (89%)	
Student	Student	31 (18%)	11 (35%)	20 (65%)	0.33	5 (16%)	26 (84%)	0.35
	Non-student	138 (82%)	37 (27%)	101 (73%)		14 (10%)	124 (90%)	
Health insurance	Yes	105 (62%)	32 (30%)	73 (70%)	0.44	14 (13%)	91 (87%)	0.32
	No	64 (38%)	16 (25%)	48 (75%)		5 (8%)	59 (92%)	
In HIV care[a]	Yes	31 (18%)	15 (48%)	16 (52%)	**0.006**	9 (29%)	22 (71%)	**<0.001**
	No	138 (82%)	33 (24%)	105 (76%)		10 (7%)	128 (93%)	
Education	High school or less	93 (55%)	26 (28%)	67 (72%)	0.89	7 (8%)	86 (92%)	0.09
	At least some college	76 (45%)	22 (29%)	54 (71%)		12 (16%)	64 (84%)	
Substance use[b]	Yes	84 (51%)	25 (30%)	59 (70%)	0.62	8 (10%)	76 (90%)	0.72
	No	80 (49%)	21 (26%)	59 (74%)		9 (11%)	71 (89%)	
	Not available	5	2	3		2	3	
Number of male partners[c]	0-1	33 (20%)	11 (33%)	22 (67%)	0.48	8 (24%)	25 (76%)	**0.008**
	>1	136 (80%)	37 (27%)	99 (73%)		11 (8%)	125 (92%)	
Prior ART[d]	Yes	32 (19%)	17 (53%)	15 (47%)	**0.001**	9 (28%)	23 (72%)	**0.001**
	No	107 (63%)	27 (25%)	80 (75%)		10 (9%)	97 (91%)	
Current ART[d]	Yes	21 (12%)	13 (62%)	8 (38%)	**<0.001**	8 (38%)	13 (62%)	**<0.001**
	No	148 (88%)	35 (24%)	113 (76%)		11 (7%)	137 (93%)	
City	Atlanta	30 (18%)	5 (17%)	25 (83%)	**0.01**	3 (10%)	27 (90%)	0.09
	Boston	14 (8%)	7 (50%)	7 (50%)		1 (7%)	13 (93%)	
	Los Angeles	41 (24%)	17 (41%)	24 (59%)		8 (20%)	33 (80%)	
	New York City	50 (30%)	10 (20%)	40 (80%)		2 (4%)	48 (96%)	
	San Francisco	10 (6%)	5 (50%)	5 (50%)		3 (30%)	7 (70%)	
	Washington, DC	24 (14%)	4 (17%)	20 (83%)		2 (8%)	22 (92%)	

Legend for Table 3.1.

P values <0.05 are bolded. Abbreviations: ART: antiretroviral treatment.

[a] Enrollment of men who reported that they were in HIV care was capped at 10 per study site.

[b] Substance use included: inhaled nitrates, smoked and power cocaine, methamphetamine, heroin, non-prescription drug use (Oxycontin, Vicodin, or Xanax), or any other hallucinogens.

[c] Surveys administered at enrollment included questions about activities in the previous six months.

[d] Thirty-two men reported prior and/or current antiretroviral drug use; all of these men reported a prior HIV diagnosis, and 19 reported that they were in HIV care at the time of study enrollment. Thirty men who reported being newly diagnosed were not asked about prior ART.

Table 3.2. ARV drugs detected in men who were HIV infected at enrollment.

ARV drug(s) detected	Total 60	Previously diagnosed[a] 34	Newly diagnosed[a] 26
Consistent with recommended ART[b]	31 (52%)	23 (68%)	8 (31%)
Consistent with PI-based regimen	24	18	6
PI alone (ATV, LPV, DRV, or RTV)	8	6	2
PI (ATV, LPV, or DRV) ± RTV + NRTI(s)[c]	16	12	4
Consistent with NNRTI-based regimen	7	5	2
NVP alone	3	2	1
EFV alone	1	1	-
EFV + FTC	3	2	1
Not consistent with recommended ART[d]	29 (48%)	11 (32%)	18 (69%)
ZDV alone	9	1	8
NFV alone	7	3	4
FTC alone	3	3	-
TFV alone	1	1	-
IDV alone	1	-	1
FTC/TFV	1	-	1
3TC + NFV	1	1	-
ZDV + NFV	1	-	1
NVP + APV	1	-	1
FTC/TFV + NFV	1	1	-
FTC/TFV + NFV + IDV	1	-	1
FTC/TFV + NFV + LPV + RTV	1	-	1
FTC/TFV + NFV + LPV + RTV + ZDV	1	1	-

Legend for Table 3.2.

Abbreviations: ARV: antiretroviral; ART: antiretroviral treatment; ATV: atazanavir; LPV: lopinavir; DRV: darunavir; RTV: ritonavir; NVP: nevirapine; EFV: efavirenz; FTC: emtricitabine; ZDV: zidovudine; NFV: nelfinavir; TFV: tenofovir; IDV: indinavir; 3TC: lamivudine; APV: amprenavir.

[a] Men were asked whether they were aware of their HIV status at the time of study enrollment.

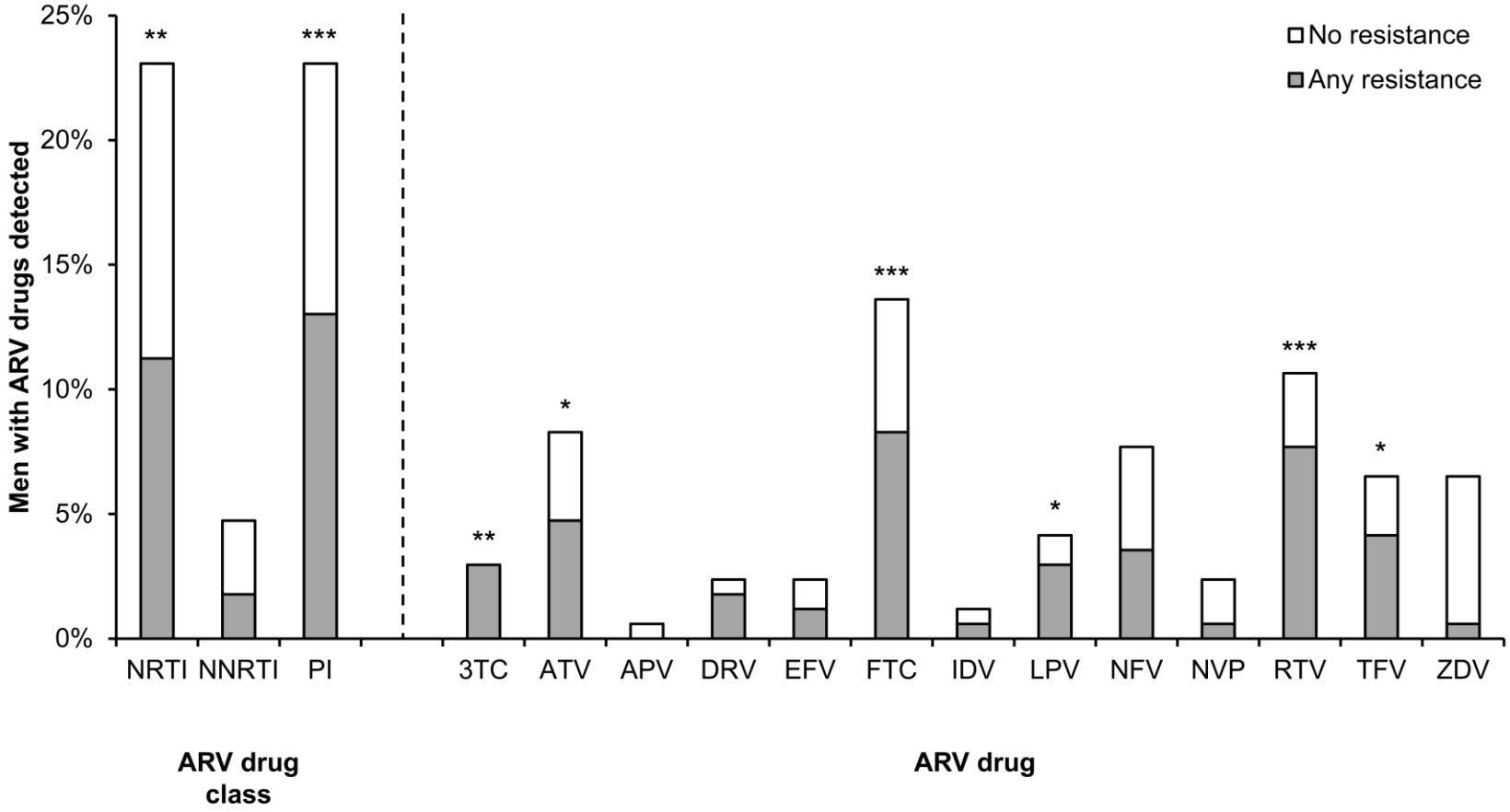
[b] Criteria used to characterize ARV drugs as consistent with recommended ART are described in the text.

[c] The NRTIs detected were either FTC alone or with TFV, or 3TC.

[d] This category includes patterns of ARV drug detection that were not consistent with recommended ART regimens. Drugs that were not recommended as part of PrEP, PEP, or ART regimens are underlined.

Figures

Figure 3.1. Association between ARV drug detection and HIV drug resistance.



Legend for Figure 3.1.

The figure shows the frequency that specific antiretroviral drugs were detected in samples from men who were tested for HIV drug resistance. Fisher's exact and chi-square tests were used to compare the proportion of men who did vs. did not have HIV drug resistance (any resistance, defined as detection of at least one DRM). P values for these comparisons are indicated (*P<0.05; **P<0.01; ***P<0.001). Abbreviations: ARV: antiretroviral; NRTI: nucleoside/nucleotide reverse transcriptase inhibitor; FTC: emtricitabine; TFV: tenofovir; ZDV: zidovudine; 3TC: lamivudine; PI: protease inhibitor; RTV: ritonavir; ATV: atazanavir; NFV: nelfinavir; LPV: lopinavir; DRV: darunavir; IDV: indinavir; APV: amprenavir; NNRTI: non-nucleoside reverse transcriptase inhibitor; EFV: efavirenz; NVP: nevirapine.

Chapter 4: Analysis of HIV tropism

This work has been submitted for publication and is reprinted here. At the journal's request, no modifications of the material in this dissertation are permitted.

Chen I, Huang W, Connor MB, Frantzell A, Cummings V, Beauchamp GG, Griffith S, Fields SD, Scott HM, Shoptaw S, del Rio C, Magnus M, Tieu H, Mannheimer S, Wheeler DP, Mayer KH, Koblin BA, and Eshleman SH. CXCR4-using HIV variants in a cohort of Black men who have sex with men: HIV Prevention Trials Network 061. *HIV Clin Trials*. Submitted.

Introduction

Individuals with X4/DM viruses often exhibit accelerated disease progression [77, 78]. While R5 viruses usually predominate early in infection, X4/DM viruses have been documented in up to 20% of recently-infected individuals [77, 79-81]. The predominance of R5 viruses during primary HIV infection is thought to result from selective advantages that favor transmission and/or replication of R5 strains [82]. The prevalence of X4/DM viruses in recent infection is higher among people who inject drugs than among those with sexually-acquired HIV infection [78, 79, 83]. This suggests that the absence of a mucosal barrier may facilitate transmission of X4/DM strains. Sexual practices (e.g., insertive vs. receptive intercourse among MSM) may also differentially impact transmission of X4/DM viruses. These issues are relevant to HIV prevention efforts, since the CCR5 coreceptor antagonist, maraviroc, is being evaluated for use in PrEP [84, 85].

This report explored whether sexual practices were associated with coreceptor tropism in HPTN 061. HIV tropism was analyzed for the subset of study participants in HPTN 061 who were HIV infected at enrollment and reported either exclusive insertive intercourse or exclusive receptive intercourse, and among men who seroconverted during the study.

Methods

Study cohort

Study participants were asked to provide detailed information on sexual practices via ACASI. Insertive intercourse was defined as insertive vaginal or anal intercourse with female, male, or transgender female/male partners; receptive intercourse was defined as receptive anal intercourse with male or transgender male partners.

Analysis of HIV tropism

HIV tropism testing was performed retrospectively using samples from two groups of men: (1) men who were HIV infected at enrollment and reported either exclusive insertive intercourse or exclusive receptive intercourse in the previous six months, and (2) men who seroconverted during the study. All samples tested had a viral load ≥1,000 copies/mL.

Statistical methods

Correlates of HIV tropism were analyzed using Fisher's exact and chi-square tests for univariate analyses. SAS, version 9.2 (SAS Institute, Cary, NC) was used for these analyses. P values <0.05 were considered to be statistically significant.

Results

In HPTN 061, 147 (42%) of the 348 men who were HIV-infected at enrollment had a viral load ≥1,000 copies/mL. HIV tropism was analyzed in samples from 51 of those men: 32 (63%) reported exclusive insertive intercourse and 19 (37%) reported exclusive receptive anal intercourse. Samples from the first HIV-positive visit were also analyzed for 21 (75%) of the 28 men who seroconverted during HPTN 061; the remaining seven men included six who had viral loads <1,000 copies/mL at the first HIV-positive visit [54] and one who had no sample available for testing. HIV tropism was determined for 63 of the 72 samples (Table 4.1). HIV tropism was not determined for nine samples due to assay failure, including samples from five men who reported exclusive insertive intercourse, three who reported exclusive receptive intercourse, and one seroconverter.

DM viruses were detected in 11 (26%) of 43 men who were HIV infected at enrollment, including eight (30%) of 27 men who reported exclusive insertive intercourse and three (19%) of 16 men who reported exclusive receptive intercourse (P=0.49; data not shown). DM viruses were also detected in 5 (25%) of 20 seroconverters. Since the proportion of men with DM viruses was similar among the HIV-infected men at enrollment and the seroconverters, these groups were combined to analyze factors associated with detection of DM viruses (Tables 4.1 and 4.2). Overall, men who had DM viruses had lower median CD4 cell counts compared to men who had R5 viruses (184 cells/mm^3 vs. 386 cells/mm^3; P=0.019; Table 4.1); all five seroconverters who had DM viruses had CD4 cell counts <350 cells/mm^3 (Table 4.3). None of the other factors evaluated was associated with detection of DM viruses in this study.

Individual *env* clones from the five seroconverters who had DM viruses were analyzed to determine whether their viral populations were comprised solely of dual-tropic viruses or mixtures of R5, X4, and dual-tropic viruses (Table 4.3). Dual-tropic

viruses were further classified as either dual-R or dual-X in these analyses [46, 86]. Dual-R viruses infect $CXCR4^+$ cells poorly and have *env* V3 loop sequences similar or identical to R5 viruses from the same individual, while dual-X viruses infect $CXCR4^+$ cells efficiently and have *env* V3 loop sequences distinct from R5 viruses from the same individual.

Mixed populations of R5 and dual-R viruses were detected in two of the five men (Cases 1 and 2, Table 4.3). There was a major subpopulation of dual-R viruses in Case 1, which had V3 sequences identical to those in one R5 clone. In Case 2, there were two proportionate subpopulations of R5 and dual-R viruses, which had identical V3 sequences. Dual-X viruses were detected in the remaining three men (Cases 3-5). All clones from Cases 3 and 4 were dual-X and had identical (Case 3) or nearly identical (Case 4) V3 sequences. The viral population in Case 5 included a major subpopulation of R5 and minor subpopulations of dual-X viruses.

Discussion

This study explored whether sexual practices were associated with HIV tropism among HIV-infected men enrolled in HPTN 061. A significant difference in HIV tropism was not observed among men who reported exclusive insertive vs. receptive intercourse at study enrollment. The sexual practices of the men included in this analysis may have been different at the time of HIV infection than those reported for the six months preceding enrollment; self-report of sexual practices may also be unreliable in some cases. In addition, HIV evolution and superinfection could also impact the tropism of the viral population over time. Overall, DM viruses were detected among 26% of the HIV-infected men at enrollment, which is consistent with other studies that found X4/DM viruses in 20% of individuals with chronic HIV infection [77].

Twenty-five percent of the seroconverters in HPTN 061 also had DM viruses. In other studies, X4/DM viruses have been observed in 2-20% of individuals with recent HIV infection [77, 79-81]. It is difficult to compare the proportion of individuals with X4/DM viruses in different studies, since the sensitivity for detecting X4/DM viruses depends on the method used for tropism testing. In this study, HIV tropism was determined using a clinically-validated, phenotypic tropism assay with enhanced sensitivity (Trofile), which can detect X4/DM variants present in 0.3% of the viral population [44]. Most genotypic tropism assays use bulk (population) Sanger sequencing; these methods typically detect minority variants present at 15-20% [87]. The sensitivity and specificity of genotypic tropism assays varies depending on the algorithms and cut-offs used to analyze V3 loop sequences [87]. Determinants outside the V3 loop may also impact HIV tropism [46, 88]. Those regions are included in the recombinant viruses analyzed in the Trofile assay but not in genotypic tropism assays. In two of the five seroconverters in this study, dual-tropic *env* clones were identified that had V3 loop sequences identical to those in R5-tropic clones from the same samples; HIV tropism would have been misclassified in these cases using a genotypic assay based on *env* V3 sequencing.

In HPTN 061, men with DM viruses had significantly lower CD4 cell counts, consistent with findings from other studies [77]. The five seroconverters with DM viruses also had low CD4 cell counts (median: 254 cells/mm^3, range: 151-335 cells/mm^3). Other studies did not find a significant difference in CD4 cell count between individuals with X4/DM vs. R5 viruses early in infection [78, 83, 89], although those with X4/DM viruses had more pronounced CD4 cell count decline over time in two of those studies [78, 83]. In another study, a lower mean CD4 cell count was observed among seroconverters with DM vs. R5 viruses (450 vs. 629 cells/mm^3) [81]. Low CD4 cell counts after seroconversion have been associated with faster HIV disease progression, independent

of HIV tropism [90, 91]. In addition, individuals starting ART with CD4 cell counts <500 cells/mm^3 may not achieve the same degree of CD4 cell count recovery as those starting ART at higher $CD4^+$ cell counts [92].

The CCR5 coreceptor antagonist, maraviroc, is currently approved for ART in the US [14], and clinical trials are exploring its use for PrEP [84, 85]. Coreceptor tropism testing is recommended before using maraviroc for ART, since maraviroc may select for X4 and dual-tropic viruses [35]. In HPTN 061, the seroconverters with DM viruses had viral populations that included either dual-tropic viruses or mixtures of R5 and dual-tropic viruses; the efficiency of CXCR4 use varied among the dual-tropic clones from each individual. The relatively high prevalence of DM viruses among Black MSM in HPTN 061 with both prevalent and incident HIV infection suggests that maraviroc may not be a suitable PrEP agent in this population.

Black MSM in the US are disproportionately affected by HIV. Infrequent HIV testing prior to enrollment and late HIV diagnosis were common in the HPTN 061 cohort [93]. X4/DM viruses have been associated with increases in viral load, more rapid CD4 cell decline, and faster disease progression [77, 78]. The high prevalence of X4/DM viruses among men in HPTN 061 highlights an urgent need to increase HIV testing frequency in this population. Further research is also needed to identify factors driving transmission and selection of X4/DM viruses and to evaluate potential associations of HIV tropism with disease progression and treatment outcomes among Black MSM.

Tables

Table 4.1. Association between HIV tropism and demographic and clinical factors.

Characteristic		**Total** 63	**DM** 16	**R5** 47	**P value**
City	Atlanta	12 (19%)	3 (19%)	9 (19%)	0.26
	Boston	5 (8%)	1 (6%)	4 (9%)	
	Los Angeles	24 (38%)	7 (44%)	17 (36%)	
	New York City	9 (14%)	1 (6%)	8 (17%)	
	San Francisco	7 (11%)	4 (25%)	3 (6%)	
	Washington, DC	6 (10%)	0 (0%)	6 (13%)	
Age	≤30 years	27 (43%)	7 (44%)	20 (43%)	0.93
	>30 years	36 (57%)	9 (56%)	27 (57%)	
Sexual identity	Homosexual/gay	30 (48%)	9 (56%)	21 (45%)	0.76
	Bisexual	16 (25%)	3 (19%)	13 (28%)	
	Other	17 (27%)	4 (25%)	13 (28%)	
Education	High school or less	34 (54%)	9 (56%)	25 (53%)	0.83
	At least some college	29 (46%)	7 (44%)	22 (47%)	
Household income	≤$30,000/year	51 (81%)	12 (75%)	39 (83%)	0.48
	>$30,000/year	12 (19%)	4 (25%)	8 (17%)	
Employment status	Employed	15 (24%)	5 (31%)	10 (21%)	0.50
	Unemployed	48 (76%)	11 (69%)	37 (79%)	
Student status	Student	18 (29%)	5 (31%)	13 (28%)	0.76
	Non-student	45 (71%)	11 (69%)	34 (72%)	
Circumcision status	Circumcised	51 (81%)	13 (81%)	38 (81%)	1.00
	Uncircumcised	12 (19%)	3 (19%)	9 (19%)	
STI at enrollment	Yes	13 (21%)	2 (13%)	11 (23%)	0.49
	No	50 (79%)	14 (88%)	36 (77%)	
Median viral load (copies/mL)		22,551	56,152	20,236	0.28
Median CD4 count (cells/mm^3)		357	184	386	**0.019**

Legend for Table 4.1.

P values <0.05 are bolded. Abbreviations: DM: dual/mixed viruses; R5: CCR5-using viruses; STI, sexually transmitted infection.

Table 4.2. Association between HIV tropism and behavioral factors.

Characteristic		Total 63	DM 16	R5 47	P value
Number of male partners	0-1	13 (21%)	5 (31%)	8 (17%)	0.29
	>1	50 (79%)	11 (69%)	39 (83%)	
Gender of partners	Male only	42 (67%)	9 (56%)	33 (70%)	0.31
	Male and female	21 (33%)	7 (44%)	14 (30%)	
Had a new male partner	Yes	51 (81%)	11 (69%)	40 (85%)	0.16
	No	12 (19%)	5 (31%)	7 (15%)	
Race/ethnicity of partners	All Black	41 (65%)	10 (63%)	31 (66%)	0.89
	Some Black	18 (29%)	5 (31%)	13 (28%)	
	None Black	4 (6%)	1 (6%)	3 (6%)	
Unprotected receptive anal intercourse	Yes	31 (49%)	6 (38%)	25 (53%)	0.28
	No	32 (51%)	10 (63%)	22 (47%)	
Unprotected insertive anal intercourse	Yes	30 (48%)	8 (50%)	22 (47%)	0.83
	No	33 (52%)	8 (50%)	25 (53%)	
Received money/goods for sex	Yes	13 (21%)	3 (19%)	10 (21%)	1.00
	No	50 (79%)	13 (81%)	37 (79%)	
Provided money/goods for sex	Yes	7 (11%)	1 (6%)	6 (13%)	0.67
	No	56 (89%)	15 (94%)	41 (87%)	
Alcohol problem[a]	Yes	14 (22%)	4 (25%)	10 (21%)	0.74
	No	49 (78%)	12 (75%)	37 (79%)	
Substance use[b]	Yes	41 (65%)	11 (69%)	30 (64%)	0.72
	No	22 (35%)	5 (31%)	17 (36%)	

Legend for Table 4.2.

Men were asked to report behaviors in the six months prior to enrollment. P values <0.05 are bolded. Abbreviations: DM: dual/mixed viruses; R5: CCR5-using viruses.

[a] Alcohol use was defined as having a score ≥8 using the Alcohol Use Disorders Identification Test.

[b] Substance use included inhaled nitrates, smoked and powder cocaine, methamphetamine, heroin, nonprescription drug use (Oxycontin, Vicodin, or Xanax), or any other hallucinogens. Injection drug use was reported in only four (6%) of 63 men included in this analysis. This included three men who were HIV infected at enrollment (two with R5 viruses and one with DM viruses) and one seroconverter (with R5 viruses).

Table 4.3. Coreceptor usage and V3 loop sequences of *env* clones from HIV seroconverters with DM viruses.

Case No.	Viral Load[a]	CD4 Count[b]	No. of Clones[c]	Tropism[d]	CCR5$^+$ cell infectivity (RLU)[e]	CXCR4$^+$ cell infectivity (RLU)[e]	V3 loop amino acid sequences
1	242,022	334	9	Dual-R	57,874-381,187	1,160-28,202	CTRPNNNTRKSIHIAPGRAFFATGDIIGDIRQAHC
			2	R5	53,141-91,918	57-72	............N.......Y....M.....
			2	Dual-R	92,523-179,041	8,182-15,549	..................Y.....
			1	R5	57,634	69	............N.......Y.....
			1	R5	56,344	65	..................Y.....
			1	R5	20,159	71	.....
			1	Dual-R	202,966	1,525	.A.....
			1	Dual-R	191,955	11,286	............V.....
			1	Dual-R	153,724	3,106	..................K..Y.....
			1	Dual-R	109,342	1,496	............M.....
2	141,411	254	8	Dual-R	50,401-229,518	302-2,472	CTRPGNNTRKSIHIGPGRAFYATGDIIGDIRQAHC
			6	R5	33,694-131,674	50-103	.....
			1	R5	38,299	79	.A.....
			1	Dual-R	229,518	2,014	............M.....
3	90,920	335	18	Dual-X	494-211,474	96,584-1,451,417	CTRPNNNTRKRMSLGPGKVFYTTGGIIGDIRKAHC
4	165,010	235	10	Dual-X	1,804-19,776	16,153-87,607	CTRPNNNTRKSIRIGPGRWSVFATGKIIGDIRQAHC
			3	Dual-X	1,783-4,628	15,345-35,774	.....E.......R...
			2	Dual-X	3,011-31,528	38,768-128,306	.....R...
			1	Dual-X	2,163	16,759	.....T.....
5	79,450	151	11	R5	30,379-154,907	55-99	CSRPNNNTRKSISIGPGRAFYATGDIIGDIRQAHC
			2	Dual-X	49,924-65,980	396,970-629,351	..........G.R.....T.....R.....
			2	Dual-X	8,888-19,161	86,732-185,976	............R.....T.....R.....
			1	R5	58,306	73	............G.....

Legend for Table 4.3.

Amino acid positions 11 and 25 in the *env* V3 loop are underlined. Abbreviations: no.: number; RLU: relative light units of luciferase output from the Trofile assay; R5: CCR5-using viruses.

[a] Viral load was measured as copies/mL.

[b] CD4 cell count was measured as cells/mm^3.

[c] The total number of clones in each individual with the indicated V3 loop sequence.

[d] HIV tropism for each *env* clone was determined using the Trofile Assay. Clones were identified as CCR5-using (R5), CXCR4-using (X4), or dual-tropic virus. Dual-tropic viruses were further classified as dual-R or dual-X according to their $CXCR4^+$ cell infectivity and V3 loop sequence.

[e] The minimum to maximum RLU is shown in cases with multiple distinct clones.

Chapter 5: Analysis of HIV diversity

Introduction

The high genetic diversity of HIV complicates the use of ARV drugs for PrEP, PEP, and ART and has hindered the development of a HIV vaccine [94, 95]. HIV diversity tends to be low early in infection, since only one or a few HIV variants typically establish infection. Rapid HIV diversification occurs after infection as the result of numerous host and viral factors [17]. Traditionally, HIV diversity is measured by sequencing clones from individual HIV variants, or by sequencing individual variants using single genome sequencing or next generation sequencing; these methods are time consuming and costly, which may limit study size and the number of genomic regions analyzed. The HRM diversity assay quantifies viral diversity without sequencing. The assay uses a nested PCR to amplify HIV DNA in the presence of a fluorescent, intercalating, duplex-dependent dye [47]. The melting range of the resulting DNA amplicons (i.e., the number of degrees over which melting occurs) is measured as a single numeric HRM score [47, 50]. HRM scores are significantly correlated with sequence-based diversity measures, including genetic diversity, genetic complexity, and Shannon entropy [47, 49].

The HRM diversity assay has been used to measure HIV diversity in adults with recent and established infection and in HIV-infected infants and children [29, 48, 96, 97]. Higher HRM scores (i.e., higher levels of diversity) were observed in adults with a longer duration of HIV infection [48, 96] and in older children, where age acts as a surrogate for the duration of infection [29, 97]. HIV diversity may also impact disease progression and ART outcome. Higher HRM scores in the HIV *gag* and *pol* regions were associated with decreased 5-year survival in children [97], and lower HRM scores were observed in children who experienced prolonged exposure to non-suppressive ART (i.e., genetic bottlenecking) [29]. Better ART outcomes, including shorter time to virologic suppression

and longer time to virologic failure, were associated with higher HRM scores in the HIV *pol* region in African children [98].

In this study, the HRM diversity assay was used to evaluate the relationship between HIV diversity and demographic and clinical factors in men enrolled in HPTN 061.

Methods

Study cohort

The HRM diversity assay used to characterize HIV in samples from HIV-infected men in HPTN 061 who had viral loads >400 copies/mL; this included men who were HIV infected at enrollment and men who seroconverted during the study.

Laboratory and statistical methods

Six regions of the HIV genome were analyzed in this study using the HRM diversity assay: two in *gag* (GAG1 and GAG2), one in *pol* (POL), and three in *env* (ENV1, ENV2, and ENV3; see Chapter 2). Median regression analyses were used to evaluate associations between HRM scores and demographic and clinical characteristics for each HRM region; P values <0.05 was considered statistically significant. For men who were HIV infected at enrollment, multiple median regressions were performed for multivariate analyses of variables that were significantly associated with HRM score at the $P<0.05$ level in univariate analyses. Wilcoxon signed-rank tests were used to analyze the change in HRM score from enrollment to end of study in a paired analysis. Associations between change in HRM score and demographic and clinical characteristics were also assessed using median regression analyses.

Results

Study cohort

In HPTN 061, 348 men were HIV infected at study enrollment. The HRM diversity assay was performed for 168 (48%) of the men (Figure 5.1); these men had enrollment samples available for additional testing with a viral load >400 copies/mL. The HRM diversity assay was also performed for 23 (82%) of 28 men who seroconverted during the HPTN 061 study; the remaining five men included four who had viral loads <400 copies/mL at the first HIV-positive visit [54] and one who had no sample available for testing. HRM scores for all six regions were obtained for 146 (87%) of the 168 men who were HIV infected at enrollment and 21 (91%) of 23 seroconverters. A summary of demographic and clinical characteristics for the men included in the following analyses is shown in Table 5.1.

Association of HIV diversity with recent HIV infection

The association between HRM scores and recent HIV infection was first assessed; this assessment included men who were acutely or recently infected at enrollment and seroconverters (Table 5.1). The 146 men who were HIV infected at enrollment included 3 with acute HIV infection and 13 with recent HIV infection (classified using a MAA [37], see Chapter 2); men classified as recently infected by the MAA were likely infected in the 6 months prior to study enrollment. The remaining 130 men who were HIV infected at enrollment were classified as having non-recent infection (Table 5.1). The seroconverters in HPTN 061 were infected within 6 months of their first HIV positive visit (i.e., time since their last documented seronegative study visit).

Since the seroconverters had a known time window of infection, HRM scores were first compared between seroconverters and men who had non-recent infection at enrollment. In univariate analyses, HRM scores were significantly lower in all six HRM

regions for seroconverters than for men with non-recent infection (P=0.003 for ENV2, P<0.0001 for the other regions, Figure 5.2A). Similarly, HRM scores were significantly lower in all six regions for men who had acute or recent infection at enrollment than for men with non-recent infection (P<0.0001 for all, Figure 5.2B). In multivariate analyses, HRM scores remained significantly lower for men with acute or recent infection at enrollment, when the analysis was adjusted for other variables (P=0.009 for GAG2, P<0.0001 for the other regions, Table 5.3).

Association of HIV diversity with demographic and clinical characteristics

The association between HRM scores and additional demographic and clinical characteristics was analyzed for men who were HIV infected at enrollment (Table 5.2); meaningful statistical analyses could not be performed for seroconverters, due to the small number of participants. In univariate analyses, HRM scores were significantly lower in one or more HRM region for men who were younger (≤30 years, Figure 5.2C), had HIV drug resistance (Figure 5.2D), had an ARV drug detected, or had higher CD4 cell counts (Figure 5.3). In multivariate analyses, HRM scores remained significantly lower for men who were younger (ENV1), had HIV drug resistance (GAG1 and GAG2), or had higher CD4 cell counts (GAG2); these results are shown in Table 5.3.

Analysis of HIV diversification over 12 months

In HPTN 061, men were followed for 12 months. HIV diversification during follow-up was evaluated using samples from men who were HIV infected at enrollment and had an end-of-study sample available for analysis. Fifty-two (36%) of the 146 men who had HRM scores at enrollment were lost to study follow-up and could not be analyzed (Figure 5.1). The HRM diversity assay was performed using end-of-study samples from 70 (48%) of the 146 men. HRM scores were obtained for all six regions for 62 (89%) of

the 70 men at the last study visit. The change in HRM score was calculated by subtracting the HRM score at enrollment from the HRM score at follow-up for all six HRM regions. HRM scores significantly increased over time for GAG1 and POL (P=0.03 and P<0.0001, respectively). In univariate analyses, POL HRM scores increased more among men who had acute or recent infection (P=0.03, Figure 5.4), lower HIV viral loads (P=0.02), and higher CD4 cell counts (P=0.03). In addition, ENV3 HRM scores decreased for men who had ARV drugs detected (P=0.049).

Discussion

In this study, HIV diversity was analyzed in six regions in HIV *gag*, *pol*, and *env* among HIV-infected men enrolled in HPTN 061. As expected, HIV diversity was lower overall among seroconverters and men with acute or recent HIV infection compared to men with non-recent HIV infection. Men with recent and non-recent HIV infection in HPTN 061 were classified using a MAA designed for cross-sectional incidence surveys [37]. The MAA used in this study included two serologic and two non-serologic biomarkers. The lower HIV diversity observed among men classified as recently infected at enrollment provides additional confirmation that the MAA accurately identifies individuals who recently acquired HIV infection.

After adjusting for acute or recent infection, HIV diversity at study enrollment was significantly lower in the GAG2 region among men with higher CD4 cell counts. This is consistent with a previous study of Kenyan women where lower *gag* diversity was strongly correlated with higher CD4 cell count during chronic HIV infection [99]. HIV diversity was also lower in the ENV1 region for younger men (<30 years) in this study. Collectively, these findings may reflect the evolution of the host adaptive immune response to HIV. Immune pressure from cytotoxic T lymphocyte (CTL) and antibody responses can impact HIV diversity in *gag* and *env*, respectively [99, 100]. It is possible

that the men with lower HIV diversity in the GAG2 and ENV1 regions had a shorter duration of infection and therefore, a less evolved immune response. CD4 cell counts are generally higher early in infection [101], and younger age was associated with HIV acquisition in HPTN 061 [10].

Lower HIV diversity in both *gag* regions was also associated with having HIV drug resistance to NRTIs, NNRTIs, and PIs. This finding is unusual since these ARV drug classes target enzymes encoded by HIV *pol* (protease and reverse transcriptase). In a previous study, prolonged exposure to non-suppressive ART was significantly associated with decreases in HIV diversity in *gag*, *pol*, and *env* (i.e., genetic bottlenecking) in Ugandan children [29]. Lower diversity in the GAG2 region was also associated with self-reported prior maternal ARV drug use in a cohort of African children [98]. These findings suggest that the selective pressure exerted by ARV drug use may also influence HIV diversity in untargeted regions. There was no association between HIV diversity and ARV drug detection in this study. The ARV drug assay used in this dissertation can only detect drugs taken recently. It is possible that the men who had ARV drugs detected did not experience a prolonged exposure to non-suppressive ARV drug levels (e.g., men may have only recently initiated or failed ART). Half of the men who had ARV drugs detected in HPTN 061 did not have HIV drug resistance (see Chapter 4). In this case, HIV drug resistance may more accurately reflect extended non-suppressive ARV drug use.

This study also analyzed HIV diversification over 12 months in a subset of men who had samples available at the end of the HPTN 061 study. HIV diversity increased in all six genomic regions analyzed for men with acute or recent infection. However, there was no significant difference in HIV diversification when compared to men with non-recent infection, except in the POL region. It is not clear why the HIV *pol* region diversified more in men with acute or recent infection. In one study, HIV diversity in *gag*

and *env*, but not *pol*, significantly increased for African women with recent infection compared to women with known non-recent infection (duration of infection: two to six years) [96]. Those findings are consistent with HIV diversification in response to the adaptive immune response, which targets HIV epitopes in *gag* and *env* [99, 100]. HIV epitopes in *pol* can also be targeted by the adaptive CTL response [102]. In HPTN 061, HIV *pol* also diversified more in men with lower HIV viral loads and men with higher CD4 cell counts in HPTN 061. These increases in HIV diversity may reflect a more robust immune response in these individuals. The CTL response contributes to establishing HIV viral load setpoint after acute HIV infection [103], while CD4+ T cells play a central role in antibody development.

Understanding factors associated with HIV diversification may yield new insights into the selective pressures driving viral evolution. The HRM diversity assay used in this study can analyze multiple genomic regions to provide a more complete picture of HIV diversification in response to these pressures. Additional studies are needed to explore the rate and patterns of HIV diversification in relation to ART and the immune response.

Tables

Table 5.1. Summary of study participants.

		Median (IQR)		
Subset	**Total**	**Age (years)**	**HIV viral load (log_{10})**	**CD4 cell count (cells/mm^3)**
HIV infected at enrollment	146	39 (28, 45)	4.36 (3.75, 4.82)	356 (196, 519)
Acute or recent infections	16	25 (20,36)	4.64 (4.24, 5.29)	525 (381, 804)
Non-recent infections	130	41 (31, 46)	4.32 (3.73, 4.81)	321 (193, 499)
HIV seroconverters	21	23 (20, 28)	4.90 (4.29, 5.22)	576 (324, 707)

Legend for Table 5.1.

Abbreviations: IQR: interquartile range.

Table 5.2. Association of HIV diversity with clinical and demographic factors at study enrollment (univariate analyses).

Characteristic	GAG1	GAG2	POL	ENV1	ENV2	ENV3
Acute or recent infection	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
Age (years)	**0.049**	**0.031**	0.333	**0.002**	0.380	0.144
HIV drug resistance	0.110	**0.023**	0.145	1.000	0.755	**0.028**
ARV drug detected	0.520	0.351	**0.047**	1.000	1.000	0.086
STI	0.820	0.795	0.577	0.160	0.575	0.736
HIV viral load (log_{10})	0.163	0.534	0.341	0.417	0.793	1.000
CD4 cell count (cells/mm^3)	0.426	**0.040**	0.054	**0.031**	**<0.0001**	0.050

Legend for Table 5.2.

P values <0.05 are bolded. Abbreviations: STI: sexually transmitted infection; ARV: antiretroviral.

Table 5.3. Multivariate analyses of HIV diversity at study enrollment.

Covariate	Region	Estimate[a]	95% CI	P value
Acute or recent infection	GAG1	-1.11	-1.51, -0.70	**<0.0001**
	GAG2	-1.46	-2.54, -0.37	**0.009**
	POL	-1.08	-1.42, -0.73	**<0.0001**
	ENV1	-0.90	-1.18, -0.61	**<0.0001**
	ENV2	-1.25	-1.85, -0.66	**<0.0001**
	ENV3	-1.90	-2.76, -1.05	**<0.0001**
Age ≤30 years	GAG1	0.05	-0.43, 0.52	0.85
	GAG2	-1.06	-2.13, 0.01	0.05
	POL	-0.23	-0.55, 0.08	0.15
	ENV1	-0.29	-0.57, -0.005	**0.046**
	ENV2	-0.07	-0.64, 0.50	0.81
	ENV3	-0.39	-1.24, 0.46	0.37
HIV drug resistance	GAG1	-0.54	-0.99, -0.10	**0.017**
	GAG2	-1.69	-2.57, -0.80	**0.0003**
	POL	-0.19	-0.55, 0.17	0.29
	ENV1	-0.08	-0.38, 0.23	0.61
	ENV2	-0.31	-0.87, 0.26	0.28
	ENV3	-0.70	-1.49, 0.10	0.09
ARV drug detected	GAG1	-0.14	-0.56, 0.28	0.50
	GAG2	-0.13	-0.96, 0.70	0.76
	POL	-0.04	-0.36, 0.28	0.80
	ENV1	0.09	-0.19, 0.36	0.53
	ENV2	0.19	-0.32, 0.70	0.47
	ENV3	-0.10	-0.85, 0.65	0.80
CD4 cell count	GAG1	-0.0002	-0.0012, 0.0008	0.71
	GAG2	-0.0009	-0.0018, 0.00	**0.049**
	POL	-0.0002	-0.0007, 0.0003	0.45
	ENV1	-0.0001	-0.0007, 0.0005	0.66
	ENV2	-0.0007	-0.0014, 0.0001	0.096
	ENV3	-0.0004	-0.0017, 0.0008	0.49

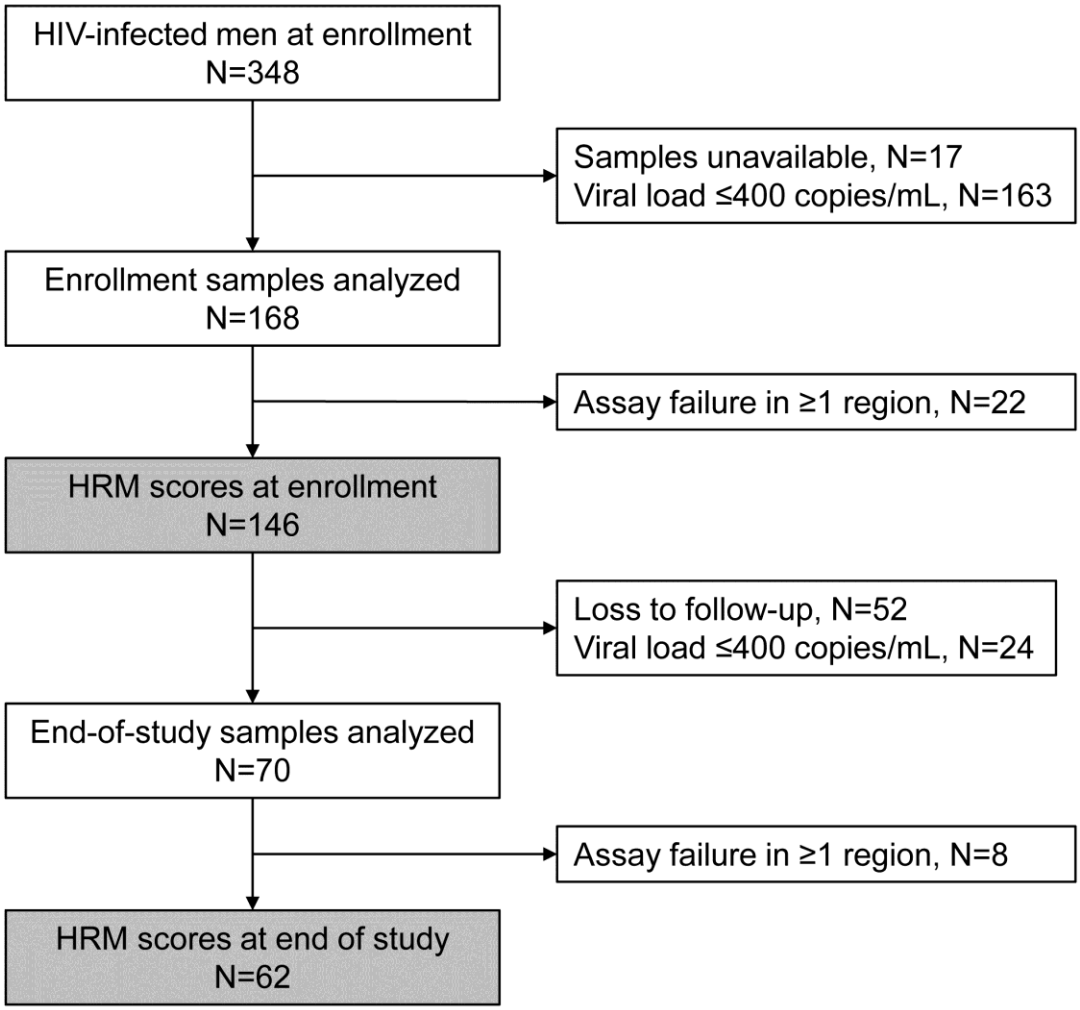
Legend for Table 5.3.

P values <0.05 are bolded. Abbreviations: CI: confidence interval.

[a] The estimate shows the difference in median HRM score for each factor after adjusting for covariates.

Figures

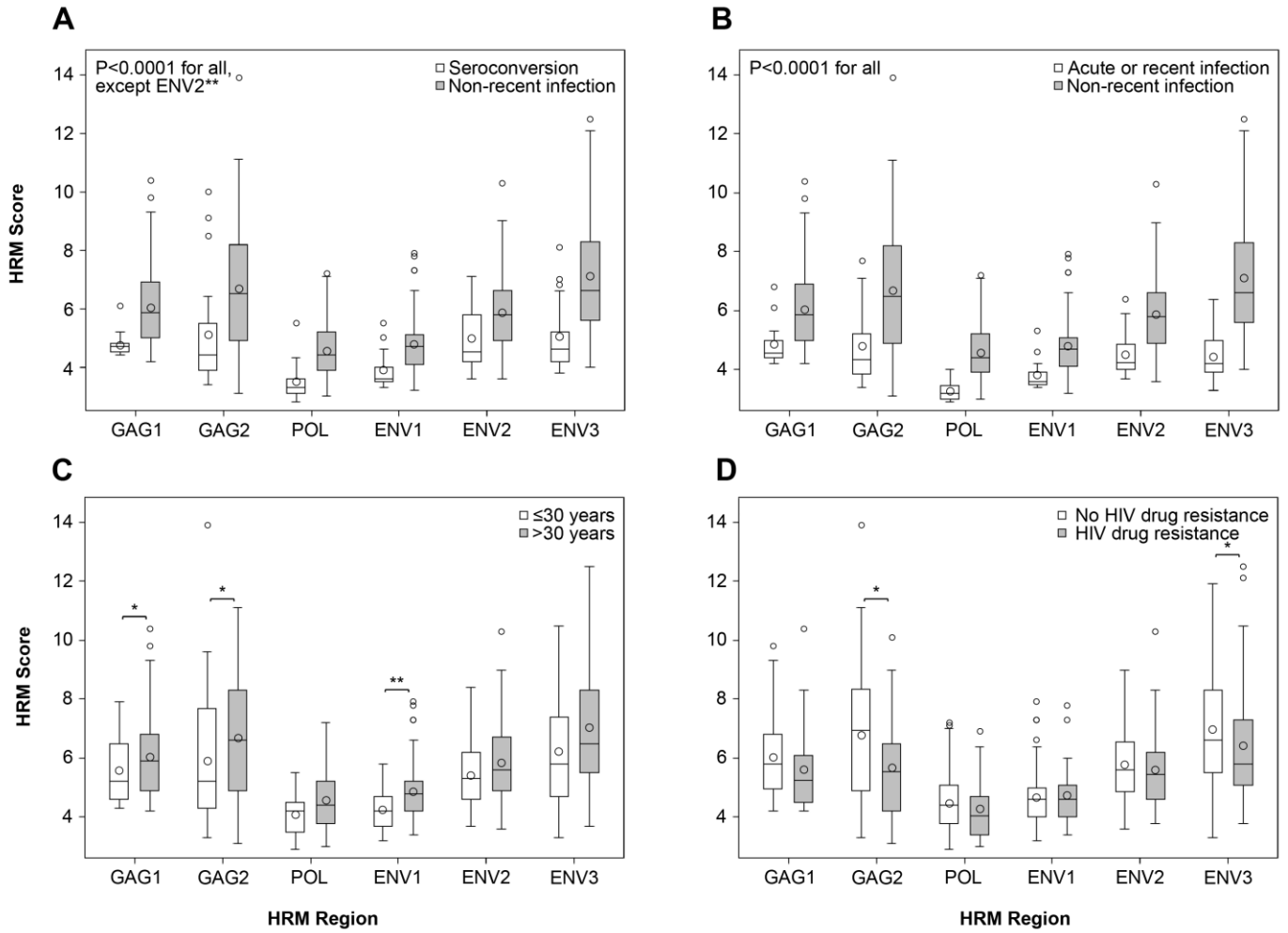
Figure 5.1. Study cohort.



Legend for Figure 5.1.

The high resolution melting (HRM) diversity assay was used to analyze samples from men who were HIV infected at enrollment, had a sample available for additional testing, and had a viral load >400 copies/mL. Men who had HRM scores for all six regions analyzed were included in analyses of HIV diversity at enrollment and in analyses of HIV diversification over time (shaded boxes).

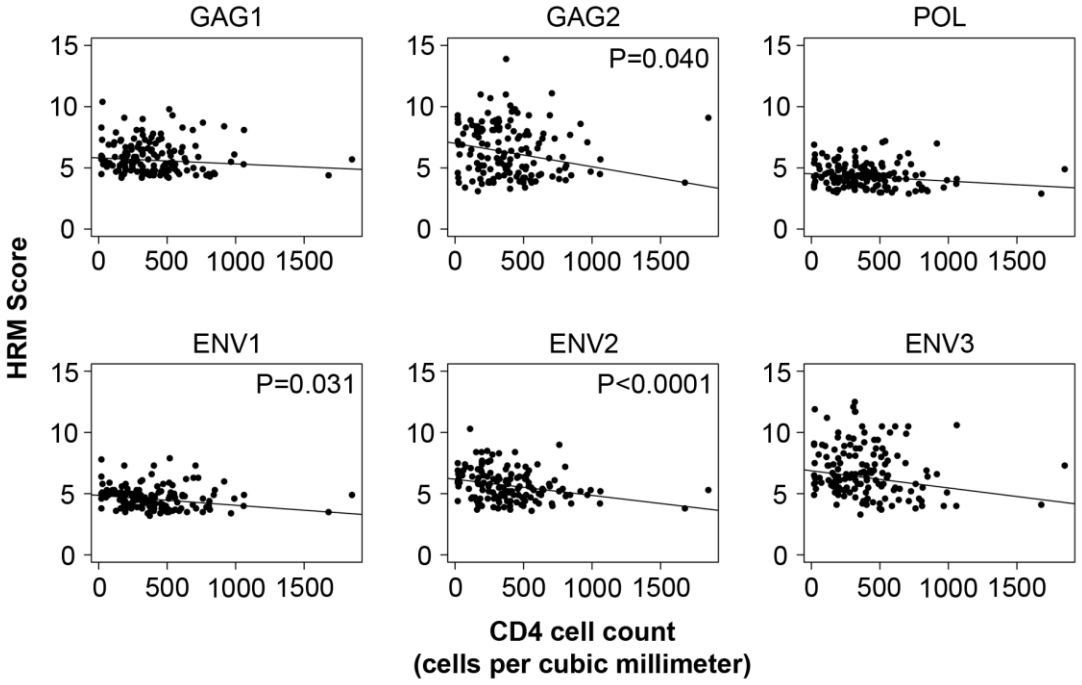
Figure 5.2. HRM scores for men who were HIV infected at enrollment and men who acquired HIV infection during the HPTN 061 study (seroconverters).



Legend for Figure 5.2.

Box and whisker plots show the distribution of high resolution melting (HRM) scores for all six regions analyzed for HIV seroconverters (A) and men who were HIV infected at study enrollment (A-D). For each column, the mean (circle), median (inner line), interquartile range (box), lower inner and upper outer fences (whiskers), and outliers are shown. Men who were HIV infected at enrollment were classified as having recent or non-recent HIV infection using a MAA. Univariate median regression analyses were used to compare HRM scores between the indicated groups; P values for these comparisons are shown (*P<0.05; **P<0.01).

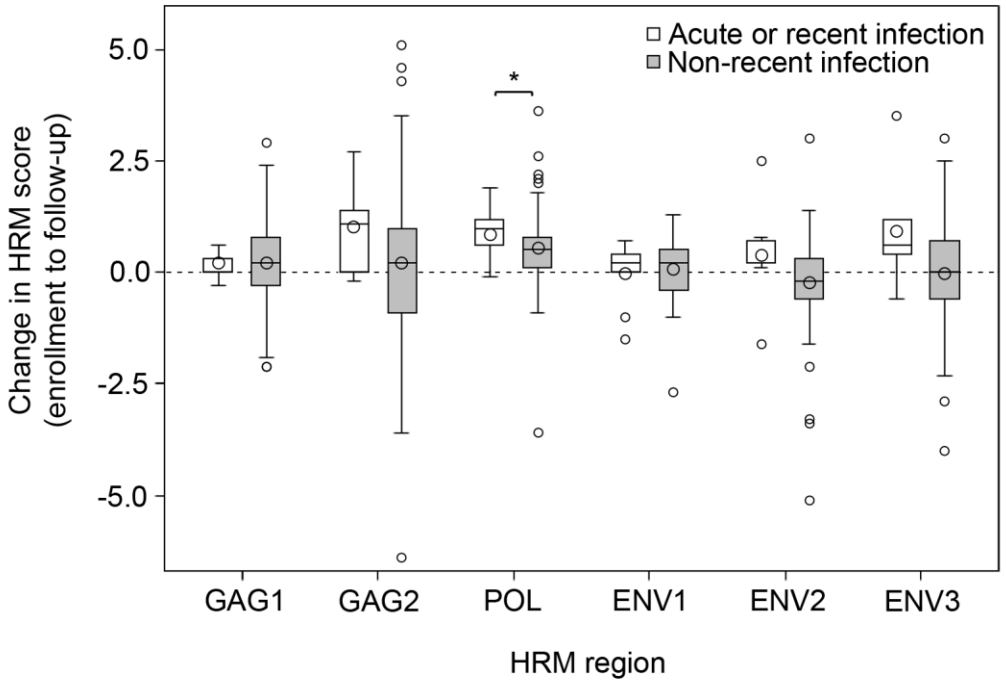
Figure 5.3. Association of HIV diversity and CD4 cell count in men who were HIV infected at enrollment.



Legend for Figure 5.3.

Scatterplots show the relationship between high resolution melting (HRM) score and CD4 cell count for men who were HIV infected at enrollment. P values <0.05 are shown.

Figure 5.4. Association between change in HRM score with HIV infection stage (paired analysis).



Legend for Figure 5.4.

Box and whisker plots show changes in HRM score over 12 months for men by HIV infection stage (acute or recent vs. non-recent). For each column, the mean (circle), median (inner line), interquartile range (box), lower inner and upper outer fences (whiskers), and outliers are shown. The change in HRM score was calculated by subtracting HRM scores obtained for the enrollment visit from those obtained for the 12-month study visit. P values <0.05 are shown (*P<0.05).

Chapter 6: Conclusions and future directions

Conclusions

The HPTN 061 study provided key insights into the HIV epidemic among Black MSM in the US. The men enrolled in HPTN 061 were all at a high risk for HIV transmission or acquisition. The two groups most at risk for transmitting HIV in HPTN 061 were analyzed in these studies: HIV-infected men who were not virally suppressed at enrollment and HIV seroconverters. Maintaining viral suppression in HIV-infected individuals on ART is crucial to improving health outcomes and preventing onward HIV transmission [104, 105]. To achieve viral suppression, individuals must first know they are infected, engage in HIV care, and then be prescribed and adhere to ART [106]. The work in this dissertation revealed pressing concerns about the availability and quality of HIV care for Black MSM at each of these stages in the HIV care continuum.

HIV treatment

Approximately half of the HIV-infected men in HPTN 061 were not virally suppressed at the time of study enrollment; this included men who were newly diagnosed, previously diagnosed but not in HIV care, and previously diagnosed and in HIV care. Many of these men had HIV drug resistance to NRTIs, NNRTIs, and PIs; 11% had MCR to two or more of those ARV drug classes. NNRTI and PI resistance was also detected in nearly a quarter of newly-infected men, including men with acute or recent infection at enrollment and men who seroconverted during the study. Notably, none of the men in HPTN 061 had INSTI resistance. This is promising since current first-line ART regimens in the US are comprised of two NRTIs (abacavir/3TC or FTC/TFV) with either an INSTI or RTV-boosted PI [51]. Unlike NNRTIs and INSTIs [107-109], NRTIs still possess partial ARV activity despite the presence of NRTI-associated DRMs [110]. Therefore, although NRTI resistance was common in HPTN 061, the men with drug-resistant HIV may still be able to take an INSTI-based ART regimen.

A third of the men who were not virally suppressed also had ARV drugs detected; these men were at risk for developing additional HIV drug resistance. Both HIV drug resistance and ARV drug detection were associated with self-reporting previous or current ART, which suggests some engagement in HIV care. However, ARV drug detection was not associated with self-reporting being in HIV care. It is possible that the men who had ARV drugs detected and reported current ART had recently initiated treatment and had not yet achieved viral suppression. However, it is not clear why men who reported previous but not current ART had ARV drugs detected; these men may have been previously engaged but not retained in HIV care. Many men also had unusual patterns of ARV drugs detected that were not recommended for ART [51-53]. Together, these findings suggest that men in HPTN 061 were taking ARV drugs without medical supervision for ART or other reasons. Additional studies are needed to understand the quality of HIV care and loss to ART follow-up for Black MSM as well as how they acquire and use ARV drugs.

HIV prevention

PrEP uptake among Black MSM has the potential to decrease HIV incidence in this population. Currently, Truvada® (FTC/TFV disoproxil fumarate) is the only ARV drug combination approved by the US FDA for PrEP [24]. In HPTN 061, the second most common DRM detected was M184V/I, which confers HIV drug resistance to FTC but increases HIV susceptibility to TFV. It is unclear whether drug-resistant viruses with the M184V/I mutation would impact PrEP efficacy. Transmission of drug-resistant HIV to an individual taking PrEP has been documented [111]. The drug-resistant variant in this case had MCR to NRTIs, NNRTIs, and INSTIs; notably, numerous NRTI-associated resistance mutations in addition to M184V were detected. As PrEP uptake continues to

increase, careful monitoring of prevalent as well as transmitted HIV drug resistance is needed in at-risk populations.

Several ARV drugs are also being evaluated as potential PrEP agents, including maraviroc, rilpivirine, and cabotegravir. Maraviroc-based regimens for PrEP were shown to be promising in phase II studies in MSM in the US [85]. However, DM viruses were frequently detected among the HIV seroconverters in HPTN 061; therefore, maraviroc-based regimens may not be appropriate for PrEP among Black MSM at a high risk for HIV acquisition. The NNRTI rilpivirine and the INSTI cabotegravir are under consideration as long-acting injectable forms of PrEP that can be administered every few months [75, 76]. Rilpivirine resistance was predicted in two men in HPTN 061 at enrollment; both men had MCR. In contrast, INSTI resistance was not predicted for any of the men in HPTN 061, including seroconverters. These findings are promising if clinical studies can demonstrate the efficacy of long-acting rilpivirine and cabotegravir for PrEP. Routine HIV drug resistance surveillance among Black MSM in the US will be needed as rilpivirine and INSTI use for ART continues to increase.

HIV diagnosis

As PrEP uptake continues to increase, studies are needed to characterize new HIV infections that may occur in individuals who take PrEP. Macaques that acquire simian immunodeficiency virus infection while taking ARV drugs for PrEP or PEP can experience an attenuated course of infection [112, 113]. Attenuated infection has also been observed in a case study of a clinical patient who acquired HIV infection while taking PEP [114]. In HPTN 061, ARV drugs were detected in one man with acute HIV infection and nine men with recent HIV infection at enrollment, which suggests that ARV drug use occurred among men who likely thought they were HIV uninfected. None of the HIV seroconverters had ARV drugs detected at their first HIV-positive visit. However, a

separate study revealed that one man had DRV and tipranavir detected at enrollment (i.e., while he was HIV uninfected) [54]. In that study, six (21%) of the 28 HIV seroconverters in HPTN 061 who had low or undetectable levels of HIV RNA at their first HIV-positive visit were characterized [54]. It is possible that ARV drug use at or near the time of HIV acquisition contributed to the unusual prevalence of low or undetectable viremia observed among these men. Two seroconverters who had undetectable viral loads were missed by several HIV tests, including fourth-generation assays [54], which suggests that such scenarios may complicate HIV diagnoses.

Infrequent HIV testing and late diagnoses were common in HPTN 061 [93]. It is possible that the HIV-infected men who received false-negative HIV test results would not have retested for HIV within 12 months if they had not been enrolled in the study. Viral load rebounds can occur in individuals with low or undetectable HIV RNA [115-117], which would increase their risk for HIV transmission. Diagnosing and engaging such individuals in care is important so their viral loads can be monitored. A quarter of the HIV seroconverters in HPTN 061 also had DM viruses and CD4 cell counts <350 cells/mm^3. DM viruses and low CD4 cell counts are both associated with accelerated disease progression [77, 78, 90, 91]. Diagnosing these men soon after HIV infection would facilitate earlier ART initiation, which can lead to better health outcomes [118, 119]. These findings further highlight the urgency of expanding HIV testing efforts among Black MSM.

Future directions

Additional studies are needed to understand the unusual patterns of ARV drug use among both HIV-infected and HIV-uninfected Black MSM. ARV drug testing could not be performed for the HIV-uninfected men enrolled in HPTN 061. However, in a separate study, ARV drugs were detected in 39 samples from HIV-uninfected women at

a high risk for HIV infection in the US [120]. Multiple PIs and outdated PIs were detected among these women, which was similar to that observed in HPTN 061. ARV drug testing in future cohorts of HIV-uninfected Black MSM may provide additional insight into the patterns of and motivations for ARV drug use in this population.

Interventions designed to increase HIV awareness, prevention, and detection are urgently needed to control the HIV epidemic among Black MSM in the US. These interventions must expand the availability and quality of health services for diagnosing and treating HIV in this population and ensure that these health services are acceptable and effective for Black MSM.

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Curriculum Vitae

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Educational History

Ph.D. expected	2016	Program in Cellular and Molecular Medicine Mentor: Dr. Susan Eshleman, M.D., Ph.D.	Johns Hopkins University School of Medicine
B.S.	2011	Biology	University of North Carolina at Chapel Hill

Other Professional Experience

Assistant Research Program Coordinator	2014-2016	Generation Tomorrow, Center for AIDS Research, Johns Hopkins University
Editor	2012-2014	American Eagle Editing Office
Undergraduate Commendation Research	2008-2011	Lab of Dr. Sarah Liljegren, University of North Carolina at Chapel Hill
Research Assistant	2007-2008	Lab of Dr. David Ryugo, Johns Hopkins University School of Medicine

Academic and Other Honors

2015	Young Investigators' Scholarship	Conference on Retroviruses and Opportunistic Infections
2014	Young Investigators' Scholarship	Conference on Retroviruses and Opportunistic Infections
2014	Excellence in Clinical Research	Pathology Young Investigators' Day, Johns Hopkins University School of Medicine
2011	Francis J. LeClair Award	Department of Biology, University of North Carolina at Chapel Hill
2010	Summer Undergraduate Research Fellowship	The American Society of Plant Biologists

Publications

Peer-reviewed articles

Chen I, Connor MB, Clarke W, Marzinke MA, Cummings V, Breaud A, Fogel JM, Laeyendecker O, Fields SD, Donnell D, Griffith S, Scott HM, Shoptaw S, del Rio C, Magnus M, Mannheimer S, Wheeler DP, Mayer KH, Koblin BA, Eshleman SH (2015) Antiretroviral drug use and HIV drug resistance among HIV-infected Black men who have sex with men: HIV Prevention Trials Network 061. J Acquir Immune Defic Syndr. 69:446-452.

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