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HIV-1 Genetic Diversity, Evolution and Neuropsychological Impairment

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

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by

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PUBLICATIONS

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ABSTRACT OF THE DISSERTATION

HIV-1 Genetic Diversity, Evolution and Neuropsychological Impairment

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HIV-1 associated neurocognitive disorders (HAND) remain a significant problem worldwide and can range in severity from disabling dementia to asymptomatic cognitive, motor and behavioral changes. Clinical assessments are the gold standard for detecting impairment and have defined the scope and severity of HAND; however, the viral determinants of HAND are not well understood. To further characterize the viral determinants of HAND, we studied viral evolution and disease by analyzing HIV-1 RNA extracted from blood and CSF samples along with participant specific clinical and neuropsychological assessments. HIV-1 RNA was amplified with Polymerase Chain Reaction (PCR) and sequenced using the Sanger Method. Neuropsychological assessments

were administered and scored by trained psychometrists and included seven ability domains: learning, delayed recall, verbal fluency, processing speed, attention/working memory, abstraction/executive functioning and motor speed.

HIV-1 population diversity was positively associated with disease, specifically AIDS and neuropsychological impairment. Further, antiretroviral resistance was associated with lower CSF viral loads and with better neuropsychological performance. Our findings suggest that HIV-1 virulence is most likely associated with the capacity of a viral population to maintain genetic diversity and not simply a specific and predominant genotypic variant. Although, the exact underlying mechanism for this remains unclear, complex phenotypes such as neuropsychological impairment may result from different HIV-1 variants operating in a coordinated manner to cause disease.

INTRODUCTION

HIV associated neurocognitive disorders (HAND) range in severity from disabling dementia to asymptomatic cognitive, motor and behavioral changes. With the widespread use of antiretroviral therapy (ART) in economically privileged countries, the incidence of HIV-associated dementia, characterized by severe neuropsychological (NP) impairment and inability to perform activities of daily living, has significantly decreased [1-2]. Despite a decrease in incident dementia, less severe forms of HAND have persisted [3-6] and may actually be increasing as HIV-infected individuals live longer [7-8]. Clinical assessments are the gold standard for detecting impairment and have defined the scope and severity of HAND; however, the viral determinants of HAND are not as well characterized.

HIV-1 crosses the blood brain barrier soon after transmission and the neurocognitive disorders associated with HIV-1, likely result from the direct and indirect consequences of viral replication [9-12]. Early studies of HIV-1 viral load in the CSF provided evidence of a direct relationship between viral load and neurocognitive impairment [13-15]; however more recent studies indicate HAND is likely determined by a complex interplay between cellular targets, viral factors and the host immune response [16-17]. To further characterize the viral determinants of disease, we studied: 1) HIV-1 *pol* evolution following acute infection, 2) HIV-1 population diversity and HAND and 3) HIV-1 antiretroviral resistance mutations and HAND.

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CHAPTER 1

HIV-1 *pol* Evolution Following Primary Infection

ABSTRACT

Efforts aimed at characterizing HIV-1 *pol* genotypic variation have largely focused on the frequency and occurrence of mutations associated with resistance to antiretroviral therapy (ART); however, how HIV-1 *pol* evolves within an individual is not well understood. To further characterize intra-individual HIV-1 *pol* evolution, we examined longitudinally collected blood samples from antiretroviral naïve individuals who were identified during early HIV-1 infection. HIV-1 *pol* evolution occurred at a rate of 4.181×10^{-3} (SD=2.570×10^{-3}) nucleotide substitutions per site per year and was time dependent, with evolution occurring at a faster pace during early stages of infection ($p<.0001$). Even after 7 years of follow-up, using the TN94 model to infer genetic distance, no participant had greater than 2% divergence between any two sequences. Further, the rate of HIV-1 *pol* evolution was not associated with HLA haplotype, CD4 or viral load. Taken together, these findings may provide useful information as more and more studies use HIV-1 *pol* sequences to assess transmission events, transmission networks and the presence of HIV-1 dual infection.

Introduction

Within a newly infected individual, HIV-1 quickly evolves from small number of homogenous variants into a highly diverse viral population [1-3]. The capacity of HIV-1 to rapidly evolve is attributed to a low fidelity reverse transcriptase (2.5-3.4 x 10^{-5} per site per generation), high replication rate (10^{10} virions produced daily), high recombination rate and viral proteins that maintain functionality despite extensive amino acid variability [4]. Estimates of evolution within HIV-1 *env*, suggest HIV-1 is one of the fastest evolving organisms [5-7]; however, mutation rates and the proportion of nucleotide sites that tolerate mutations differ both within and between HIV-1 coding regions [8-10]. These observed differences are likely shaped by the functional constraints of encoded proteins, cellular restriction factors and specific host immune responses [11-13]. HIV-1 *env* and *gag* provide two well characterized but contrasting examples of HIV-1 evolution. HIV-1 *env* is highly variable with the fixation of nucleotide variants driven by escape from neutralizing antibody [7, 14-16], while HIV-1 *gag* is relatively conserved and evolution is associated with CTL escape [9, 12, 17].

Efforts aimed at characterizing HIV-1 *pol* genotypic variation have largely focused on the frequency and occurrence of mutations associated with resistance to antiretroviral therapy (ART). Advances in characterizing these mutations have served to improve the clinical management of HIV-1 infection and expand *pol* sequencing. To date, HIV-1 *pol* may be the most sequenced gene from any organism (eg. as of 6/7/11 there were139,890 sequences in the

Stanford HIV Drug Resistance Database). In addition to providing important information for the treatment of HIV infected individuals, the generation and collection of HIV-1 *pol* sequence data has enhanced our understanding of the epidemiology of HIV-1 in time and geography [18-20]. While, access to large HIV-1 sequence databanks has facilitated the characterization of HIV-1 genotypic variation within large epidemics, how HIV-1 *pol* evolves within an antiretroviral naive individual following acute infection, is not as well characterized. The present study characterized intra-individual HIV-1 subtype B *pol* evolution using longitudinally collected blood samples from a well-defined cohort of antiretroviral naïve individuals.

Methods

Participants

Participants included in this study were enrolled in the San Diego Primary Infection Cohort from July 1, 1996 through May 30, 2007. Inclusion was limited to participants that remained antiretroviral naïve through the collection of at least two blood samples, had >1 population-based *pol* sequence and found not to be dually infected while followed for this study. Dual infection was assessed by previously published methods [21-22]. Additional participant data included demographics, estimated date of infection, reported HIV risk behaviors, HLA haplotype, antiretroviral resistance, CD4 counts and blood plasma viral load. Duration of infection for participants at enrollment was estimated using a standardized protocol [23-24].

Population-based HIV-1 pol sequencing and Sequence analysis

For the primary infection cohort, the ViroSeq™ HIV genotyping system (Applied Biosystems, Foster City, CA) was used for population-based *pol* sequencing per manufacturer instructions. The system generated a 1500 base pair amplicon including the entire protease and first 2/3 of the reverse transcriptase. Sequencing was performed on an ABI 3100 Genetic Analyzer, and sequences were manually reviewed and edited employing the ViroSeq™ genotyping software (Version 2.4.2). Genotypic drug resistance was interpreted

by the algorithm available with the ViroSeq™ program. Mixed bases were determined by both the basecaller program in the Viroseq™ package and through manual interrogation of the sequence electropherograms. Sequences were initially compiled, aligned, and edited in BioEdit using CLUSTAL W alignment tool [25]. The alignment was then manually edited to preserve frame insertions and deletions. HIV-1 subtype was determined using SCUEAL [26].

Estimating intra-individual HIV-1 pol genetic distance

For each participant a longitudinal measure of HIV-1 *pol* genetic distance was measured by comparing a participant's earliest and latest available population-based *pol* sequence. For each pairwise comparison two different measures of HIV-1 genetic distance were calculated, one based on a weighted count of nucleotide changes (Tamura-Nei 94 model) and the second based on unweighted (hamming distance) counts of both nucleotide substitutions and changes to mixed bases. To normalize genetic distance estimates, measures were divided by sequence length.

Estimating intra-individual HIV-1 pol evolution.

HIV-1 *pol* evolution was determined by dividing intra-individual *pol* genetic distances by the time elapsed between sample collection dates. First, intra-individual estimates of evolution were calculated using data that covered a participant's total time from follow-up. Additional analysis was limited to

participants with ≥ 3 population-based *pol* sequences and included *pol* velocity estimates for each available sequence from baseline. To compare these HIV-1 *pol* evolution estimates across the cohort, measurements were grouped by time from estimated date of infection (EDI), with the largest grouping of participants corresponding to ≤2 years EDI and >2 years EDI. Intra-individual early and late estimates were compared using a non-parametic paired t-test.

Results

Participant Characteristics.

Between January 2006 and May 2007, 115 individuals were enrolled in the San Diego primary infection cohort. All participants were diagnosed with primary HIV-1 infection and were antiretroviral naïve at the time of enrollment. In total, 25 of the 115 enrolled participants were excluded from this study: seven because only one *pol* sequence was available, seven started ART before at least one follow-up sample was taken, three were infected with non-subtype B HIV-1, and seven were found to be intraclade dually infected [22]. The 90 individuals who meet eligibility criteria were all male, and the majority were non-Hispanic white (74%) and reported sex with other men (97%) as their major risk factor for acquiring HIV. Following enrollment, participants were observed for a median of 2.74 years (min=0.49, max=8.97) since primary infection.

At least one major antiretroviral resistance mutation found in 17% of participants, which was consistent with results reported previously from this cohort [23]. The most common resistance associated mutations were identified within reverse transcriptase.

Intra-individual estimates of HIV-1 pol genetic evolution and disease.

HIV-1 *pol* evolution was determined by conducting a pairwise comparison between a participant's earliest and latest available *pol* sequence. For each participant, two different measures of HIV-1 genetic distance were calculated, one based on a weighted count of nucleotide changes (Tamura-Nei 94 model) and the other an unweighted count (Hamming distance). Estimates of genetic distance based on unweighted counts were significantly higher than weighted estimates. The higher genetic distances seen with the normalized unweighted counts were largely attributed to variation at sites with mixed bases. Under the TN94 model, mixed bases were treated as non-informative and excluded from analysis, per standard procedure [27]. Since more than half of the genetic distance measures using the TN94 model based were zero, we measured HIV-1 *pol* evolution using the approach that included counts of mixed bases. HIV-1 *pol* evolution occurred at a faster rate during the earlier stages of infection ($p<.0001$) but was not associated with HLA haplotype, CD4 count, viral load or the presence of transmitted resistance associated mutations.

Conclusions

Understanding how HIV-1 evolves within an infected person is important for understanding drug resistance and likely also important for vaccine design and molecular epidemiology. The humoral arm of the adaptive immune response explains most of the evolution observed over the course of infection within HIV-1 *env* but not for other coding regions [7, 15, 16]. On the other hand, approximately two-thirds of all mutations in non-envelope regions are likely secondary to CTL selection [8, 28-30]. Further, evidence from well-characterized epitopes, such as gag p24, suggests intra-individual patterns of CTL escape mutations vary dramatically between coding regions [9, 12, 17].

To further characterize HIV-1 subtype B *pol* genetic variability, we studied HIV-1 *pol* evolution over the course of infection. We measured intra-individual genetic distance using two different approaches. Genetic distance measures using conventional approaches were zero for most individuals. The second approach, which was unweighted and counted changes in mixed bases as nucleotide substations, estimated HIV-1 *pol* evolution occurred at a rate of 4.181×10^{-3} nucleotides per site per year.

In this study, intra-individual genetic divergence remained low, even after 7 years of follow-up. HIV-1 *pol* evolution appeared to occur at a faster pace during the early stages of infection but overall was not associated with HLA haplotype, CD4 count, viral load or the presence of transmitted resistance associated mutations. Taken together, these measures may provide useful

information as more and more studies use HIV-1 *pol* sequences to assess transmission events, transmission networks and the presence of HIV-1 dual infection.

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CHAPTER 2

Higher HIV-1 Genetic Diversity, AIDS and Neuropsychological Impairment

ABSTRACT

Standard methods used to estimate HIV-1 population diversity in the setting of natural infection are not well suited for use with large study cohorts (e.g., single genome amplification, clonal amplification and pyrosequencing). Additional approaches are needed to address unresolved questions regarding the relationship between intra-individual HIV-1 genetic diversity and disease. With a small cohort of individuals, we validated a method for measuring HIV-1 population diversity and then used this approach investigate disease in large cohort (n=187) of participants who underwent neuropsychological and clinical assessments (n=187). We found HIV-1 population diversity was associated with both a diagnosis of AIDS and neuropsychological impairment.

Introduction

HIV-1 is usually transmitted as a small number of relatively homogenous variants, but exists within an infected individual as a highly diverse viral population [1-4]. In vitro studies of viral RNA model systems suggest that maintaining this highly diverse viral population may provide an adaptive advantage and can alter virulence [5-10]. Additional study is needed to better characterize both HIV-1 diversity within an individual and the mechanistic underpinnings of HIV-1 virulence.

Standard methods used to estimate HIV-1 population diversity in the setting of natural infection are resource intensive (e.g. single genome amplification, clonal amplification, pyrosequencing and heteroduplex assay), which likely explains why past studies that examined intra-individual HIV-1 population diversity and disease progression relied on relatively small cohorts (range n=6-44) [11-18]. Additionally, these studies arrived at conflicting results. Several observed an association between increased intra-individual HIV-1 population diversity and disease, such as progression to acquired immunodeficiency syndrome (AIDS) [11, 15-18], while others did not [12-14].

To enhance the study of HIV-1 diversity and address these unresolved questions, we validated a method for estimating HIV-1 population diversity that relies on HIV-1 *pol* sequence data generated as part of standard clinical care [19-22]. After validating this approach, we studied the relationship between intra-individual HIV-1 population diversity and disease in a large cohort of clinically

well-characterized HIV-1 infected individuals who underwent neuromedical and comprehensive neuropsychological testing examinations.

Methods

Study Design.

Our study utilized two different participant cohorts. From the UCSD Primary Infection Cohort, we used blood samples to investigate if mixed base counts provide reliable estimates of HIV-1 diversity. From the CNS HIV Antiretroviral Therapy Effects Research (CHARTER) Cohort, we used blood and cerebrospinal fluid (CSF) samples to investigate the relationship between intra-host HIV-1 population diversity and disease.

Participants enrolled in the UCSD Primary Infection Cohort were included in this study if they were antiretroviral naive, had two or more longitudinally collected blood samples, and single genome and population-based sequences were available from blood samples collected within a month of each other. Blood samples from dates at which an individual was suspected of dual infection with previously published criteria were excluded [21]. Using a longitudinal cohort allowed us to screen for dual infections and decrease any irregularities in our validation studies that might be attributable to dual infection. When two or more sample dates were eligible from the same participant, the last date was used.

All participants enrolled in the CHARTER Cohort were included in this study if viral loads were high enough (~500 copies/ml) to generate population-based sequences of HIV-1 *pol*, as described below. CHARTER participants received neuromedical, neuropsychological, and laboratory examinations. Standardized neuropsychological assessments tested seven ability domains

(learning, delayed recall, verbal fluency, processing speed, attention/working memory, abstraction/executive functioning, motor speed), as previously described [23]. Results were summarized using global ratings that ranged from 1 (above average) to 9 (severely impaired) with a global score of 5 or higher used to categorize participants as impaired [24-25]. These methods conform to the recently published international guidelines for classifying HIV-1 associated neurocgnitive disorders [26]. Also, for each participant, the “Frascati” criteria (incidental, contributing, confounding) were used to classify non-HIV-1 related conditions that increase risk for neurocognitive impairment [25]. Infection duration in this cohort was available only as self-reported information. Among CHARTER participants, AIDS diagnosis was based on the 1993 Centers for Disease Control guidelines [www.cdc.gov/hiv/resources/guidelines].

The Human Research Protection Program of the University of California San Diego approved the use of all participant data and samples. In accordance with the Human Research Protection Program of the University of California, informed consent was provided by all study participants.

Sample Collection and Processing.

For both cohorts, blood plasma was collected by venipucture and CSF by lumbar puncture, and stored at −80 °C. For blood samples collected from participants enrolled in the UCSD Primary Infection Cohort, HIV RNA was extracted using the QIAamp ViralRNA Mini Kit (Qiagen, Hilden, Germany) per manufacturer’s instructions. For blood and CSF samples collected from

CHARTER Cohort participants, HIV RNA was extracted using the ViroSeq v.2.0 HIV genotyping system (Applied Biosystems, Foster City, CA, USA).

Single Genome Amplification and Sequencing.

Extracted HIV RNA was used to generate cDNA using random decamers and the RETROscript kit (Ambion, Applied Biosystems, Foster City, CA, USA) per manufacturer's instructions. As previously described [27], the first round of nested PCR was performed with 10 uL of diluted cDNA template and 40 uL of reaction mix, which included the following outer primer set (CI-Pol 5', 3RT 3'). The second round PCR, was performed with 5 uL of the first round product and 45uL of reaction mixture which included the following inner primer set (5RT 5', 3RT 3'). Reactions were performed in parallel on a 96-well plate, and the second-round products were visualized to ensure no more than 30% of the reaction wells were positive [28].

Population-based Pol Sequencing.

The ViroSeq HIV genotyping system (Ambion Applied Biosystems) was used for population-based HIV-1 *pol* sequencing per manufacturer's instructions. Following cDNA synthesis, polymerase chain reaction (PCR) amplification was used to generate a 1500-bp amplicon including the entire protease and first two thirds of the reverse transcriptase. The resulting PCR product was sequenced using the ABI 3100 Genetic Analyzer, sequences were manually edited and

resistance associated mutations were determined using the ViroSeq genotyping software.

Evaluating and Comparing Measures of Intra-individual HIV-1 Diversity.

Using HIV RNA extracted from the blood samples of participants enrolled in the UCSD Primary Infection Cohort, we evaluated HIV-1 population diversity with three different methods: 1) Shannon Entropy using single genome, 2) average pairwise distance (APD) using single genome sequences and 3) counts of mixed bases (i.e., ambiguous nucleotides) in population-based sequences.

A batch file implemented in HyPhy [29] was used to provide a count of total mixed bases, synonymous mixed bases and nonsynonymous mixed bases in population-based *pol* sequences. Each count was divided by sequence length to provide a normalized index: Total Mixed Base Index (TM-Index), Synonymous Mixed Base Index (SM-Index) and Amino Acid Residue Mixed Base Index (RM-Index). Shannon Entropy was measured for nucleotide sequences and amino acid sequences using the HIV Los Alamos National Laboratory (LANL) Entropy-One tool (http://www.hiv.lanl.gov, accessed April 2010). For each participant‘s set of single genome sequences, Entropy-One results were summarized as the number of sites with a Shannon Entropy value ≥ .3 divided by the number of positions in the alignment. APD was measured by analyzing participant specific single genome sequences with HyPHy software using the TN93 model [29].

Estimating Cost and Time.

Estimates of the comparative costs and labor required for population-based sequencing, Single genome sequencing (SGS) and Ultra deep sequencing (UDS) were based on previously published analysis [21]. The estimated and anticipated sequencing costs for measuring HIV-1 population diversity were calculated from the costs of reagents, disposable materials, kits and sequencing runs. The estimated and anticipated labor was calculated as the labor time plus instrument time required to perform sequencing.

Statistical Analysis.

All tests were two-sided and interpreted at 5% significance level. Tests for associations between two continuous variables were performed using Spearman rank correlation. Logistic regression analyses were used to investigate the relationships between HIV-1 diversity (measured by the TM-Index, SM-Index and RM-Index) and AIDS diagnosis and between HIV-1 diversity and neuropsychological impairment. Additional variables considered in multivariable logistic regression analyses, where appropriate, included HIV RNA levels in CSF and blood, duration of infection, current and nadir CD4, and ART history. The model investigating neuropsychological impairment also controlled for AIDS and non-HIV-1 related conditions that increase risk for neurocognitive impairment. The odds ratios were calculated for a 0.01 unit increase in each mixed base index.

Results

Study Participants.

Participants from the San Diego Primary Infection cohort (n=16) selected to investigate the use of mixed base indices, were mostly white men in their early 30s. The three major reasons participants enrolled in the Primary Infection Cohort did not meet inclusion criteria were: 1) the absence of longitudinal samples to assess the potential of dual infection, 2) the absence of single genome and population-based sequences performed on blood samples collected within a month of each other, and 3) HIV-1 dual infection as identified by previously published methods [21].

The participants from the CHARTER cohort (n=187) selected to investigate the relationship between HIV-1 population diversity and disease were in large part black (49%) men (80%) in their early 40s and 23% were receiving antiretroviral therapy at the time of sampling. Median blood and CSF HIV RNA levels were 4.6 and 3.6 $\log_{10}$ copies/ml, 43% had an AIDS diagnosis, and the median current and nadir CD4 counts were 362 and 251 cells/ul (Table 1). Drug resistance mutations were not common; M184V and K103N were the two most prevalent. The M184V mutation was detected in 8 blood samples (5 %) and 7 CSF samples (4 %). The K103N mutation was detected in 13 blood samples (7 %) and 14 CSF samples (8%).

Table 2.1: CHARTER participant demographics and clinical characteristics

CHARTER participants (n=187) who met study criteria were in large part black (49%) men (80%) in their early 40s and 23% were receiving antiretroviral therapy at the time of sampling. Values are reported as percentages or medians with interquartile (IQR) ranges in parentheses.

Variable (N = 187)	**Median (IQR) or %**
Male	80%
Black	49%
Hispanic	8%
White	41%
Age (years)	42 (37 – 47)
Estimated Duration of infection (months)	113 (39 – 173)
HIV RNA CSF (log_{10}copies/ml)	3.56 (3.14 – 4.05)
HIV RNA Blood (log_{10}copies/ml)	4.55 (4.11 – 4.88)
Current CD4 (cells/ul)	362 (224 – 532)
Nadir CD4 (cells/ul)	251 (141 – 378)
AIDS	43%
Blood Total Mixed Base Index	0.013 (0.009 – 0.018)
Blood Synonymous Mixed Base Index	0.010 (0.005 – 0.014)
Blood Residue Mixed Base Index	0.003 (0.002 – 0.005)
CSF Total Mixed Base Index	0.014 (0.008 – 0.019)
CSF Synonymous Mixed Base Index	0.009 (0.005 – 0.014)
CSF Residue Mixed Base Index	0.003 (0.002 – 0.005)

Mixed Base Counts and Evaluating Intra-individual HIV-1 Diversity.

Using single genome and population-based sequences generated from UCSD Primary Infection Cohort participants, we measured and compared Shannon Entropy of nucleotide sequences and APD to two mixed base indices structured to estimate nucleotide diversity (TM-Index and SM-Index). Also, Shannon Entropy of amino acid sequences was compared to the mixed base index structured to measure amino acid diversity (RM-Index). In brief, all mixed base indices strongly correlated with other measures of viral population diversity (figures 1 and 2). Specifically, the TM-Index correlated with both Shannon Entropy (nucleotide) (rho = 0.76, p = 0.001) (figure 1a) and with APD (rho = 0.80, $p < 0.001$) (figure 2a). The SM-Index also correlated with both Shannon Entropy (nucleotide) (rho = 0.72, p = 0.002) (figure 1b) and with APD (rho = 0.75, p = 0.001) (figure 2b). The RM-Index correlated with Shannon Entropy (amino acid) (rho = 0.59, p = 0.015) (figure 1c).

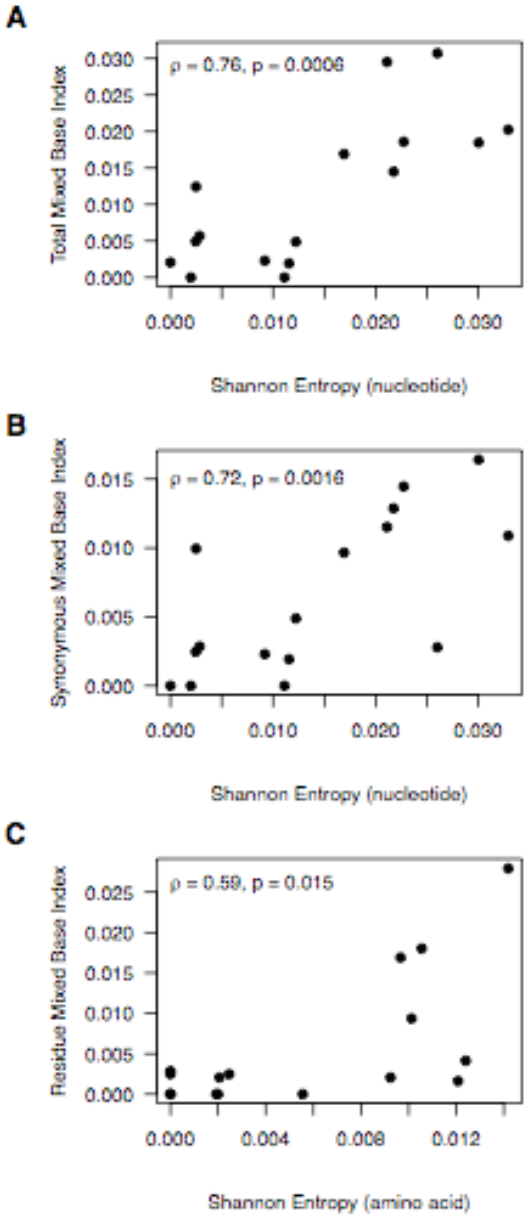

Figure 2.1: Comparing Shannon Entropy to mixed base indices

For participants with both single genome and population-based sequences, we measured and compared Shannon Entropy to mixed base indices structured to estimate nucleotide and amino acid diversity. Shannon Entropy of nucleotide sequences correlated with the (A) Total Mixed Base Index (rho = 0.76, p = 0.001) and (B) Synonymous Mixed Base Index (rho = 0.72, p = 0.002). Also, Shannon Entropy of amino acid sequences correlated with the (C) Residue Mixed Base Index (rho = 0.59, p = 0.015).

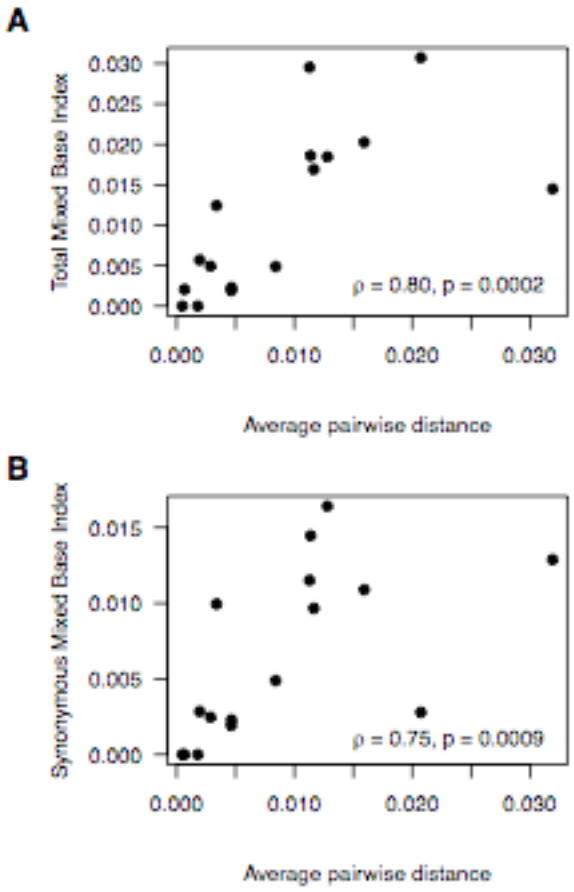

Figure 2.2: Comparing average pairwise distance (APD) to mixed base indices

For participants with both single genome and population-based sequences, we measured and compared APD to mixed base indices structured to estimate nucleotide diversity. APD correlated with the (A) Total Mixed Base Index (rho=0.80, p=0.002) and (B) Synonymous Mixed Base Index (rho=0.75, p<0.001).

HIV-1 Population Diversity, AIDS and Neuropsychological Performance.

Using population-based HIV-1 *pol* sequence data, AIDS diagnosis and standard classifications of neurocognitive status from CHARTER cohort participants, we tested a number of statistical models that, where appropriate, included HIV RNA levels in CSF and blood, duration of infection, current and nadir CD4, ART history and co-morbidity status (incidental, contributing, confounding). We found a diagnosis of AIDS was independently associated with: (i) TM-Indices in blood (blood OR = 2.0, p = 0.015) and (ii) RM-Index in blood (OR = 6.5, p = 0.018) (Table 2). A trend for an association between AIDS diagnosis with the SM-Index in blood was also observed (OR = 1.8, p = 0.067). In modeling neuropsychological impairment, nadir CD4 was initially included but eventually excluded because current CD4 was a stronger predictor of impairment. Concerning neuropsychological impairment, we found that it was independently associated with the TM-index in blood (OR = 1.9, p = 0.01) and RM-Index in blood (OR = 11.4, p = 0.001) (Table 3). There was also a trend for the CSF TM-Index (p = 0.08) to be associated with neuropsychological impairment. For all of these models, the association between viral diversity and neurocognitive impairment was independent of diagnosed comorbid dieases, as determined by “Frascati” criteria [25].

Table 2.2: Multivariable regressions modeling AIDS

A diagnosis of AIDS was independently associated with the Total Mixed Base Index measured in blood, (OR = 2.0, p = 0.015, Model A) and the Residue Mixed Base Index measured in blood (OR = 6.5, p = 0.018, Model B). Variables that are independently significant at p<0.05 are in bold.

Model A: AIDS as a function of Blood Total Mixed Base Index, infection duration, plasma VL and ART history				
	Estimate	Std Error	Adjusted OR	**p-value**
Blood Total Mixed Base Index	0.666	0.275	1.95	**0.0154**
Infection Duration (years)	0.103	0.036	1.11	**0.0039**
Blood Plasma VL (log_{10})	0.669	0.267	1.95	**0.0121**
ART History (Current Use)	3.046	0.604	21.0	**<0.0001**
ART History (Past Use)	0.901	0.467	2.46	0.0539
Model B: AIDS as a function of Plasma Residue Mixed Base Index, infection duration, plasma VL and ART history				
	Estimate	Std Error	Adjusted OR	**p-value**
Blood Residue Mixed base Index	1.865	0.788	6.46	**0.0179**
Infection Duration (years)	0.123	0.035	1.13	**0.0004**
Blood Plasma VL (log_{10})	0.685	0.268	1.98	**0.0107**
ART History (Current Use)	2.886	0.594	17.9	**<0.0001**
ART History (Past Use)	0.765	0.465	2.15	0.0998

Table 2.3: Multivariable regressions modeling neuropsychological performance

Neuropsychological impairment was independently associated with the Total Mixed Base Index measured in blood (OR = 1.9, p = 0.01, Model A) and the Residue Mixed Base Index measured in blood (OR = 11.4, p = 0.001, Model B). Variables that are independently significant at p<0.05 are in bold. [a] Odds ratio (OR) per 10 units increase in CD4 count.

Model A: Neurocognitive impairment as a function of Blood Total Mixed Base Index, infection duration, AIDS, current CD4, comorbidity status and ART History				
	Estimate	Std Error	Adjusted OR	p-value
Blood Total Mixed Base Index	0.649	0.256	1.9	**0.013**
Infection Duration (years)	-0.105	0.037	0.90	**0.004**
Current CD4 count	-0.015	0.01	0.99[a]	0.140
AIDS	-0.758	0.493	0.47	0.123
Comorbidity (Confounding)	2.36	0.541	10.6	**< 0.001**
Comorbidity (Contributing)	1.01	0.406	2.8	**0.013**
ART History (Current Use)	1.06	0.569	2.9	0.062
ART History (Past Use)	0.066	0.437	1.1	0.879
Model B: Neurocognitive impairment as a function of Blood Residue Mixed Base Index, infection duration, AIDS, current CD4, comorbidity status and ART History				
	Estimate	Std Error	Adjusted OR	p-value
Blood Residue Mixed Base Index	2.43	0.759	11.4	**0.001**
Infection Duration (years)	-0.088	0.035	0.92	**0.013**
Current CD4 count	-0.015	0.010	0.98[a]	0.136
AIDS	-0.891	0.507	0.41	0.079
Comorbidity (Confounding)	2.46	0.551	11.7	**< 0.001**
Comorbidity (Contributing)	1.08	0.413	2.9	**0.009**
ART History (Current Use)	1.03	0.585	2.8	0.077
ART History (Past Use)	0.037	0.452	1.0	0.935

Intra-individual HIV-1 Diversity and Viral Dynamics in Blood and CSF.

We further clarified the relationship between HIV diversity and the sampled compartments. HIV-1 population diversity in blood plasma correlated with diversity in the CSF for all mixed base indices (Figures 3a-c). In addition, for most individuals, the RM-Index was higher for viral populations derived from the CSF than for blood (0.004 vs. 0.037, p=0.059), but this was not observed for the TM-Index (CSF 0.0143 vs. blood 0.0137, p=0.115) and SM-Index (CSF 0.010 vs. blood 0.010, p=0.263). These findings were not explained by pleocytosis, as defined by >5 white blood cells/ml of CSF [30]. The correlation between blood TM-index and CSF TM-Index did not differ between participants with or without pleocytosis (rho=0.641 vs. rho=0.728, p= 0.321). When we examined nucleotide diversity in blood and CSF in relation to time, we found that the duration of infection had linear relationships with both blood TM-index ($p < 0.001$) (figure 4a) and blood SM-Index ($p < 0.001$) (figure 4b). In contrast, the relationship between duration of infection and amino acid diversity in blood, as measured by the RM-Index, was best described by quadratic regression ($p = 0.07$), where the RM-Index steadily increased until approximately year 12 of infection and then steadily declined (figure 4c).

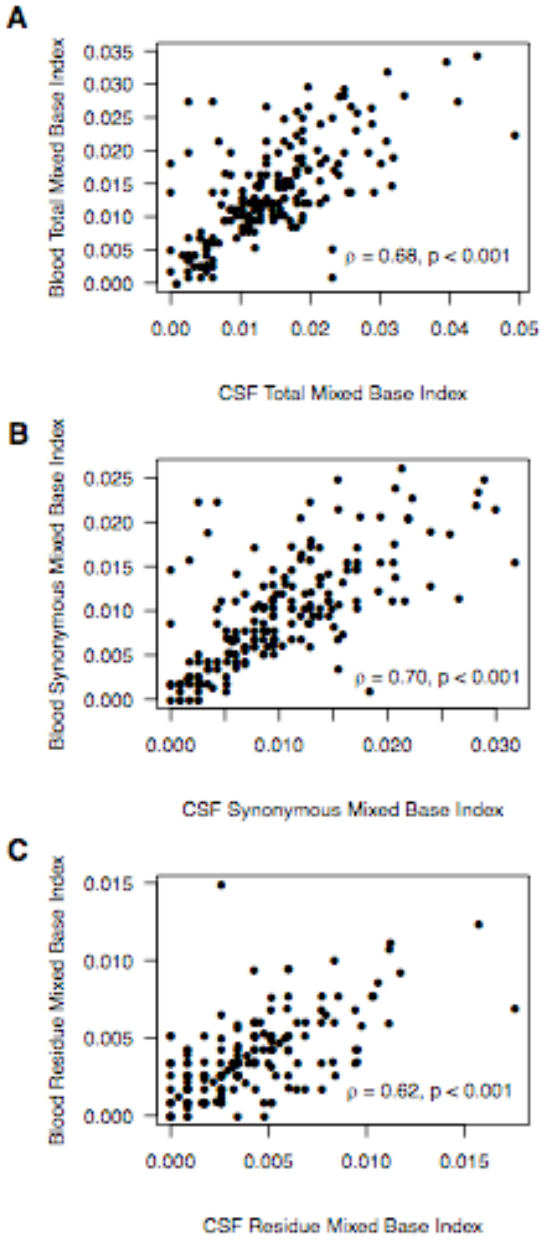

Figure 2.3: HIV-1 population diversity in blood and CSF

HIV-1 population diversity was evaluated in blood and CSF using normalized counts of mixed bases from population-based sequences. HIV-1 population diversity in blood correlated with diversity in CSF as measured by the (A) Total Mixed Base Index (spearman rho=0.68, p<0.001), (B) Synonymous Mixed Base Index (spearman rho=0.70, p<0.001) and (C) Residue Mixed Base Index (spearman rho=0.62, p<0.001).

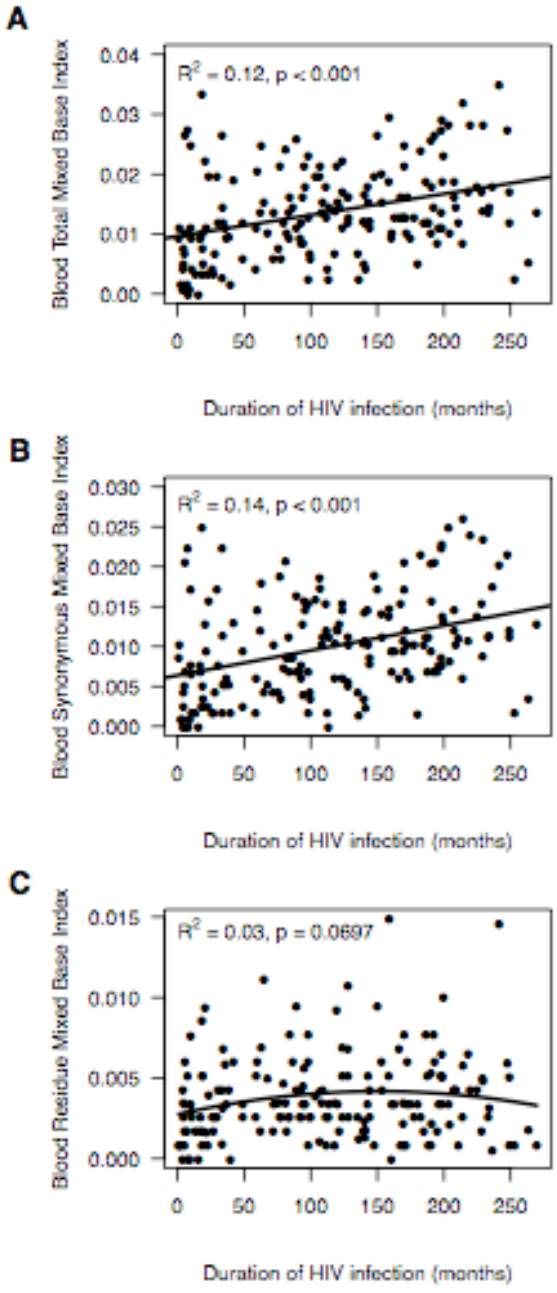

Figure 2.4: HIV-1 population diversity in blood with relation to time

Duration of infection had linear relationships with viral population diversity in blood as measured by the (A) Total Mixed Base Index ($p < 0.001$) and (B) Synonymous Mixed base Index ($p < 0.001$), but the trend was quadratic for the (C) Residue Mixed base Index ($p = 0.07$).

Comparing Cost and Time.

Using population-based HIV-1 *pol* sequences to investigate HIV viral population diversity in both blood and CSF for the CHARTER cohort instead of SGS or UDS saved approximately $298,078 ($390,830 for SGS and $298,078 for UDS) and 2,431 hours of labor (14,586 hours for SGS and 2,431 hours for UDS). The costs saving are based on the estimated cost per sample of $278 for population-based sequencing, $1,323 for SGS, and $1,075 for UDS. The labor savings are based on the estimated labor time per sample of 3 hours for population-based sequencing, 42 hours for SGS, and 9.5 hours for UDS.

Conclusions

This study investigated the use of a novel approach for measuring intra-host viral population diversity and the relationship between viral genetic diversity and HIV-1 disease state. The approach evaluated HIV-1 population diversity by providing a normalized count of mixed bases from population-based sequences. Three different measures of nucleotide variability were evaluated: Total Mixed Base Index (TM-Index), Synonymous Mixed Base Index (SM-Index) and Amino Acid Residue Mixed Base Index (RM-Index). Since the mixed base methods required only one population-based sequence to evaluate population diversity, they were well suited to analyze HIV-1 RNA populations in blood and CSF from a relatively large study cohort (n=187).

The time and cost associated with performing standard methods of evaluating HIV-1 genotypic population diversity likely explain why previous studies have used relatively small sample sizes [11-18]. To address this methodological limitation, we investigated the use of population-based sequences and a normalized count of mixed bases to evaluate HIV-1 population diversity. We found the mixed base method was comparable to Shannon Entropy and APD, two widely used methods for measuring HIV-1 population diversity. This approach builds on previous studies that have used the occurrence of mixed bases in population-based sequences to screen for HIV-1 dual infection [20-21] and as a site-specific marker of variation [19, 22].

Using the normalized mixed base approach, we found that increased HIV-1 population diversity was associated with: AIDS (TM-Index measured in blood and RM-Index in blood) and neuropsychological impairment (TM-Index measured in blood and RM-Index in blood). These associations between HIV-1 population diversity and disease state, suggest that HIV-1 virulence is most likely associated with the capacity of a viral population to maintain genetic diversity and not simply a specific and predominant genotypic variant, although the exact underlying mechanism for this remains unclear. For complex phenotypes such as neuropsychological impairment, HIV-1 variants may work in a coordinated manner to cause disease. As a hypothesis, neuropathogenesis may require multiple distinct viral variants [8-10]. For example, one variant with high viral replication in the CNS may trigger inflammation and prime other cells for infection, while a second variant replicates in a specific tissue that disrupts a process essential for optimal neuropsychological function. This hypothesis builds on studies of poliovirus that link viral diversity and pathogenesis [8-10], but these and other hypotheses need to be tested to better characterize the mechanisms of HIV-1 virulence in relation to viral population diversity.

In this study, we observed a steady increase in genetic variability (i.e. mixed bases) at synonymous sites over time, as measured by the SM-Index, in both CSF and blood plasma. In contrast, diversity at nonsynonymous sites (i.e. amino acid residues), as measured by the RM-Index, in blood plasma steadily increased until about year 12 of duration of infection and then steadily declined. The observed rise and decline in diversity at nonsynonymous sites in blood likely

results from the diminishing capacity of the immune system. During the earlier 'asymptomatic' phases of infection, strong CD8+ T-lymphocytes selection may drive diversity by selecting for relatively rare variants that facilitate immune escape [31-34]. As the immune system weakens during the late stages of infection, the fitness advantage of these rare variants may decline and concomitantly their frequency. Similarly, a number of studies have observed that the frequency of HLA allele specific CTL escape mutations tend to decline when transmitted to an HLA allele discordant individual [34-36]. While this pattern was seen in the blood, diversity at nonsynonymous sites in the CSF continued to steadily increase over time, suggesting that immune and potentially other selection pressures in the CNS differ from those in the blood.

To further investigate intra-individual differences between HIV-1 variants circulating in the blood and CSF, we compared measurements of HIV-1 population diversity in the blood and CSF. For most participants, HIV-1 population diversity was higher in CSF than blood plasma, suggesting that HIV-1 in the CNS likely originated from multiple variants and not a consequence of monoclonal amplification. Additionally, pleocytosis, a marker of lymphocyte trafficking from the blood to the CSF, was not associated with changes in viral diversity in the CSF. This lack of difference may be related to the unusually high proportion of cases demonstrating pleocytosis in this cohort (118/174; 68%), and is consistent with the relatively high CSF viral loads observed, which was a condition of eligibility in this study. Since it is not feasible to amplify virus at low levels, these patients were excluded from the study.

In this study, neuropsychological impairment was *negatively* correlated with reported duration of infection, which was unexpected given that loss of CD4+ T lymphocytes is associated with greater duration of infection [37-38], and lower CD4 counts have been associated with neuropsychological impairment [25, 39-40]. This may point to a limitation of the study, and is most likely a bias in the study design and cohort, which concerns factors like cross-sectional design, survival bias and differences in antiretroviral therapy use, effectiveness and duration. An obvious example of survival bias for all HIV cohorts is that HIV-infected people who live longer and with less disease would be more likely to participate in study cohorts. This factor is further obscured by differential use of antiretroviral therapy and its effectiveness. A prospective longitudinal study would most likely provide additional and more conclusive insight, especially in relation to duration of infection, which was only by self-report in the CHARTER cohort.

The present study was limited to the HIV-1 *pol* coding region. Viral diversity in other coding regions, like *env,* are likely to be important in neuropathogenesis [42-45], and this should be evaluated in future studies. However, investigating *pol* did allow us to evaluate resistance-associated mutations in the context of HIV-1 diversity and disease, but the low prevalence of these mutations in our study population, specifically M184V and K103N, prevented meaningful conclusions. An additional limitation of this study is that we did not screen for dual infections among CHARTER cohort participants included in the analysis of neuropsychological impairment. It is likely that some participants in the CHARTER cohort were dually infected, which could influence

both intra-host viral diversity measures and disease progression [20-21]. Although the mixed base approach is less resource intensive than other methods used to evaluate diversity, it does not allow for the identification or quantification of specific variants and this may be necessary for characterizing dual infection or specific neurovirulent phenotypes. Future studies will be needed to evaluate these issues.

Measuring mixed bases in population-based sequences can be used as less expensive and time-consuming method to evaluate HIV-1 population diversity, and is a method especially suited for evaluating intra-host diversity for large cohorts. Using this approach to investigate diversity in a relatively large well-defined cohort, we demonstrate that HIV-1 population diversity is positively associated with disease, specifically AIDS and neuropsychological impairment.

Acknowledgements

Chapter 2, in full, is a reprint of the material as it appears in Virology. Hightower GK, Wong JK, Letendre SL, Umlauf AA, Ellis RJ, Ignacio CC, Heaton RK, Collier AC, Marra CM, Clifford DB, Gelman BB, McArthur JC, Morgello S, Simpson DM, McCutchan JA, Grant I, Little SJ, Richman DD, Smith DM and the CHARTER Study Group. Higher HIV-1 genetic diversity is associated with AIDS and neuropsychological impairment. The dissertation author was the primary investigator and author of this material.

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CHAPTER 3

Antiretroviral Resistance Mutations and Neuropsychological Performance

ABSTRACT

When antiretroviral therapy does not fully suppress HIV replication, it can select for variants that are resistant to antiretroviral therapy. These variants can exhibit reduced replication capacity and result in lower viral loads in blood. Our study evaluated antiretroviral resistance mutations in blood and CSF and neuropsychological (NP) performance. Neuropsychological performance was assessed for all participants and drug susceptibility of HIV RNA populations in blood and cerebrospinal fluid were determined from *pol* sequences. We found *a*ntiretroviral resistance was associated with lower CSF viral loads ($p<0.01$) and better NP performance ($p=0.04$). These findings suggest that HIV variants with decreased antiretroviral susceptibility may be less neurovirulent than wild-type HIV.

Introduction

HIV associated neurocognitive disorders (HAND) range in severity from disabling dementia to asymptomatic cognitive, motor and behavioral changes. With the widespread use of antiretroviral therapy (ART) in economically privileged countries, the incidence of HIV-associated dementia, characterized by severe neuropsychological (NP) impairment and inability to perform activities of daily living, has significantly decreased [1-2]. Despite a decrease in incident dementia, less severe forms of HAND have persisted [3-6] and may actually be increasing as HIV-infected individuals live longer [7-8]. In addition, comorbidities that are common in individuals infected with HIV, like hepatitis C virus (HCV) infection and methamphetamine (Meth) abuse, are also associated with NP impairment and may make it difficult to distinguish the contribution of each to NP impairment [9-13].

Effective ART, as assessed by suppression of blood plasma viral load, is considered the standard of care for HAND; however, poor penetration into the central nervous system (CNS) by some antiretroviral medications suggest that suppression of blood plasma viral load may not be an adequate guide when selecting treatment options for HAND and raises the concern that suboptimal antiretroviral concentrations could select for resistance-associated mutations [14-15]. In addition, these mutations can affect HIV replication and fitness in the presence or absence of ART. Studies have examined the relationship between resistance-associated mutations, in vivo viral load and HIV disease [16-22].

These studies, however, were limited to blood viral loads and focused on indicators of HIV disease in the blood, like CD4+ cell counts. Considerably less is known about the impact of antiretroviral resistance on cerebrospinal fluid (CSF) viral load and the brain. To this end, we investigated the relationships between resistance-associated mutations, viral loads in blood and CSF, and NP performance.

Methods

Study Participants and Clinical Evaluations.

We investigated ninety-four participants enrolled in a research study at the University of California San Diego's HIV Neurobehavioral Research Center. The primary objective of this National Institute on Drug Abuse funded research study was to determine the impact of Meth use on HIV disease of the immune and nervous systems. All subjects provided informed consent according to a protocol approved by the UCSD Human Research Protections Program, including consent to future uses of their specimens and data. Each participant underwent a comprehensive neuromedical evaluation that used structured clinical assessments of medical history, ART and other medication use and neurological and general physical signs of disease [11]. Volunteers were selected for inclusion in this analysis if they had at least 500 HIV RNA copies/ml of blood plasma. Since Meth users are more likely to be co-infected with HCV and since the treatment for HCV affects the immune and nervous systems, volunteers were excluded if they reported past or current treatment with interferon-alpha.

Neuropsychological Testing.

Participants underwent standardized NP assessments of seven ability domains (learning, delayed recall, verbal fluency, processing speed, attention/working memory, abstraction/executive functioning, motor speed), as previously described [9, 23]. All NP tests were administered and scored by

trained psychometrists using demographically corrected normative data. Results were summarized by a neuropsychologist using global ratings that range from 1 (above average) to 9 (severely impaired), based on the demographically adjusted test scores in the seven ability domains. A global score of 5 or higher denotes NP impairment that is present in at least two ability areas [23].

Meth dependence was determined with the Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders version IV [24]. Inclusion in the parent study from which the Meth users were drawn, required lifetime dependence and evidence of use within the previous eighteen months, as well as a minimum of ten days of abstinence prior to NP testing.

Laboratory Measures.

Blood was collected by venipuncture and CSF was collected by lumbar puncture. HIV and HCV infections were diagnosed by serology and HIV RNA levels (viral loads) in blood plasma and CSF were measured (Amplicor HIV-1 Monitor; Roche Diagnostics, Branchburg, NJ). The ultrasensitive assay was used for CSF (lower limit level of detection of 50 copies/ml) and the standard assay was used for blood (lower limit level of detection of 400 copies/ml). A fluorescence-activated cell sorter quantified CD4 lymphocytes. At the time of their NP testing and clinical sample collection, the urine of each participant was screened with a point-of-care test for common recreational drugs, including amphetamines, cocaine, barbiturates, tetrahydrocannabinol, opiates, benzodiazepines, and phencyclidines (Rapid Response; Biotechnostix, Inc.,

Markham, Ontario, Canada). In addition, participants received a *Breathalyzer* test to evaluate alcohol intoxication (Alcohol Countermeasure Systems, Toronto, Ontario, Canada). NP testing was rescheduled if alcohol was detected or if the participant's urine was positive for non-prescribed substances, with the exception of cannabis, given its long elimination period. Likewise, participants were not tested if they appeared to be intoxicated or in withdrawal.

Antiretroviral Resistance Genotyping.

The ViroSeq HIV genotyping system (Applied Biosystems, Alameda, CA) was used for population-based *pol* sequencing of HIV RNA extracted from blood plasma per manufacturer instructions [25]. Genotyping of the reverse trancriptase coding region of CSF-derived HIV RNA included cDNA synthesis with RETROscript kit (Applied Biosystems, Alameda, CA) using random decamers according to manufacture's protocol. Followed by two rounds of amplification with Taq polymerase (Invitrogen, Carlsbad, California) as previously described elsewhere [26], using primers CI-Pol 1 and 3RT at cycling parameters:
95 °C × 2 min; 95 °C × 30 s, 50 °C × I min, 72 °C × 1 min for 35 cycles;
72 °C × 10 min and primers M13F-5RT and M13R-3RT at cycling parameters:
95 °C × 2 min; 95 °C × 30 s, 50 °C × I min, 72 °C × 1 min for 35 cycles;
72 °C × 10 min. All assays included negative controls with PCR products visualized by agarose gel electrophoresis, and were conducted in conditions to minimize PCR contamination. Sequencing was performed on an ABI 3100 Genetic and sequences were manually reviewed using BioEdit and ViroSeq

genotyping software (version 2.4.2; Applied Biosystems, Alameda, CA). The Stanford HIV Resistance Database [http://hivdb.stanford.edu, March 2009] was used to interpret drug resistance from genotypic data.

Statistical Analysis.

HIV RNA levels in blood and CSF were log transformed to stabilize variances. Demographic and medical characteristics of study participants were summarized, both overall and stratified by presence of resistance-associated mutations. The demographic and medical characteristics of study participants for whom CSF HIV RNA were available were compared to the other study participants to assess the informativeness of the missing data. Fisher's exact tests were used to compare categorical or binary measures and Wilcoxon rank sum tests were used to compare continuous measures. For those participants who had successful lumbar puncture, univariate and multivariate predictors of CSF HIV RNA were examined using Tobit analyses, which is a type of regression that adjusts for censored data and is useful for these evaluations since HIV RNA are subject to limit of detection censoring [27].

Multiple regression analyses were used to investigate the relationship between presence of antiretroviral resistance or specific resistance-associated mutations, HIV RNA in CSF, and NP performance. Specifically, regressions were performed using either HIV RNA in CSF or the Global Clinical Rating as the continuous outcome and antiretroviral resistance as the main predictor of interest. Other variables used in this model included past and current ART use,

methamphetamine dependence, estimated duration of infection and HIV RNA levels. Statistical analyses were performed using JMP (version 5.0 for Mac, SAS Institute, Cary, NC, USA) and R version 2.3.1 (R Development Core Team 2006).

Results

Study Participants and Antiretroviral Resistance.

Participants were mostly Caucasian men in their mid 30s (median 35 years). The median HIV RNA levels were 4.7 (blood) and 2.9 (CSF) $\log_{10}$ copies/ml. The median blood CD4+ cell count was 319/μl, and 25% of participants had a positive serology for HCV. As expected, Meth use was common in the cohort with 57% of participants reporting a history of abuse or dependence (Table 1). Lumbar punctures were successfully performed on 73% of participants (69 of 94). Clinical and demographic characteristics of participants with successful lumbar punctures resembled participants without lumbar punctures (data not shown), except that participants who did not undergo lumbar puncture had longer estimated durations of HIV infection (mean 8.5 years vs. 5.5 years, $p = 0.02$).

At the time of study evaluation, 63% had a past history of ART use and 29% were receiving ART at the time of participation and sampling. One or more resistance-associated mutations were detected in the blood plasma of 48 of the 94 study participants (51%). The most common mutations, M184V and K103N, were detected in (22%) and (16%) of participants respectively (Supplementary Table 1). Univariate analyses demonstrated that individuals with antiretroviral resistance (DR+) differed from those with no antiretroviral resistance (DR-) in duration of HIV infection, levels of CSF and blood plasma HIV RNA, past ART use, and current ART use (Table 1). Participants with resistant virus did not differ

in current CD4+ cell count, CD4+ cell nadir, HCV serostatus, or diagnosis of AIDS (Table 1).

We were also able to complete resistance profiles for the reverse transcriptase coding region derived from HIV RNA derived from CSF for twenty-six participants. Median CSF viral loads for these participants were higher than for those participants for whom we were unable to complete resistance profiles (3.16 $\log_{10}$ copies/ml vs. 2.6 $\log_{10}$ copies/ml, p=.013); but did not differ in regards to demographic characteristics, current CD4+ cell count, CD4+ cell nadir, HCV serostatus, or diagnosis of AIDS (data not shown). Resistance-associated mutations in the reverse transcriptase coding region from HIV RNA populations in the CSF were identified in eleven of these twenty-six participants. Genotypic discordance between blood and CSF, defined as the presence of one or more resistance-associated mutation in blood but no evidence of resistance in the CSF or vice-versa, was found in five of these eleven participants (45%). In all but one case, participants with M184V and/or K103N present in blood also had the mutation in the CSF. The five participants with genotypic discordance between blood and CSF were not significantly differ from the seven participants with genotypic concordance with respect to demographic characteristics, blood viral load, CSF viral load, current CD4+ cell count, CD4+ cell nadir, HCV serostatus, or diagnosis of AIDS.

Table 3.1: Participant demographics and clinical characteristics

P-values are based on univariate analyses. Individuals with drug resistance (DR+) in blood differed from those with no drug resistance (DR-) in duration of HIV infection, CSF HIV RNA, plasma HIV RNA, past antiretroviral (ARV) use, and current ARV use.

	Overall	**DR+ (range)**	**DR- (range)**	**p-value**
Sample Size	**94**	**48**	**46**	
Age (years)	35	34 (23-39)	35 (21-51)	> 0.10
Sex (male)	92%	96%	88%	> 0.10
Ethnicity (non-Caucasian)	37%	38%	37%	> 0.10
Education (years)	12	12 (9-20)	12 (6-18)	> 0.10
Duration of HIV (years)	**5.3**	**8.1 (.052-16.4)**	**4.1 (0-16.9)**	**< 0.01**
HIV RNA, CSF (log10 c/mL)*	**2.9**	**2.6 (1.7-4.4)**	**3.3 (1.7-6.2)**	**< 0.01**
HIV RNA, Blood Plasma (log10 c/mL)	**4.7**	**4.4 (2.8-6)**	**4.8 (3.3-6.3)**	**< 0.05**
CD4 Count, Current (/μL)	319	340 (4-1188)	308 (3-1296)	> 0.10
CD4 Count, Nadir (/μL)	216	200 (0-772)	269 (0-1296)	> 0.10
AIDS Diagnosis	47%	50%	43%	> 0.10
Past ARV Use	**63%**	**73%**	**51%**	**< 0.05**
Current ARV Use	**29%**	**50%**	**7%**	**< 0.001**
–Adherence (<95% in 4 weeks)	53%	50%	75%	> 0.10
HCV Seropositive	25%	25%	24%	> 0.10
Methamphetamine Abuse Ever	57%	51%	63%	> 0.10
*Values are medians or proportions; n = 94; *subgroup analysis*				

Antiretroviral Resistance and CSF Viral Loads.

In univariate analysis, lower CSF viral loads were associated with the presence of antiretroviral resistance in blood-derived virus, lower HIV blood viral load, and current ARV use (Table 2). Multivariate analyses, which included adjustments for current and past ART use and blood viral load, demonstrated that lower CSF HIV RNA levels were associated with the presence of antiretroviral resistance in blood (Model: $p< 0.01$). This relationship was particularly strong for participants in whom we detected the M184V mutation (Model: $p< 0.01$). Among the 26 participants with completed CSF resistance profiles of reverse transcriptase, univariate analysis did not indicate differences in CSF viral loads between individuals with and without evidence of resistance in CSF.

Table 3.2: Univariate correlates of CSF HIV RNA levels

Lower CSF HIV RNA levels were independently associated with both lower blood plasma HIV RNA levels and the presence of drug resistance in the blood, particularly the resistance associated M184V. Statistically significant values are bolded.

	p-value
Drug Resistance in Blood	**0.0025**
--M184V	**<0.001**
--K103N	>0.10
Duration of HIV (years)	>0.10
HIV RNA, Plasma (log10 c/mL)	**<0.001**
CD4 Count, Current (/μL)	0.08
CD4 Count, Nadir (/μL)	>0.10
AIDS	>0.10
Past ARV Use	>0.10
Current ARV Use	**<0.02**
--Adherence (<95% in 4 weeks)	>0.10
HCV Seropositive	>0.10
Methamphetamine Abuse Ever	>0.10
--Days of Abstinence	>0.10
--Density of Use	>0.10

Antiretroviral Resistance, Methamphetamine Dependence and Neuropsychological Performance.

Since ART can alter the relationships between HIV RNA levels and other biological measures, participants who were taking ART at the time of sampling were excluded from bivariate comparisons of HIV RNA levels and global rating. In these analyses, the correlation between global rating and CSF viral load was stronger in individuals without drug resistance (DR-) than in individuals with drug resistance (DR+), although this difference did not reach statistical significance (Figure 1). The correlation between global rating and blood viral load was also stronger in individuals without drug resistance (DR-) than in individuals with drug resistance (Figure 2). Multivariate analysis demonstrated that antiretroviral resistance in blood-derived virus (b =-0.88 ; p=0.024) was associated with better NP performance (Model: Adjusted R^2=0.12, p=0.017). The opposite was observed for methamphetamine dependence (b = 0.76; p=0.031) and duration of HIV infection (b =0.073 ; p=0.057), where each were independently associated with worse NP performance. Similar results were observed when multivariate analysis was performed on the subset of participants who had CSF samples. In this multivariate analysis, antiretroviral use, blood viral load and CSF viral load did not explain additional variance in NP performance. There was no statistically significant independent or collinear relationship between CSF HIV RNA levels and NP performance, but each was independently associated with antiretroviral resistance.

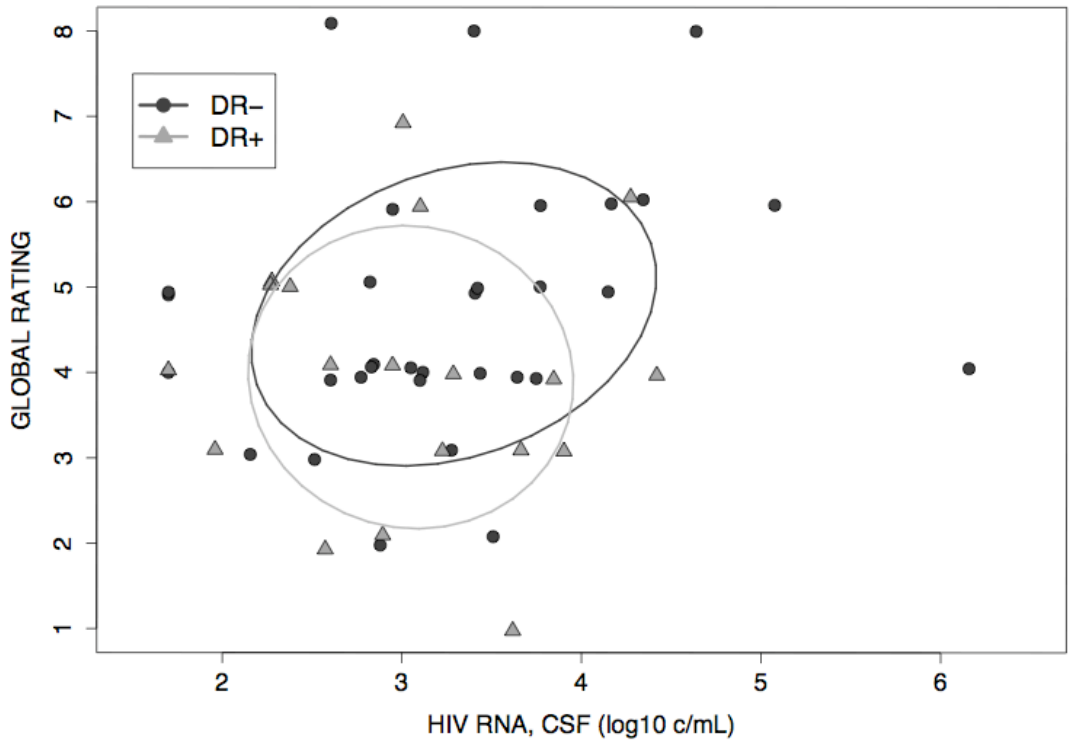

Figure 3.1: CSF HIV RNA Levels and Neuropsychological Performance

Confidence Ellipses (Global Rating vs. CSF Viral Load) for individuals without drug resistance (DR-) (rho = 0.27, p = 0.14) and individuals with drug resistance (DR+) (rho = -0.07, p = 0.78) demonstrate that the correlation between global rating and CSF viral load is stronger in individuals without drug resistance (DR-) than in individuals with drug resistance (DR+), although this did not reach statistical significance. This analysis excluded participants on ART at the time of the study.

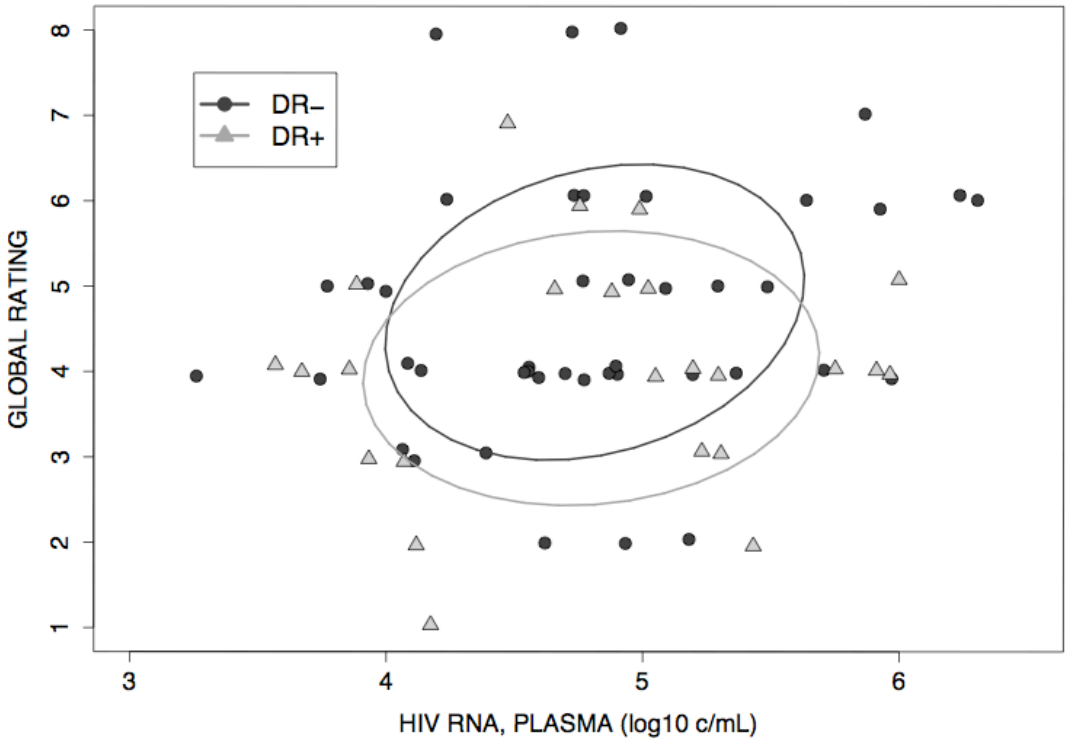

Figure 3.2: Blood HIV RNA Levels and Neuropsychological Performance

Confidence Ellipses (Global Rating vs. Plasma Viral Load) for individuals without drug resistance (DR-) (rho = 0.25, p = 0.10) and individuals with drug resistance (DR+) (rho = -0.16, p = 0.29) demonstrate that although not statistically significant, the correlation between global rating and plasma viral load is stronger in individuals without drug resistance (DR-) than in individuals with drug resistance (DR+). This analysis excluded participants on ART at the time of the study.

Conclusions

Prior to the era of highly active antiretroviral therapy, CSF viral loads were clearly associated with HAND [28-30]; but more recent studies have not replicated this finding [31-32]. Our study indicates that drug resistance may in part explain this discrepancy. Although HIV RNA levels in CSF did not correlate with NP performance overall, the presence of resistance-associated mutations was associated with lower CSF viral loads and better NP performance. This finding suggests that HIV variants harboring resistance-associated mutations are less neurovirulent than wild-type HIV, which raises the question of the mechanism by which this might occur.

In vitro studies demonstrate that single mutations within *pol* can result in reduced replication capacity when compared to wild-type [33-37]. Single amino acid substitutions in clade B virus that result in the greatest reduction in replication capacity include the Nucleoside Reverse Transcriptase Inhibitors (NRTI) associated mutations (K65R, T215Y and M184V) and the Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI) associated mutations (V106A, Y188H and G190S) [36, 38-39]. Our study extends these observations by demonstrating that resistance-associated mutations known to reduce replication capacity in vitro, particularly M184V, are associated with lower CSF viral loads, independent of past and current ART use. In contrast, the NNRTI associated mutation K103N, was not associated with lower viral loads, as might be predicted from its nominal in vitro reduction in replication capacity [40-41].

Although this investigation suggests that resistance-associated mutations may benefit the nervous system, this benefit is unlikely to exceed that from virologic suppression. Suboptimal ART may select for HIV that is less replication competent and less neuropathogenic, but ongoing HIV replication regardless of phenotype leads to brain injury in a substantial proportion of untreated individuals. To date, a standard in vitro method to characterize HIV phenotypes by neurovirulence does not exist. One approach that may yield additional insight is to use an in vitro assay that utilizes microglia or brain macrophages, likely the primary cell types infected by HIV in the brain [42], to assess the replication capacity of specific resistance associated mutations [43].

Although this study is the largest and most comprehensive to investigate the relationship between antiretroviral resistance, viral loads, and NP performance, our study has several limitations. We relied on retrospective analysis of an existing cohort and only samples from participants with detectable CSF viral loads at the time of sampling were eligible for inclusion in the analysis. While detailed analysis demonstrated that this subset did not significantly differ from the other participants in the study, there is the possibility of selection bias. In addition, analyses of antiretroviral resistance were primarily based on HIV RNA extracted from blood. We did investigate CSF resistance profiles for twenty-six participants, but genotypic assessment was limited because viral loads were low in the CSF, which limited sequencing of the reverse transcriptase coding region. In all but one case, participants with M184V and/or K103N present in blood also had the mutation in the CSF. This degree of concordance between CSF and

blood for resistance-associated mutations K103N and M184V is likely in part explained by regimen stability, as the median time on current ART regimen for these participants was 10 months. Therefore, although our analysis was nested in a well-characterized cohort and is one of largest studies of its kind, our observations should be validated in larger prospective studies with more frequent sampling.

In conclusion, our study indicates that HAND is influenced by factors including duration of HIV infection, antiretroviral resistance, and methamphetamine use. In particular, antiretroviral resistance, appears to be associated with lower CSF viral loads and with better NP performance. This may be mediated by impaired viral fitness and is likely less beneficial than complete viral suppression by ART. The findings also suggest that drug resistance may be one explanation for the weakening of the relationship between CSF viral loads and NP performance in the modern treatment era. Further study of drug resistance and HIV neuropathogenesis may contribute to an improved understanding of HIV disease in the CNS and perhaps improvements in treatment options for HAND.

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Chapter 3, in full, has been submitted for publication. Hightower GK, Letendre SL, Cherner M, Gibson SA, Ellis RJ, Wolfson TJ, Gamst AC, Ignacio CC, Heaton RK, Grant I, Richman DD, Smith DM and HNRC Group. Select resistance-associated mutations are associated with lower CSF viral loads and improved neuropsychological performance. The dissertation author was the primary investigator and author of this material.

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