Resistance and immune activation profiles in HIV-1 subtype C-infected subjects failing antiretroviral therapy

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Declaration

I, Glen Pierre Malherbe, declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Pretoria. It has not been submitted before for any degree or examination in any other Technikon or University. Confidentiality of patient's identity has been followed according to the ethical rules as prescribed by the Faculty of Health Science Research Ethics Committee, University of Pretoria.

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Summary

HIV-1 subtype C is the most prevalent subtype globally. Most HIV research has historically focussed on subtype B, creating a paucity of information regarding HIV-1 subtype C, which is the focus of this work. HIV-1 treatment failure and associated drug resistance were central themes throughout three broad areas of investigation.

Firstly, the prevalence and type of drug resistance mutations were compared between South African subjects failing first-line antiretroviral therapy in a rural and an urban setting, in order to determine if treatment setting had any impact on failure time, the development of drug resistance mutations and the effectiveness of second line therapy. Results indicated that subjects in the rural area had been failing therapy for a longer time and subsequently had a greater number and more significant drug resistance mutations at genotype. Nevertheless, despite worse drug resistance profiles, second-line protease inhibitor-based therapy would most likely be as effective in the rural cohort as in the urban treatment programme.

Secondly, the effect of highly-active antiretroviral therapy (HAART) on immune activation and the development of drug resistance mutations was investigated to identify potential biomarkers of drug resistance. Results indicated that CXCL9, CXCL10, β2M, TGF-β1, IFN-γ, IL-6, TNF and sCD14 were significantly elevated in HIV-1-infected subjects compared to controls, as well as during therapy failure. The persistently elevated levels of CXCL9, CXCL10 and β2M in subjects failing therapy in the setting of a marked reduction in these markers in subjects on successful HAART, suggests that they may be useful not only to monitor immune activation during HAART, but also to distinguish between good and poor responders. Ongoing monocyte-macrophage activation was identified even during HAART-induced virological suppression by sCD14 and TGF-β1 maintaining their elevated status. Adjuvant anti-inflammatory therapy may therefore be useful for subjects on HAART to achieve lower levels of immune activation.

Thirdly, the contribution of suboptimal APOBEC3G-editing to genetic diversity and drug resistance was investigated using RNA virus from plasma in this novel study, to determine whether suboptimal APOBEC3G hypermutation could be a factor in the development of drug resistant HIV-1 subtype C in South Africa. There was no clear evidence that hA3G

contributes to significantly higher G→A substitution in resistance sequences compared to therapy-naïve sequences, although it would appear that hA3G may be contributing to elevated genetic diversity at hA3G-preferred substrates in HIV-1 subtype C.

These studies could make a major contribution to what is known about HIV-1 subtype C infection, drug resistance, immune activation and innate immunological defence.

General Introduction

The HIV-1/AIDS Pandemic

Sub-Saharan Africa is the region most severely affected by the Human Immunodeficiency Virus type 1 (HIV-1)/Acquired Immunodeficiency Syndrome (AIDS) pandemic, and a staggering 70% of all new infections occurred in this region in 2012.[1] However, according to the UNAIDS Report on the Global AIDS Epidemic 2013[1], the situation may be improving with decreases in the region's contribution to global infections and AIDS-related deaths. Most prominently, new infections have declined (see Table 1) most likely due to increased treatment access. Other regions profoundly affected by HIV-1/AIDS remain south-east and central Asia, Central and South America and Eastern Europe.

Table 1: The change in the contribution of sub-Saharan African infections to global HIV statistics. 2001 versus 2012.

Statistic	Sub-Saharan contribution to global statistics 2001 versus 2012
People living with HIV	72.3% → 70.8%
New infections	76.5% → 69.6%
AIDS-related deaths	78.9% → 75.0%

The AIDS pandemic is particularly severe in the southern-most part of the African continent where 31% of all new infections and 34% of all AIDS-related deaths occur in only 10 countries. Approximately 40% of adult women with HIV-1 live in southern Africa. South Africa has the largest number of infections (~ 6,100,000 in 2012[2]). In South Africa, most maternal deaths are AIDS-related and HIV-1/AIDS accounts for 35% of all deaths in children less than five years of age. Access to antiretroviral therapy (ART) has increased approximately 17-fold in just 7 years (from 2% to 37% coverage in 2009) and, if current programs are sustained and expanded, antiretroviral treatment (ART) should continue to have a strong positive impact on the local epidemic (UNAIDS Report on the Global AIDS Epidemic 2013[1])

The "Main" or M group of HIV-1 viruses has evolved into nine major subtypes (A-D, F-H, J and K) and at least 16 different circulating inter-subtype recombinant forms, as well as numerous unique recombinant forms[3,4]. Subtypes A and C account for the majority of global infections, with C being the predominant subtype in southern Africa (including South Africa), Ethiopia, India and Nepal. Since these geographic regions are also heavily populated, it is not surprising that approximately 50% of all global infections are of the C subtype[5-7]. Although largely unconfirmed and the subject of considerable debate, subtype C has been linked to faster disease progression and higher rates of heterosexual and mother-to-child transmission relative to other HIV-1 subtypes[8,9], although subtype D infections have been linked to a faster decline in $CD4^{+}$T-lymphocytes when compared to other subtypes[10]. Despite the importance of subtype C at the global level, C viruses remain a relatively understudied viral subtype compared to the HIV-1 subtype B viruses that predominate in North America, Europe and Australia[11]. This study addresses several unresolved questions relating to the therapeutic response and development of drug resistance in African subjects infected with HIV-1 subtype C.

Origins and Unique Properties of HIV-1

HIV-1 continues to have a devastating impact on global population health. The virus entered the human population approximately 50-100 years ago through a process involving multiple cross-species transmissions or zoonoses. It is generally believed, based on phylogenetic analysis, that at least three independent zoonotic transmissions of chimpanzee Simian Immunodeficiency Virus (SIV) gave rise to the three groups or lineages of HIV-1: Main (M), Outlier (O) and Non-main, non-outlier (N)[12]. A fourth genetically distinct lineage, designated HIV-1 group P, was recently identified in a Cameroonian woman living in France. Since then, only a few group P viruses have been detected in the human population. Phylogenetic evidence suggests that HIV-1 P viruses originated from gorillas rather than chimpanzees[13].

A characteristic feature of all retroviruses is their ability to integrate in host cell deoxyribonucleic acid (DNA)[14]. As a result, retroviruses cannot be easily eradicated from infected cells. HIV-1, however, is also a particularly complex retrovirus. In addition to the three structural genes (*gag*, *pol*, and *env*) found in all viruses, HIV-1 contains six regulatory genes that allow for increased infectivity by modulating interactions between HIV-1 and its

host[15]. The genome, which consists of two copies of single-stranded ribonucleic acid (ssRNA), contains the following genes, 5' → 3', some of which are encoded by shared nucleotides – *gag* (group-specific antigen), *pol* (polymerase) (which encodes the protease, reverse transcriptase and integrase proteins), *vif* (virion infectivity factor), *vpr* (viral protein R), *rev* (regulator of virion), *tat* (Transcriptional Transactivator), *vpu* (viral protein U), *env* (Envelope) and *nef* (Negative Factor). Although HIV-1 has a short genome (the subtype B hxb2 reference strain is 9719nt in length), the virus' ability to use all three reading frames and synthesize proteins from overlapping reading frames, is remarkable. The six regulatory and accessory proteins play an important role in allowing HIV-1 to evade the host immune system: Tat upregulates viral transcription from the 5'-Long Terminal Repeat (LTR)[16] transports viral proteins from the nucleus to the cytoplasm[7], Vpu promotes degradation of $CD4^+$ T-lymphocytes (Cluster of Differentiation 4)[18], Vif overcomes the inhibitory effects of the host innate immunological protein APOBEC (Apolipoprotein B, mRNA editing, catalytic polypeptide)[19], Vpr promotes the infection of host macrophages[20] and Nef promotes the down regulation of surface $CD4^+$T-lymphocytes and class I MHC (Major Histocompatability Complex) expression, blocks apoptosis and enhances virion infectivity[21].

One the most salient characteristics of HIV-1 is its ability to infect and destroy $CD4^+$ helper T lymphocytes, a process that in the absence of effective ART, leads to severe immunodeficiency, progression to AIDS and death[22]. Virus entry into susceptible target cells is mediated through a complex sequence of interactions between the gp120 subunit of Env and cellular receptors, primarily CD4 and either CCR5 (CC chemokine receptor 5) or CXCR4 (CXC chemokine receptor)[23]. Trimers of HIV-1 gp120 recognize and bind to CD4 inducing a conformational change in gp120, exposing the co-receptor binding sites. Depending on the tropism of the virus, which is determined by the V3 (variable region 3) loop of gp120 (glycoprotein 120), the cellular co-receptor CCR5 or CXCR4 is engaged. This, in turn, triggers a subsequent conformational change in gp41 (glycoprotein 41), promotes the formation of a stable six-helix bundle structure and completes membrane fusion by providing the force required for the viral core to enter the cell. Co-receptor usage is not random. CCR5 is used exclusively during acute/early infection and it is the main receptor used by the majority of HIV-1 isolates worldwide[24]. CCR5 is also used to gain entry into CD4-expressing macrophages and dendritic cells (DC), cells that play a critical role in maintaining innate immunity and in the cross talk that occurs between the innate and adaptive immune systems[22,25]. HIV-1 envelopes that use CCR5 typically have a low number of positively

charged V3 amino acids, whereas CXCR4-using envelopes contain an excess of positively charged amino acid residues, most notably at positions 11 and/or 25[26]. Following release of the viral core into the cytoplasm, viral genetic material is delivered to the nucleus for integration into the host genome. This involves: i) uncoating of the viral capsid and release of the viral ribonucleoprotein complex (RNP), ii) reverse transcription into a double-stranded viral DNA intermediate, and iii) formation of a pre-integration complex (PIC) that is transported into the nucleus via the cell's microtubule transport system[27]. During this time, host restriction factors such as APOBEC3G (Apolipoprotein B, mRNA editing, catalytic polypeptide-like 3G) and TRIM5α (tripartite motif 5 alpha) can act to prevent cross-species transmission[28].

Course of HIV-1 Infection

The early understanding of HIV-1 pathogenesis was based on studies of $CD4^+$ T lymphocyte depletion in peripheral blood and of changes in HIV-1 RNA (ribonucleic acid) viral load (VL) in plasma[29]. Based on changes in circulating $CD4^+$ T lymphocyte and VL levels, HIV-1 infection was divided into three phases: i) an acute/early phase characterized by a burst of plasma viremia and a modest, but short-lived decrease in circulating $CD4^+$ T lymphocytes that is curtailed and partially controlled by cell-mediated immunity[30,31]; ii) a chronic or asymptomatic phase that typically lasts for up to 10-12 years and is characterized by a gradual, but profound loss of $CD4^+$ T lymphocytes (an average of 100 cells/mm^3 of blood per year) leading ultimately to iii) a symptomatic phase involving total collapse of the host immune system, the emergence of AIDS-related malignancies, opportunistic infections and death[22]. Based on this early model of HIV-1 pathogenesis, $CD4^+$ T lymphocytes that were directly killed by HIV-1 were replaced by new cells generated in the thymus or through homeostatic proliferation. In this "tap and drain" model[32], progression to AIDS was attributed to a negative imbalance between cell death and regeneration as a result of immune exhaustion and an inability to generate new $CD4^+$ T cells[33]. However, the actual number of infected $CD4^+$ T cells in peripheral blood during chronic infection is very low[34,35] and, over time, it became apparent that direct cytolytic killing could not account for the sustained loss of $CD4^+$ T cells that occurs during disease progression[36]. As a result of this, and other discrepancies, investigators began to focus their attention on immune activation as a cause rather than a consequence of $CD4^+$ T cell depletion[37]. This view was supported by *in vitro* studies showing

that cytokines can modulate viral replication[38,39] and that chronic infection is associated with increased production of pro-inflammatory cytokines [40,41], as well as increased numbers of activated B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes (Cluster of Differentiation 8)[42,43]. As with the virus model, the immune model was based on events occurring in peripheral blood.

HIV-1 as a Disease of Persistent Hyper-Immune Activation

Although there is clearly a broad immunological response to HIV-1 that attempts to control and clear the infection, these responses are insufficient and occur too late to eliminate the virus[44]. The reduction in the plasma viremic peak to a "steady state" level during the first few weeks of infection coincides with a vigorous HIV-1-specific immune response involving cell mediated immunity ($CD8^+$ CTL (cytotoxic T-lymphocyte) and $CD4^+$ T-helper HIV-1-specific responses) in addition to antibody production, all of which are believed to play a role in the initial control of infection[45]. Unfortunately, this intense selection pressure, when combined with high rates of viral replication, leads to the generation of escape mutants and the establishment of persistent chronic infection, the level of which correlates with subsequent disease progression[22]. A major breakthrough in the understanding of this process came from studies of mucosal membranes[46]. These studies revealed that the early targets of SIV and HIV-1 are activated $CD4^+$ T memory cells that carry the CCR5 HIV-1 co-receptor, the majority of which (>80% of the body's entire $CD4^+$ T cell population) are found in the gastrointestinal tract (GIT)[47-49]. Irrespective of the route of transmission, productive HIV-1/SIV infection is detectable in the GIT, followed at day 5-10 by an intense burst of viral replication in the *lamina propria* and a massive (70-90%) depletion of the "recently" or "partially-activated" $CCR5^+$ memory $CD4^+$ T cells[50]. Although this process also occurs during non-pathogenic SIV, it appears that the key determinant of disease progression in HIV-1 and pathogenic SIV infection, is the persistence of chronic immune activation[22]. This process is driven, in large measure, by virus-induced damage to the epithelial membrane and increased translocation of microbial products from the lumen of the intestine into the circulation[51]. Because of its constant exposure to bacterial, viral and parasitic pathogens, the GIT plays a role, not only in fuelling systemic immune activation, but also in the recruitment, activation and infection of new $CCR5^+CD4^+$ target cells. Thus, during the chronic phase of infection, $CD4^+$ T cells continue to decline not only due to direct cytolytic killing of infected cells but also due to the activation induced death of uninfected bystander cells[52,53]. In

addition, activated $CD4^+$ and $CD8^+$ T cells, and LPS-activated macrophages, produce a variety of different pro-inflammatory cytokines and chemokines that enhance viral replication and further exacerbate the situation. ART is only partially effective at suppressing microbe-driven immune activation and the extent of persistent immune activation during ART is strongly predictive of therapeutic failure and progression to AIDS[54-56].

Treatment and the Evolution of Drug Resistant HIV-1

Highly active antiretroviral therapy (HAART) has had an enormous impact on HIV-1-related morbidity and mortality and, although difficult to measure, on the transmission of infection [57-63]. In the United States, it is estimated that more than 3 million years of life have been saved since 1989, as a direct result of progress in AIDS care and treatment, including prophylactic treatment for the prevention of opportunistic infections (OIs) and treatment with ART[64]. In addition, it is estimated that efforts towards the prevention of mother-to-child transmission (PMTCT) in the United States have averted at least 2,900 childhood infections, the equivalent to an additional 137,000 years of survival benefit. Similar advances are now being observed in the developing world[65]. The Doha declaration in 2001, which allowed developing countries to circumvent patent rights, combined with funding from PEPFAR (President's Emergency Plan for AIDS Relief) and the Global Fund for AIDS, have resulted in a dramatic increase in the use of ART in resource-poor developing countries, with the greatest increase in subjects on treatment being in sub-Saharan Africa[66].

Although early results have been promising with 78% (10,351/13,288), 76% (7413/9794) and 67% (3840/5690) of subjects showing virological suppression after 6, 12 and 24 months, respectively, there are still major challenges associated with further scale-up of treatment programs in Africa and the developing world[67]. These challenges relate to the high cost of drugs in the developing world, sustained access to ART, the lack of diagnostic and laboratory monitoring and the shortage of health care workers. Another concern relates to the risk of developing drug resistance and the limited availability of alternative second-line drugs. HIV-1 belongs to the *Lentivirus* genus, a rapidly evolving family of viruses with short generation times[68]. Due to the high replication rate of HIV-1, and the error prone nature of its reverse transcriptase enzyme (which has no proof-reading ability)[69], it is estimated that a 95% adherence to ART regimens is required to prevent the emergence of drug resistance strains of HIV-1[70]. Strict adherence is therefore a major requirement for achieving virological

suppression, immune restoration, and changing HIV-1 from an acute illness to a chronic treatable infection. Most existing studies on drug resistance and virological failure in African populations are cross-sectional of a single population and are limited. The World Health Organization[71] has reported that the prevalence of acquired drug resistance among adult Africans failing a first-line non-nucleoside reverse transcriptase inhibitor (NNRTI) -based HAART regimen after a median of 12 months is approximately 62%.

The scope of this thesis

South Africa, with its estimated 6.1 million infections, laboratory infrastructure, standardized NRTI/NNRTI (nucleos(t)ide reverse transcriptase inhibitor/non-nucleoside reverse transcriptase inhibitor)-based national treatment program, and rural and urban treatment settings, serves as the world's largest clinical trial for the study of drug resistance in individuals infected with HIV-1 subtype C. Rural and urban settings covered by the same ART programme (but with different treatment challenges) provide an ideal opportunity to quantify and understand the evolution of drug resistance at the population level. This information is needed to assess efficacy and cost effectiveness, and guide future directions of the program. The current study was designed to assess the prevalence and patterns of HIV-1 drug resistance in rural compared to urban populations within South Africa and answer clinically relevant questions relating to the selection of first-line antiretroviral drugs, as well as the role of immune activation and APOBEC3G in promoting the development of HIV-1 drug resistance. An understanding of these factors may provide new insights for the development of adjunct treatment strategies that target immune activation and for the early identification of therapy failure and/or drug resistance.

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Differential HIV-1 subtype C drug resistance profiles in subjects failing first line antiretroviral therapy in urban and rural settings in South Africa

Abstract

The development of drug resistance mutations threatens the significant improvements in morbidity and mortality association with successful HAART. A paucity of studies comparing the outcome of rural versus urban treatment programmes, and the possible lower genetic barrier of HIV-1 subtype C to certain resistance mutations, led to this study in South Africa. Comparisons of clinical and population-based genotyping data, and the resulting Genotypic Susceptibility Scores, in a rural and urban district of South Africa, revealed that subjects failing first-line therapy in the rural area were more likely to; i) be on d4T-containing regimens, ii) be on first-line treatment for a longer period, iii) have TAMs, and iv) have more than two reverse transcriptase drug resistance mutations; compared to the urban area. However, predicted virus susceptibility to second-line protease inhibitor-based HAART were highly similar for the urban and rural areas. This study is novel as it compares an urban and rural treatment programme but still supports the notions that i) increased duration on failing HAART increases drug resistance, and ii) second-line protease inhibitor-based HAART can still be effective after first-line failure – independent of treatment programme intensity or location.

Introduction

Successful highly-active antiretroviral therapy (HAART) significantly decreases morbidity and mortality associated with HIV-1 infection, transforming it from an inevitably fatal illness into a manageable, chronic condition[1-4]. Significant increases in subject lifespan and quality of life are a result of reduced HIV-1 viremia in the setting of partial reconstitution of the immune system. The development of drug resistance mutations (DRM) in the presence of suboptimal drug levels, however, poses a threat to the long-term success of HIV treatment programmes as it limits treatment options, increases cost and results in a reservoir of resistant virus that can be transmitted to other hosts[5].

Similar responses to HAART have been reported across HIV-1 subtypes[6]. Most data on drug resistance, however, concern subtype B, the predominant subtype found in North America, Europe and Australia. In contrast, there is still relatively little information on subtype C, which accounts for the majority of infections worldwide[7] and is predominantly found in sub-Saharan Africa and India. More research is needed, especially since it has been reported that subtype C might have a higher propensity for the development of DRM to common first line drugs [8,9]. For instance, it has been shown that subtype C can be polymorphic at codon 106 that facilitates a after selection with efavirenz (EFV), which confers high-level cross-resistance to the class of non-nucleoside reverse transcriptase inhibitors (NNRTIs)[10]. In addition, deletions within codon 65 of subtype C can cause frameshifts which result in transcription errors in this region with the subsequent rapid development of resistance to tenofovir (TDF)[11].

Even a relatively small increase in DRM in subtype C might be significant given the magnitude of the treatment program in countries such as South Africa, where approximately 2.4 million people were accessing HAART by the end of 2013[12]. It would appear that up to 85.4% of subtype C-infected subjects failing 1st line HAART have evidence of at least one drug resistance-associated mutation[13,14]. Overall, based on data from 12 studies in 8 countries, between 3.7% and 49% of individuals on first-line HAART in sub-Saharan Africa fail virologically with evidence of DRMs[15].

Even though various groups have published results of HIVDR patterns in South Africa, these have predominantly been from urban centres, and mostly from research sites or

academic institutions that are not necessarily representative of the treatment programs in the country. In fact, the UNAIDS Report[16] indicates that 79.5% of surveys in South Africa originate from urban or semi-urban centres, while only 17.5% originate from rural areas. South Africa has a few, large, highly-populated urban centres with most of the country being of a rural nature. Worldwide, rural areas are known to pose different, and possibly problematic, challenges to healthcare, such as: i) fewer treatment sites; ii) fewer experienced physicians; iii) longer distances travelled to access healthcare facilities coupled with inadequate access to transportation; and iv) different drug allocation strategies[17-19]. Challenges specific to HAART programmes include: i) the availability of HAART and the reliability of drug supply; ii) the availability of adequate virological monitoring to detect therapy failure; iii) distance to central laboratories that might impair sample integrity; iv) increased stigma in rural communities; and v) the availability and quality of adherence counseling[15,20]. The latter factor is possibly the most important as it has been repeatedly shown that poor adherence to ART accounts for the majority of treatment failures[3,21].

Very few studies have investigated whether treatment outcomes, and specifically DRM, are different in rural and to urban treatment settings. The aim of this study was therefore to describe and compare selected clinical and DRM profiles of subjects failing first-line HAART at two sites in South Africa – one urban and one rural. The urban site is a district hospital in central Pretoria (Tshwane District Hospital), while the rural site is located in KwaZulu Natal (Africa Centre for Health and Population Studies).

Methods

The terms 'urban' and 'rural' were defined according to Urban Influence codes as described by Larson & Fleishman (2003)[22]. The urban group is from a large metropolitan statistical area of more than 1 million people, while the rural group is located adjacent to a small metropolitan statistical area (<1 million people) where the population size of the largest city is more than 10,000 people.

All subjects had started HAART after 2004 as part of the South African National HIV Treatment Plan and were treated according to the National Department of Health HIV Guidelines operative at that time[23]. HIV-1 viral loads (VLs) were measured routinely at 6-monthly intervals and virological failure was defined as two VL measurements of more than 1000 RNA copies/ml plasma at least 8 weeks apart, despite adherence counseling. Genotypic drug resistance testing (DRT) became available to the urban group in 2008 as part of a research project and to the rural group in December 2010 as part of the implementation of a treatment model in 17 primary healthcare clinics in the area[24]. Subjects were only referred for DRT when interventions to improve adherence had failed to result in virological suppression. Participating clinicians sent 3 EDTA tubes of blood to the Department of Immunology at the University of Pretoria or to the HIVDR laboratory in Durban, together with clinical information consisting of all available CD4+ T-cell counts, VLs, HAART history and a limited social history. Only subjects failing on a first-line NNRTI-based regimen were selected for inclusion in this study.

Subjects in the urban group were managed by medical doctors, while nurses fulfilled a supporting role i.e. providing counseling and carrying out routine observations. Doctors being trained in medical virology visited the clinic on a weekly basis and subjects could easily be referred to the specialized services provided at the academic hospital adjacent to the district hospital complex. After 2008, nurses started repeating prescriptions for subjects who had undetectable VLs and were clinically stable. The clinic also had a full-time pharmacist, a dietician who performed anthropometric measurements and provided dietary advice and a social worker who was responsible for preparatory and adherence counseling, training of lay counselors, defaulter tracing and home visits. The laboratory was located in close proximity to the hospital and samples were collected from the clinic twice daily when necessary. In contrast, the rural clinics were run by nurses and medical doctors only visited the clinics on a

weekly or bi-weekly basis to see complicated subjects. These clinics generally did not have specialized staff, such as pharmacists, dieticians and social workers, and hospital referral was complicated by large travel distances. The laboratory was located in Durban, approximately two hours' drive from the clinics.

In both groups, the same DRT methodology was applied, namely population-based genotyping by means of the same in-house drug resistance assay[24]. Briefly, RNA was extracted from 200µl of plasma using the Nuclisens miniMAG extraction kit (bioMéRIEUX, SA). A PCR product containing 1315bp was generated from the HIV-1 subtype C *pol* region. Sequencing was performed on HIV-1 protease and the first 300 codons of the reverse transcriptase (RT) gene. RNA was reverse transcribed into cDNA with the Superscript III kit (Invitrogen Corporation, Carlsbad, CA) and a gene-specific primer: RT21. Platinum taq polymerase (Invitrogen Corporation) and a nested PCR protocol were used to amplify the protease and RT gene regions from cDNA. The amplicon was sequenced using 4 primers. Sequencing reactions were run on an automated 3500xl, 24 capillary Genetic Analyzer (Applied Biosystems Inc, Forster City, CA). Sequences were assembled and manually edited using CLC DNA Workbench 5.7.1 software (CLC bio, Denmark) and then submitted to Stanford HIV Drug Resistance (HIVDB) website (http://hivdb.stanford.edu) to identify HIV-1 drug resistance mutations, while the HIV-1 subtype was assigned on the Stanford database using the Rega HIV-1 subtyping tool. Frequencies of mutations were determined and resistance patterns analyzed with emphasis on clinical relevance. Data were anonymized and captured into a relational database (SATuRN RegaDB).

Genotypic susceptibility scores (GSS) were calculated for each antiretroviral agent using the Stanford HIVSeq algorithm version 6.0.5 (http://hivdb.stanford.edu) and a total score was then summed for the standard second-line regimens. This was done to assess the impact of observed drug resistance mutations on the predicted effectiveness of standard second-line regimens. Total GSS for the standard second-line regimen was calculated depending on the subject's treatment history; for participants on d4T or AZT at the time of genotyping, GSS was calculated for a regimen of TDF, 3TC and lopinavir/ritonavir (LPVr); while for those on TDF at the time of genotyping, GSS was calculated for a regimen of AZT, 3TC and LPVr. These standard second-line regimens were consistent with the recommendations in the 2012 South African National ART Guidelines. For the purposes of this analysis, a compromised second-line regimen was defined as GSS<2.

The urban and rural group data were exported from SATuRN RegaDB into Excel 2010 files. Pivot tables with IF/AND and VLOOKUP functions were used to calculate variable categories. Excel data were then exported to Stata version 12 and descriptive statistics calculated. Data were also exported to Statistix version 9 and p-values calculated for differences between variables. For count data, Two-Proportion tests were used, and the Wilcoxon Rank Sum test for continuous data. Alpha (α) was set on the 95th percentile and a p-value $\leq$ 0.05 was considered significant.

The study was approved by the research ethics committees of the Faculty of Health Sciences at the University of Pretoria (46/2011), the Biomedical Research Ethics Committee of the University of KwaZulu Natal (BF052/10) and the Health Research Committee of the KwaZulu Natal Department of Health (176/10). No personal participant information was entered in the database and all participants were allocated an unique identifying code. Results of the DRT were made available to the treating doctors and nurses in real time and support was given regarding selection of next regimens and access to newer medication, as needed.

Results

Demographic and clinical characteristics of the two subject groups are presented in Table 1. There was a large difference in size, 492 in the rural versus 103 in the urban groups. Gender ratios and ages were not significantly different. In the urban group, the median baseline CD4+ T-cell count was significantly lower than that of the rural group (98 versus 126 cells/µl, p=0.05); however, the range was large in both groups. Similarly, the median baseline VL for the urban subjects was significantly lower than that of the rural subjects (3.18 versus 4.23 $\log_{10}$, p<0.01). A similar proportion of subjects had achieved virological suppression to below 1000 RNA copies/ml plasma (75.8% versus 70.1%, p=0.37) and below 50 RNA copies/ml plasma (60.2% versus 54.4%, p=0.40).

At the time of genotyping, the difference in CD4+ T-cell counts and the proportions of subjects failing immunologically in the two groups were not significant: the median CD4 count was still very low (in the 130-140 range) and over 41% of subjects were failing immunologically in both groups. Both groups reported VL values above $\log_{10}$ 4.0; however, the urban group had a higher median value than the rural group (4.18 versus 4.08 $\log_{10}$, p=0.03). The urban group had more intense monitoring as evidenced by the significantly larger number of VL tests performed on them per year (3 versus 1.4, p< 0.01). They had, conversely, been failing for a shorter time: the majority in the urban group had less than 6 months of treatment failure (calculated as the time between the estimated start of treatment failure and referral for genotyping), while the majority of subjects from the rural group had been failing for more than 24 months before being referred for genotyping.

Table 2 shows the comparison of the HAART regimens, number of subjects with resistance mutations and GSS in the two groups. Consistent with the duration of treatment failure, the duration of treatment in the two environments was significantly different. The median for the urban group was 25.9 months while that for the rural group was 49.2 months with over 50% of those in the latter group falling into the >48 month category. Initial ART regimens were essentially comparable although a larger proportion of rural subjects had been started on regimens consisting of d4T/3TC/EFV and TDF/3TC/EFV, while a larger proportion of their urban counterparts had been started on AZT-based regimens. Similarly, at the time of genotyping, a significantly larger proportion of rural subjects was still on d4T-containing regimens, while more urban subjects were on AZT-based regimens. Urban

subjects were more likely to have changed regimen, either in terms of a NRTI substitution or both NRTI and NNRTI substitutions ($p<0.01$). Urban subjects also had a significantly shorter time before the first treatment change and had more regimen changes during the course of treatment ($p<0.01$).

At genotyping, a significantly larger proportion of urban subjects had no HIVDR mutations detected, indicating "wild-type" virus (35.9% versus 11.2%; $p<0.01$). Furthermore, a larger proportion of urban subjects had fewer than 2 reverse transcriptase mutations detected, while rural subjects were more likely to have between 2 and 6 mutations. The proportion with more than 6 mutations was, however, comparable in both groups (14.6% in urban versus 18.7% in rural group; $p=0.54$). Rural subjects were more likely to have TAMs (30.9% versus 14.6%; $p<0.01$), including those with multiple (three or more) TAMs (12.6% versus 3.9%; $p=0.02$). Resistance mutations are depicted in Figure 1. The most common mutations in both groups were: M184V, K103NS and V106AM. Interestingly, the predicted GSS for second line regimens was essentially identical with 98.2% of the rural and 97.6% of the urban group having scores equal or above 1.5.

Discussion

This is one of the very few studies which has compared HIV treatment outcomes, and, to our knowledge, the first to compare HIVDR profiles in subjects failing first-line NNRTI-based HAART in urban and rural settings. Urban subjects had lower CD4+ T-cell counts and VLs at baseline (first viral load measurement after presentation at the respective clinics) and were more aggressively managed than their rural counterparts: they had more therapy switches within a shorter duration of therapy, more VL tests per annum and had been failing for a shorter time before being referred for DRT. Distinguishing between those subjects who reached virological suppression and those who did not was based on the VL < 1000 copies/ml plasma threshold.

The HIVDR results seem to reflect these different approaches to therapy in the two groups: there were several significant differences in the absolute numbers of subjects with no resistance mutations, with NRTI and NRTI mutations and the total number of mutations of any type. Although the individual percentages were different in the urban and rural environments, the overall patterns of resistance formation were similar. Taken together with the findings shown in Figure 1, the underlying trend is the accumulation of similar mutations with increasing duration of treatment in both environments, with the rural group having accumulated more mutations due to the longer time spent with actively replicating virus while on ART. This is in line with other studies that observed that prolonged ART failure leads to the accumulation of drug resistance[25,26].

The more aggressive management of urban subjects might be due to the different management approaches employed by medical doctors and nurses, with doctors being more confident in ordering laboratory tests and treatment changes, and having more scope to work outside national guidelines. The urban group further had easier access to alternative medications, i.e. didanosine (ddI) was only prescribed in this group. Differential management might also be due to the ease of referring subjects to tertiary services and requesting blood investigations, due to the proximity of the referral hospital and laboratory in the urban setting.

A Ugandan study assessing pediatric responses to ART similarly found that urban children were more likely to be switched to second-line regimens than their rural counterparts[27].

It is interesting to note that despite more significant HIVDR in the rural group, both groups had comparable predicted GSS for second-line therapy. This is in keeping with other data that show that second line protease inhibitor-based therapy is effective in suppressing VL, at least in the short term, even in the absence of a fully active NRTI backbone[28,29].

This study has important limitations. It did not assess the larger groups receiving ART at the different sites, or all the subjects failing ART in the clinics, but rather focused on the sub-groups that had been referred for HIVDR; hence, there are no clinic denominators or mortality statistics. Important variables that might have impacted on the development of HIVDR, such as adherence and use of traditional medication or alcohol, were not routinely collected and could thus not be compared between the groups. In addition, the study has not completed follow-up of subjects after change to second line regimens; the ultimate treatment responses are therefore still unknown for these two groups. Despite these limitations, this study presents one of the largest datasets of HIVDR results in sub-Saharan Africa and addresses an important, yet understudied, area of HIV research.

In conclusion, this study showed that urban and rural subjects received significantly different HIV care, with urban subjects being more aggressively managed. This translated into fewer subjects with evidence of HIVDR and, specifically, severe HIVDR at the time of genotyping. Despite these differences, the expected response to second-line ART remained comparable between the groups. These findings have important implications for management of subjects in large treatment programs where financial constraints may limit the feasibility of intense subject monitoring. It seems that a balance can be obtained between limited virological monitoring on the one hand and prevention of severe, treatment-limiting HIVDR on the other.

Table 1. Comparison of subject demographic and clinical characteristics from the rural and urban groups

Characteristic	Unit	Rural group (N=492)	Urban group (N=103)	p-value Ł
Sex, male	N(%)	136(27.7)	26(27.4)	0.95
Female		355(72.3)	69(72.6)	
Age	years, median	36(31-42)	36(31-40)	0.27
< 20	(IQR)	19(3.9)	1(1.1)	
20-29	N(%)	90(18.3)	19(20.0)	
30-39		212(43.2)	53(55.8)	
40-49		105(21.4)	19(20.0)	
50+		19(3.9)	3(3.2)	
Baseline CD4+count	cells/µl,median	126(60-191)	98(40-185)	0.05
<50	(IQR)	98(19.9)	30(29.7)	
50-99	N(%)	97(19.7)	21(20.8)	
100-149		94(19.1)	12(11.9)	
150-199		95(19.3)	16(15.8)	
200-249		36(7.3)	12(11.9)	
250+		72(14.6)	10(9.9)	
CD4+ at genotype	cells/µl median	138(110-173)	131(103-162)	0.12
<50	(IQR)	51(10.4)	17(16.5)	
50-99	N(%)	4(0.8)	0(0)	
100-149		237(48.2)	53(51.5)	
150-199		128(26.0)	17(16.5)	
200-249		31(6.3)	9(8.7)	
250+		41(8.3)	7(6.8)	
*Immunological failure at time of genotype	N(%)	203(41.3)	43(41.7)	0.56
VL at baseline	log copies/ml median (IQR)	4.23(3.11-4.92)	3.81(1.40-4.23)	<0.01
VL at time of genotype	log copies/ml median (IQR)	4.08(3.08-4.48)	4.18(3.19-5.11)	0.03
VLs per subject per year	median	1.4(1.1-1.8)	3.0(1.9-5.4)	<0.01
Viral suppression,				
< 1000 copies/ml	N(%)	344(70.1)	78(75.8)	0.37
< 50 copies/ml		267(54.4)	62(60.2)	0.40
**Duration of virological failure	months median (IQR)	20.0(10.8-33.2)	8.2(3.1-17.7)	<0.01
<6	N(%)	48(9.8)	38(36.9)	<0.01
6-12		84(17.1)	26(25.2)	0.07
13-24		168(34.2)	26(25.2)	0.10
>24		192(39.0)	13(12.6)	<0.01

IQR = interquartile range

*Immunological failure was defined according to WHO criteria:

i. Decline of CD4+ cell count to lower than or equal to the baseline
ii. Persistent CD+ cell count of less than 100 cell/ µl
iii. A decline of 50% or more from the on-treatment peak value.

**Virological failure was estimated from:

i. Duration from the first VL > 1000 copies/ml until the date of the genotype,
ii. If there was a VL < 50 copies/ml between the above dates, duration was then measured from the next VL > 1000 copies/ml.
iii. If all VL values were > 1000 copies/ml then the duration was taken from the baseline until the date of the genotype

Ł Note: p-values were either the Wilcoxon rank sum test or Two-proportion tests

Table 2. Comparison of ART Regimens, Genotype Susceptibility Score and Number of Resistance mutations in subjects from the rural and urban groups

Characteristic	**Unit**	**Rural group**	N(%)	**Urban group**	**N(%)**	**p-value Ł**
Duration of ART (months)	median (IQR)	49.2(36.7-61.0)		25.9(14.2-41.5)		< 0.01
<24			54(11.0)		40(43.5)	< 0.01
24-48			179(36.4)		39(42.4)	0.47
>48			259(52.6)		13(14.1)	< 0.01
Initial ART regimen		d4T/3TC/EFV	297(60.4)	d4T/3TC/EFV	53(53.1)	0.05
		d4T/3TC/NVP	131(26.6)	d4T/3TC/NVP	24(23.5)	0.73
		TDF/3TC/EFV	48(9.8)	TDF/3TC/EFV	3(3.1)	0.05
		TDF/3TC/NVP	8(1.6)	TDF/3TC/NVP	1(1)	0.97
		AZT/3TC/EFV	5(1.0)	AZT/3TC/EFV	11(10.8)	<0.01
		AZT/3TC/NVP	3(0.6)	AZT/3TC/NVP	8(7.8)	<0.01
				ddI/3TC/EFV	2(1)	
		Total	492(100)	Total	102(100)	
ART regimen at time of genotype		d4T/3TC/EFV	218(44.3)	d4T/3TC/EFV	23(24.5)	<0.01
		d4T/3TC/NVP	109(22.2)	d4T/3TC/NVP	10(9.8)	<0.01
		TDF/3TC/EFV	99(20.1)	TDF/3TC/ EFV	12(11.8)	0.07
		TDF/3TC/NVP	22(4.5)	TDF/3TC/NVP	7(6.9)	0.44
		AZT/3TC/EFV	32(6.5)	AZT/3TC/EFV	22(23.6)	<0.01
		AZT/3TC/NVP	12(2.4)	AZT/3TC/NVP	16(15.7)	<0.01
				ddI/3TC/EFV	12(7.8)	
		Total	492(100)	**Total**	102(100)	
Number of subjects:						
with all switches			362/492(74.0)		87/102(85.3)	<0.01
with NRTI substitutions			105(21.3)		57(56.4)	<0.01
with NNRTI substitutions			65(13.2)		18(17.8)	0.31
with both NRTI and NNRTI substitutions			19(4.0)		13(11.3)	<0.01
time to 1st switch (months)	median (IQR)	20.0(10.8-33.2)		13.0(6.0-13.0)		<0.01
Any regimen changes	median (min-max)	2(1-4)		3(1-6)		<0.01
Subjects with mutations:						
none (wild type)			55/492(11.2)		37/103(35.9)	<0.01
with NNRTI mutations			415/492(84.3)		57/103(55.3)	<0.01
with NRTI mutations			411/492(83.5)		57/103(55.3)	<0.01
with any RT mutations						
<2			82(16.7)		39(47.6)	<0.01
2-3			255(51.9)		28(34.1)	0.01
4-6			62(12.7)		3(3.7)	0.03
>6			92(18.7)		12(14.6)	0.54
with TAMs			152/492(30.9)		15/103(14.6)	<0.01
0			343/492(69.7)		87/103(84.5)	<0.01
1-2			90/492(18.3)		11/103(10.7)	0.08
≥3			62/492(12.6)		4/103(3.9)	0.02
with PI mutations			4/492(0.8)		3/103(2.9)	0.45
Predicted 2nd line GSS	median (IQR)	2.0(2.0-2.0)		2.0(2.0-3.0)		
<1.5			9(1.8)		2(2.4)	0.75
≥1.5			482(98.2)		81(97.6)	1.0

IQR = interquartile range, d4T = stavudine, 3TC = lamivudine, EFV = efavirenz, NVP = nevirapine, TDF = tenofovir, AZT = zidovudine, DDI = didanosine, GSS = genotypic susceptibility score, NNRTI = non-nucleoside reverse transcriptase inhibitor, NRTI = nucleos(t)ide reverse transcriptase inhibitor, RT = reverse transcriptase, TAMs = thymidine analogue mutations, PI = protease inhibitor

Ł p-values were either the Wilcoxon rank sum test or Two-proportion tests

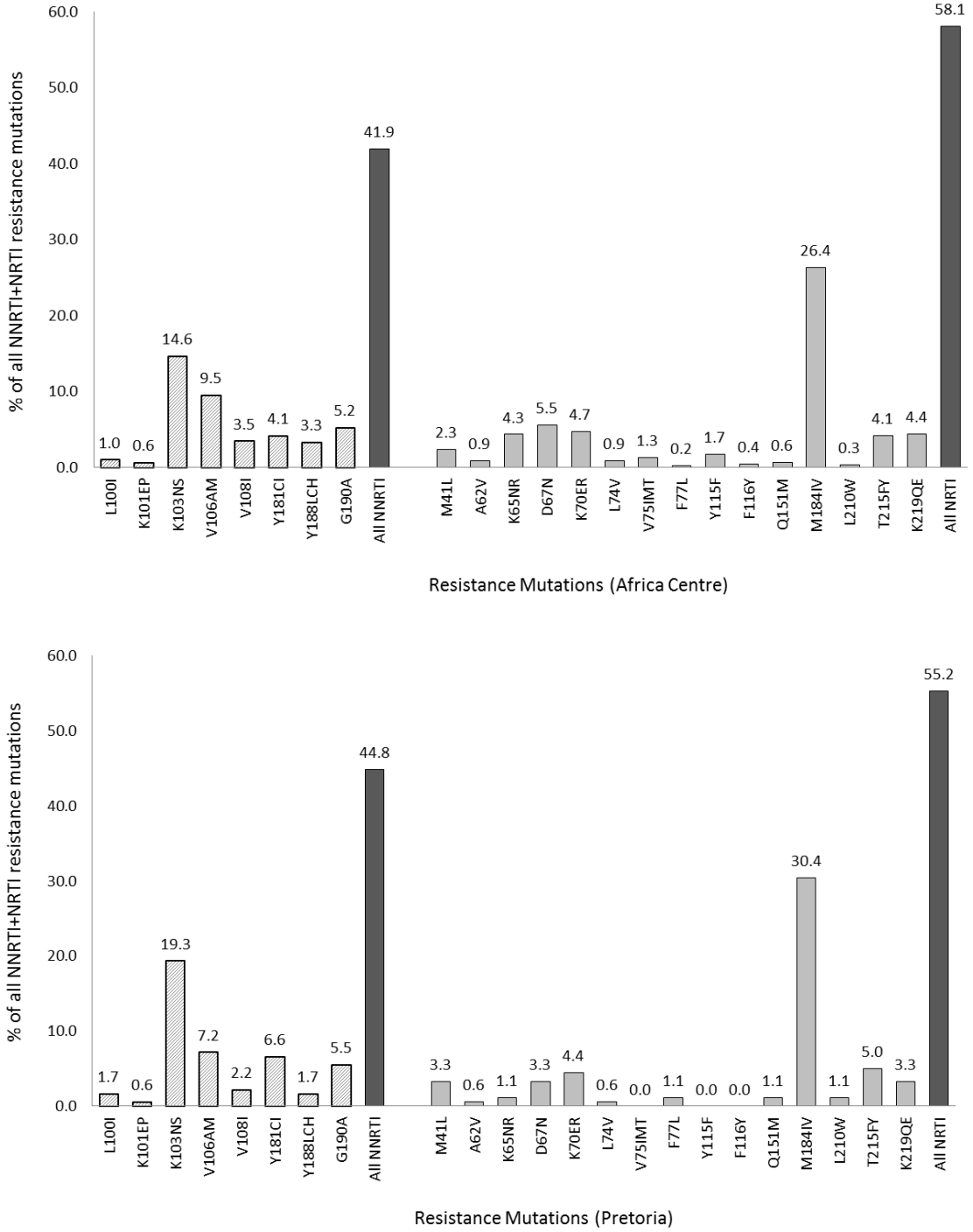

Figure 1. The patterns of resistance mutation formation in the rural group and urban group

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Circulating biomarkers of immune activation distinguish viral suppression from non-suppression in HAART-treated subjects with advanced HIV-1 subtype C infection

Abstract

Few studies have examined immune activation profiles in subjects with advanced HIV-1 subtype C infection, or assessed their potential to predict responsiveness to HAART. BioPlex, ELISA and nephelometric procedures were used to measure plasma levels of inflammatory biomarkers in HIV-1 subtype C-infected subjects sampled before and after 6 months of successful HAART (n=20); in subjects failing HAART (n=30) and in uninfected controls (n=8), in order to determine if biomarkers for therapy failure and/or the development of drug resistance could be identified. Prior to HAART, CXCL9, CXCL10, β2M, sTNF-R1, TGF-β1, IFN-γ, IL-6, TNF and sCD14 were significantly elevated in HIV-1-infected subjects compared to controls ($p<0.01$). All of these markers, with the exception of sTNF-R1, were also elevated in subjects failing HAART ($p<0.05$). The persistently elevated levels of CXCL9, CXCL10 and β2M in subjects failing therapy in the setting of a marked reduction in these markers in subjects on successful HAART, suggests that they may be useful not only to monitor immune activation during HAART, but also to distinguish between good and poor responders. In the case of sCD14 and TGF-β1, the levels of these biomarkers remained persistently elevated despite HAART-induced virological suppression, a finding that is consistent with ongoing monocyte-macrophage activation, underscoring a potential role for adjuvant anti-inflammatory therapy.

Introduction

In HIV-1 infection, depletion of T cells is caused by productive virus infection and Fas-mediated apoptosis of infected and uninfected cells[1,2]. In addition, chronic immune activation, especially of cells of the innate immune system, together with accompanying, counteracting endogenous anti-inflammatory mechanisms, further contribute to T cell depletion[3,4]. These mechanisms include chronic activation of plasmacytoid dendritic cells and monocytes/macrophages. HIV-infection of plasmacytoid dendritic cells causes persistent activation, resulting in excessive production of pro-apoptotic interferon (IFN)-α, as well as immunosuppressive indoleamine-2,3-dioxygenase and transforming growth factor (TGF)-β[4-13]. In the case of monocytes/macrophages, translocation of microbial products, especially lipopolysaccharide and DNA, across the damaged intestinal epithelium, results in persistent systemic activation of these cells due to interaction with Toll-like receptors 4 and 9, as well as with cytosolic pathogen nucleic acid sensors[14-23]. The resultant production of pro-inflammatory cytokines, especially TNF, drives T cell activation and activation-induced cell death[6,21,22]. Sustained immune activation is associated with disease progression, AIDS and death[24]. While highly active antiretroviral treatment (HAART) is able to suppress viral replication to levels of <25 copies/ml plasma and partially restore circulating $CD4^+$ T cells, it is unable to normalize immune activation[21,25].

Immune activation in HIV infection is associated with the presence of circulating pro-inflammatory/anti-inflammatory and anti-viral cytokines/chemokines, as well as with other biomarkers of immune activation, which vary qualitatively and quantitatively with disease progression[26-31]. However, relatively little is known about the profile of circulating biomarkers of immune activation in the setting of advanced HIV-1 subtype C infection, as well as the usefulness of its measurement, not only in monitoring response to HAART, but also as a strategy to detect virologic treatment failure. These issues are the focus of the current study.

Methods

Black, adult (≥18 years) participants attending the Antiretroviral Clinic at a district hospital in Pretoria, South Africa, were included in this study. Ethics approval was granted by The Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Ethics Committee Approval No. 46/2011). All participants gave informed consent and whole blood samples were collected in EDTA vacutainers, processed within 24 hours to separate the plasma component by centrifugation, and stored at -70°C for up to 37 months. $CD4^+$ T-lymphocyte counts ($CD4^+$) (Beckman Coulter SA (Pty) Ltd) and HIV-1 RNA (VL) (Nuclisens HIV-1 Viral Load Assay v1.2 or v2.0) were measured by standard flow cytometric and PCR-based procedures respectively, according to manufacturer's instructions. HIV subtype was determined at the time of population-based genotyping using an in-house assay described in Chapter V, and submitting the sequences to the Stanford database using the Rega HIV-1 subtyping tool.

Twenty HIV-infected participants were followed from pre-treatment to approximately 6 months on HAART. Pre-treatment samples were taken prior to the initiation of HAART in subjects presenting with $CD4^+$ counts ≤200 cells/µl blood or WHO stage 4 disease. All subjects were started on HAART, were clinically stable and did not develop clinical signs of immune reconstitution inflammatory syndrome during the first six months of treatment. All subjects were clinically screened for the presence of opportunistic infections, including tuberculosis. All subjects were virologically suppressed (VL <50 copies/ml plasma) at approximately 6 months of HAART (suppressed group). Drug regimens consisted of two nucleos(t)ide reverse transcriptase inhibitors (NRTIs) (stavudine (d4T) + lamuvidine (3TC), n=18, or tenofovir (TDF) + 3TC, n=2) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz (EFV), n=14 or nevirapine (NVP), n=4). Two subjects were started on ritonavir-boosted lopinavir (LPV/r) for clinical reasons.

A second group consisted of 30 participants failing HAART as evidenced by two successive VL results of >1000 copies/ml plasma at least eight weeks apart despite intensive adherence counselling (failing group). Drug regimens consisted of two NRTIs (d4T + 3TC, n=23 or zidovudine (AZT) + 3TC, n=7) and one NNRTI (EFV, n=20 or NVP, n=10). Participants had been referred for drug resistance testing and study samples were taken at the

time of referral. They had been on HAART for a median time of 30 months (range 9 – 97 months) and had been failing treatment for a median of 15.5 months (range 5 – 38 months). Five subjects (17%) had been referred from peripheral clinics and the duration of treatment failure could not be determined. Three subjects (10%) had experienced treatment interruptions at some time before treatment failure and 13 (43%) never had a suppressed VL while on HAART. All subjects with $CD4^+$ ≤200 cells/µl (n=21) were on cotrimoxazole or dapsone prophylaxis.

A third group (n=8) of black, HIV-uninfected, healthy control subjects was also included in the study. The median ages of the control, suppressed and failing groups were 29 (range 24-49), 41.5 (25-63) and 40.5 (27-55) years respectively, and the corresponding male:female ratios 1:0.6, 1:4 and 1:4.

Circulating biomarkers of immune activation

These were selected on the basis of being largely representative of T cell, monocye/macrophage, dendritic cell and natural killer cell activation. Circulating cytokines/chemokines were measured using: i) the BioPlex® suspension array system (Bio-Rad Laboratories Inc., Hercules, CA, USA) (IL-6, IL-10, IFN-γ, TNF-α, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CXCL10/IP-10); or ii) conventional ELISA namely IFN-α (eBioscience Inc., San Diego, CA, USA); TGF-β1 total (Biolegend, San Diego, CA, USA); CXCL9/MIG and sTNF-R1 (Raybiotech Inc., Norcross, GA, USA); and sCD14 (Abcam, Cambridge, MA, USA). CRP and β2M were assayed by nephelometry (Siemens healthcare Diagnostics, BN Prospec Nephelometer, Newark, USA). Previously published ranges for each of these parameters together with supporting references are shown as supplementary data.

Data analyses and statistics

As participant groups consisted of ≤30 individuals, data were considered to be non-parametric and distribution-free statistical tests implemented in Stata v11.2 (StataCorp). Continuous data were analysed according to the median, minimum and maximum concentrations. Median concentrations of each parameter were compared between cohorts using the Wilcoxon-Mann-Whitney test for independent groups and Wilcoxon signed rank sum test for matched groups. Correlations between parameters were determined using the

Spearman correlation test for the HIV-infected pre-HAART group (n=20), as well as for this group combined with the group failing HAART (n=50). Statistical significance was set at $p \leq 0.05$.

Results

Circulating CD4$^+$ T-lymphocyte counts, HIV-1 viral loads, cytokines/chemokines, β2-microglobulin, sCD14 and CRP in the control, suppressed (pre- and post-therapy) and failing groups

CD4+ counts, HIV-1 VL, and levels of inflammatory biomarkers are shown in Table 1. As expected, plasma VL decreased from a median of 53,000 to <50 RNA copies/ml plasma and there was a significant increase in the circulating CD4$^+$ count (83 to 208 cells/µl; $p<0.0001$) in the suppressed group. In the failing group, the median plasma VL was 7,300 copies/ml and CD4+ count 126 cells/µl. With respect to the circulating biomarkers of immune activation, CXCL9, CXCL10, TGF-β1, sTNF-R1, β2M, and sCD14 were significantly elevated ($p<0.03$) and CCL4 significantly decreased ($p=0.04$) in the pre-HAART group relative to the control group, while IFN-γ was moderately increased, but not significantly so ($p=0.07$). Following 6 months of HAART, CXCL9, CXCL10, β2M, IFN-γ, IL-6, TNF-α and sTNF-R1 were significantly decreased ($p<0.01$), CCL4 increased ($p=0.00$), while TGF-β1 and sCD14 remained elevated despite undetectable plasma VL. It is difficult to attribute major significance to the decreases in TNF-α and IL-6 as the pre-therapy values for both were low. No difference was observed in IFN-α, CCL3 and CRP either between the HIV-infected and uninfected control group or the virologically suppressed group pre- and post-HAART.

In the failing group, the same 5 biomarkers (CXCL9, CXCL10, TGF-β1, β2M and sCD14) were also significantly elevated compared with the control group ($p<0.02$), the values for CXCL10 and β2M being somewhat lower than those of the pre-HAART group ($p<0.03$), while those of CXCL9, TGF-β1 and sCD14 were essentially comparable ($p>0.5$). Although the value for CCL2 was significantly lower, and that of IL-10 higher, than the corresponding values of the control group, interpretation is difficult as these values were low in both groups.

Analysis of correlations between variables

Correlations between $CD4^+$, VL and the various biomarkers in the pre-HAART group are shown in Table 2. $CD4^+$ counts correlated negatively and significantly with VL and with sTNF-R1 and CCL2. Positive correlations were observed between VL and sCD14 and β2M. Significant positive correlations were also observed between several of the biomarkers including, but not limited to, IFN-γ, CXCL10, CCL2, CCL3 and TNF-α. Although not shown, correlations for the composite group (consisting of the pre-HAART and failing groups) were generally comparable, albeit weaker, with the exception of $CD4^+$ count with VL ($r=-0.64$, $p<0.00$), while the following modest correlations were found between: i) CCL4 with CXCL9, IL-6, CCL3, and IFN-γ ($r=0.30$ - 0.43 respectively; $p<0.03$ – $p=0.00$); and ii) β2M with CD4 counts, IL-6, and IFN-γ ($r=-0.28$ - 0.43 respectively; $p<0.05$ – $p<0.02$).

Discussion

Our findings in subjects infected with HIV-1 subtype C are consistent with the co-existence of distinct mechanisms of immune activation, which appear to be differentially affected by successful HAART[21,25]. Although only moderately elevated pre-HAART, it is likely that IFN-γ underpins the increases in CXCL9 and 10, a contention supported by the strong, positive inter-correlation between IFN-γ and CCL10, as well as that of CCL9 with CCL10. Other cell types such as dendritic cells and monocytes may also contribute to the increases in these cytokines pre-HAART following exposure of the cells to alternative activators such as IFN-λ1[11,32,33]. The unexpectedly low level of IFN-α, as well as those of CCL2 and 3, may be due to advanced immunosuppression in the setting of high levels of TGF-β1 in this group of subjects[34]. In the case of β2M, CXCL 9 and 10, and TNF-R1 (a surrogate for TNF), HAART-associated decreases most likely reflect efficient viral suppression and consequent decreased turnover and reactivity of both CD4+ and CD8+ T cells.

The absence of effects of HAART on plasma sCD14, as previously reported by us and others[17,21], as well as the increase in CCL4, is consistent with ongoing chronic inflammation due to sustained activation of monocytes/macrophages, even in the face of virally suppressive therapy, and may persist for several years[21,35]. In this setting, the persistent activation of monocytes/macrophages, predominantly the subtype which co-expresses CD14 and CD16, is most likely driven by the process of microbial translocation[21,24,36]. The consequence is sustained generation of pro-inflammatory mediators and cytokine-driven T cell death pathways. Interestingly, Sandler *et al.*[37] recently reported significant positive correlations between plasma sCD14, IL-6, CRP, serum amyloid A and D-dimer in subjects infected with HIV-1 subtype B[38]. Subjects with the highest quartile of plasma sCD14 concentrations had a 6-fold higher risk of death than those in the lowest quartile[37].

In addition, and supported by the findings of the current study, endogenous, monocyte/macrophage-targeted, anti-inflammatory mechanisms are also likely to contribute to ongoing immunosuppression with TGF-β1 appearing to play a pivotal role. Notwithstanding platelets, plasmacytoid dendritic cells, macrophages of the M2 phenotype and immunoregulatory $CD8^+$ T cells, immunosuppressive and pro-fibrotic TGF-β1 is likely to originate predominantly from regulatory T cells[38,39]. In this context it is noteworthy that

extensive fibrosis of the T cell zone of lymphoid tissue appears to be a significant factor in the failure of T cell reconstitution following successful HAART[13]. Persistently elevated plasma levels of TGF-β1 and sCD14, even in the setting of ostensibly successful HAART, may therefore identify a subset of subjects at highest risk of a poor outcome.

In the group of subjects failing HAART, the circulating concentrations of CXCL9, CXCL10 and β2M were also significantly higher than those of the control group and, with the exception of CXCL9, significantly lower than the pre-HAART values for the suppressed group. The circulating concentrations of sCD14 and TGF-β1 in the failing group were comparable to those of the suppressed group both pre- and post-therapy. Although persistent elevations, or a rebound following an earlier decrease, in plasma CXCL9, CXCL10 and β2M appear to be associated with a poor response to HAART, suggesting that serial measurement of these biomarkers may be a useful adjunctive strategy. Nevertheless, measurement of VL clearly remains the definitive strategy in the clinical setting.

With respect to previous studies, our findings are generally in agreement with a recent study by Kamat *et al.* in which elevated circulating concentrations of CXCL9, CXCL10, sCD14 and soluble IL-2 receptor (sIL-2R) represented a profile which distinguished viremic and aviremic subjects infected with HIV-1 subtype B from uninfected, healthy control subjects[30]. In agreement with the report of Kamat *et al.*[30], we also detected a significant, negative correlation between numbers of circulating $CD4^+$ T cells and VL, but failed to show a correlation between these disease markers and CXCL10 in the pre-HAART group. However, this correlation was detected when the pre-HAART and failing groups were combined, most likely due to increased statistical power. As mentioned above, and in agreement with Kamat *et al.*[30] we also detected a significant positive correlation between CXCL9 and CXCL10 (Spearman correlation test; 0.68, p=0.00, Table 2), while in contrast to these authors a significant, positive correlation between CXCL10 and IFN- γ was evident as can be expected in conditions of chronic inflammation.

Notwithstanding the different viral subtypes investigated, there are, however, several other important differences between the study by Kamat *et al.*[30] and the current study. Most importantly, the profile of biomarkers of immune activation measured by Kamat *et al.*[30], which did not include β2M or TGF-β1, was not measured serially in a single cohort of subjects pre- and post-HAART as done in the current study, which may account for the

observed lack of effect of HAART on IFN-γ in the former study. These are strengths of the current study.

Limitations, however, are: i) small sample sizes; ii) measurement of circulating biomarkers at a single, relatively short-term time point (6 months) following initiation of HAART in the suppressed group; iii) no pre-therapy measurement of circulating biomarkers prior to initiation of therapy in the failing and control groups; and iv) no laboratory confirmation (e.g. pharmacokinetic analyses) that patients were regularly taking antiretroviral medications. Nonetheless, the general agreement with previous studies, predominantly in the setting of HIV-1 subtype B infection, supports the reliability of our findings.

In conclusion, successful administration of HAART to subjects with HIV-1 subtype C infection is accompanied by significant decreases in circulating biomarkers associated with T cell activation and turnover (IFN-γ, CXCL9, CXCL10, sTNF-R1 and β2M). Serial measurement of 3 of these (CXCL9, CXCL1O, and β2M) may represent a useful adjunct to measurement of viral loads in monitoring responses to HAART. In addition, persistently elevated levels of sCD14 and TGF-β1, despite successful HAART, are consistent with chronic activation of monocytes/macrophages and possible risk of a poor outcome, underscoring the adjunctive therapeutic potential of monocyte/macrophage-targeted anti-inflammatory chemotherapy in subjects with advanced HIV infection.

Table 1: $CD4^+$ T-cell lymphocyte counts, HIV-1 viral loads and circulating biomarkers of immune activation, in the 3 study groups

	HIV Negative, n=8		Suppressed group, n=20				Failing group, n=30	
			Pre-Haart		Post-HAART			
Measurement	**Median**	**Range***	**Median**	**Range***	**Median**	**Range***	**Median**	**Range***
$CD4^+$ T-cell count	N/A	N/A	83	3 – 296	$208^{\#}$	86 – 537	$126^{\#}$	10 – 533
Viral load	N/A	N/A	53 000	5 700 – 330 000	$<50^{\#}$		$7\ 300^{\#}$	240 – 350 000
sCD14	4 116.7	3 185.7 – 5 169.7	$8\ 815^{+}$	4 945.1 – 11 000	$8\ 259.1^{+}$	3 424.7 – 11 000	$9\ 209.8^{+}$	430.5 – 11 000
β2-microglobulin	1.6	1.3 – 2.4	4.8^{+}	2.0 – 8.6	$2.5^{+\#}$	1.7 –- 3.6	$3.3^{+\#}$	2 - 9.1
CRP	4.0	0 – 15.1	1.8	0.1 – 43.8	3.9	0.3 – 30.2	3.2	0.5 – 97.3
TGF-β1 Total	6 458.8	1 845.8 – 12 448.1	$12\ 701.9^{+}$	4 634.8 – 37 587.2	$14\ 882.2^{+}$	4 426.7 – 50 000	$11\ 399.3^{+}$	0 – 43 434.5
IFN-γ	18.2	0 – 210.4	53.1	4.6 – 392.8	$16.9^{\#}$	0 – 74.4	40.9	0 – 403.1
CXCL10	416.1	225.7 – 688.5	$1\ 864.0^{+}$	858.3 – 66 740.7	$822.5^{+\#}$	212.2 – 1 378.0	$1\ 097.0^{+\#}$	150.2 – 3 957.1
CXCL9	849.1	246 – 2 991.8	$6\ 059.5^{+}$	1 714.9 – 12 000	$2\ 940.6^{+\#}$	1 410.2 – 12 000	$4\ 849.4^{+}$	1 318 – 12 000
IFN-α	18.0	16 – 28.2	19.4	11.7 – 64.9	16.8	14.3 – 54.7	17.3	14.3 – 120.1
IL-6	1.5	0.5 – 11.3	2.7	0.9 – 40.6	$1.2^{\#}$	0.3 – 4.0	2.3	1.1 – 32.3
IL-10	0.9	0.4 – 14.9	2.5	0.5 – 26.2	1.4	0.6 – 2.6	2.2^{+}	0.8 – 9.6
CCL2	20.3	9.6 – 28.0	13.5	2.3 – 33.2	8.3^{+}	3.8 – 32.2	$6.5^{+\#}$	1.4 – 46.9
CCL3	2.6	2.3 – 5.8	2.9	2.1 – 12.8	2.8	2.3 – 11.5	2.8	2.3 – 32.1
CCL4	39.8	23.5 – 70.4	22.8^{+}	0 – 82.0	$48.8^{\#}$	7.6 – 84.1	33.9	4.4 – 110.3
TNF-α	0	0 – 36.5	1.1	0 – 124.0	$0^{\#}$	0 – 8.5	1.7	0 – 223.6
sTNF-R1	353.2	190.9 – 470.6	512.2^{+}	212.3 – 800	$380.7^{\#}$	214.5 – 635.7	363.5	151.3 – 800

All results are presented as pg/ml with the exception of sCD14 (ng/ml), β2-microblobulin and CRP (µg/ml), circulating CD4+ T cell counts (cells/µl blood) and viral loads (copies/ml).

* Range: minimum to maximum value.

+ P<0.04 - <0.0001 for comparison with corresponding values for healthy control subjects.

# P<0.02 - <0.0001 for comparison with corresponding pre-HAART values.

Median concentrations were compared by means of Wilcoxon-Mann-Whitney test for independent groups and Wilcoxon signed rank sum test for matched groups.

Table 2: Most significant correlations in the HIV-1 infected pre-HAART group [n=20], (paired values represent the correlation coefficients uppermost with the corresponding p value)

	VL	TNF-R1	sCD14	CXCL9	IL-6	IL-10	IFN-γ	CXCL10	CCL2	CCL3	CCL4	TNF-α	CRP	β2M
CD4	**-0.45 (0.05)**	**-0.52 (0.02)**	-0.25 (0.28)	-0.04 (0.88)	-0.32 (0.17)	-0.27 (0.25)	-0.23 (0.33)	-0.18 (0.45)	**-0.59 (0.01)**	-0.28 (0.23)	-0.30 (0.20)	-0.13 (0.57)	-0.42 (0.07)	-0.30 (0.20)
VL	1.00	0.27 (0.25)	**0.58 (0.01)**	0.25 (0.28)	0.37 (0.10)	0.07 (0.78)	0.20 (0.40)	0.40 (0.08)	0.36 (0.12)	0.29 (0.22)	0.29 (0.21)	-0.06 (0.79)	0.22 (0.35)	**0.64 (0.00)**
IFN-α	0.31 (0.18)	0.33 (0.16)	**0.58 (0.01)**	0.31 (0.18)	**0.58 (0.01)**	0.26 (0.27)	**0.44 (0.05)**	0.14 (0.56)	0.20 (0.40)	**0.66 (0.00)**	-0.07 (0.77)	**0.45 (0.05)**	0.19 (0.42)	0.30 (0.20)
TNF-R1	0.27 (0.25)	1.00	**0.58 (0.01)**	0.06 (0.79)	**0.46 (0.04)**	0.29 (0.21)	0.06 (0.79)	0.04 (0.88)	0.03 (0.90)	0.25 (0.29)	-0.03 (0.90)	-0.05 (0.88)	**0.54 (0.01)**	0.39 (0.09)
sCD14	**0.58 (0.01)**	0.58 (0.01)	1.00	**0.45 (0.05)**	**0.80 (0.00)**	0.19 (0.43)	0.43 (0.06)	0.37 (0.10)	0.11 (0.60)	**0.63 (0.00)**	0.19 (0.44)	0.26 (0.28)	**0.44 (0.05)**	**0.62 (0.00)**
CXCL9	0.25 (0.28)	0.06 (0.79)	**0.45 (0.05)**	1.00	**0.50 (0.02)**	0.21 (0.37)	**0.64 (0.00)**	**0.68 (0.00)**	0.19 (0.41)	**0.64 (0.00)**	0.17 (0.46)	**0.48 (0.03)**	0.25 (0.30)	**0.67 (0.00)**
IL-6	0.37 (0.10)	**0.46 (0.04)**	**0.80 (0.00)**	**0.50 (0.02)**	1.00	**0.50 (0.03)**	**0.74 (0.00)**	0.41 (0.08)	0.37 (0.11)	**0.83 (0.00)**	0.36 (0.11)	**0.57 (0.01)**	0.38 (0.10)	0.42 (0.06)
IL-10	0.07 (0.78)	0.19 (0.21)	0.19 (0.43)	0.21 (0.37)	**0.50 (0.03)**	1.00	**0.50 (0.03)**	0.20 (0.41)	0.34 (0.14)	**0.66 (0.00)**	0.43 (0.06)	0.39 (0.09)	-0.12 (0.62)	0.16 (0.49)
IFN-γ	0.20 (0.40)	0.06 (0.79)	0.43 (0.06)	**0.64 (0.00)**	**0.74 (0.00)**	**0.50 (0.03)**	1.00	**0.68 (0.00)**	**0.60 (0.01)**	**0.79 (0.00)**	0.42 (0.06)	**0.64 (0.00)**	0.06 (0.80)	0.35 (0.13)
CXCL10	0.40 (0.08)	0.04 (0.88)	0.37 (0.10)	**0.68 (0.00)**	0.41 (0.08)	0.20 (0.41)	**0.68 (0.00)**	1.00	0.36 (0.12)	**0.48 (0.03)**	0.37 (0.11)	0.37 (0.11)	0.25 (0.30)	**0.60 (0.05)**
CCL2	0.36 (0.12)	0.03 (0.90)	0.11 (0.63)	0.19 (0.41)	0.37 (0.11)	0.34 (0.14)	**0.60 (0.01)**	0.36 (0.12)	1.00	**0.48 (0.03)**	**0.58 (0.01)**	0.28 (0.23)	0.04 (0.89)	0.22 (0.35)
CCL3	0.29 (0.22)	0.25 (0.29)	**0.63 (0.00)**	**0.64 (0.00)**	**0.83 (0.00)**	**0.66 (0.00)**	**0.79 (0.00)**	**0.48 (0.03)**	**0.48 (0.03)**	1.00	0.41 (0.07)	**0.64 (0.00)**	0.12 (0.61)	**0.44 (0.05)**

Spearman correlation test; statistically significant correlations ($p \leq 0.05$) are shown in bold.

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Impact of human APOBEC3G mutation on HIV-1 subtype C diversity and subsequent HIV drug resistance.

Abstract

Apolipoprotein B, mRNA-editing, catalytic polypeptide-like 3G (APOBEC3G or hA3G) deaminates cytosine residues of Virion Infectivity Factor (Vif)-deficient Human Immunodeficiency Virus Type 1 (HIV-1), resulting in extensive G→A substitution (hypermutation) and replication incompetent proviral deoxyribonucleic acid (DNA). Limited information exists regarding the effects of hA3G hypermutation although increased viral genetic diversity and drug resistance have been reported. Using existing data, automated online tools and manual couting methods, statistical comparisons of genetic diversity from subjects failing highly active antiretroviral therapy (HAART) to control sequences from therapy-naïve individuals was carried out to investigate the contribution of suboptimal hA3G hypermutation to increased genetic diversity and drug resistance. The findings of this study primarily show that i) there are few theoretical hA3G-preferred substrate contexts available for hypermutation at primary drug resistance codons in subtype C, ii) substitutions at h3AG substrates differ between resistant and therapy naïve datasets, iii) more guanines sites are affected in resistant viral isolates and iv) resistant viral isolates are not substituted more than therapy naïve viral isolates independent of guanine substrate. We conclude that although hA3G activity may contribute to elevated genetic diversity in plasma-derived HIV-1 subtype C, drug resistant viral isolates are not the result of preferential editing by hA3G.

Introduction

Human APOBEC3G (hA3G) is a member of the APOBEC family of proteins which are involved in innate immunological defence against several exogenous retroviruses and endogenous retroelements[1-3]. APOBEC3G is required for resistance to infection by Vif-deficient HIV-1[4-6] as has been shown in monocyte-derived dendritic cells[7]. hA3G, via hydrolytic substitution[2], causes the deamination of cytidine nucleotides in single-stranded HIV-1 DNA during reverse transcription[1,2,8] which results in the accumulation of uracils [9,10] in this first single-stranded DNA (ssDNA) and thus adenines in the second DNA strand[11]. The accumulation of uracils also results in their destruction by the base-excision repair pathway[11] via host cell virion-associated base repair enzymes such as uracil DNA glycosylase (UNG)[12] and apurinic-apyrimidinic endonuclease[2]. The net effect of successful hypermutation is increased genetic diversity and a decrease in viral expansion[11]. The editing of HIV-1 by hA3G can produce replication incompetent virions, as shown by the non-permissive phenotype conferred to HIV-1 host cells *in vitro*[1,5,10,13].

APOBEC3G shows a strong tendency to deaminate residues in a specific context on single-stranded DNA [14]. This mutational bias is not linked to hotspots along the target, but is preferential at the CC dinucleotide context[4,15-17]. The deamination at these preferred sites, which translates to AG in the proviral protein-coding DNA strand after deamination of the first minus strand DNA, seems to be very important in selecting for mutants that have premature stop (termination) and start codons (methionines)[18] which can alter the open reading frames of coding sequences[2].

Recent evidence suggests that mutations at drug resistance-sensitive sites can be caused by APOBEC3G[19] operating suboptimally. The net effect of any possible suboptimal hypermutation could be the production of new infectious virions with increased genetic diversity – rather than uninfectious virions – and may result in drug resistance[20]. Kim *et al* (2010)[20] investigated sequence diversity in the presence of lamivudine (3TC) and APOBEC3G and found that G→A mutations can be important in HIV-1 drug resistance evolution. Indeed, sub-lethal editing of HIV-1 and the resulting increased genetic diversity could even contribute to viral evolution and immune escape[21].

HIV-1 subtype C is the most prevalent strain of HIV-1 globally[22]. However, little is known about the role of suboptimal APOBEC3G-driven hypermutation on the development of drug resistance. This study, which focuses on HIV-1 subtype C originating from plasma viral RNA quasispecies isolates from different individuals was conducted to determine; i) if any drug resistant viral isolates were statistically significantly hypermutated, ii) if drug resistant viral isolates show a higher level of G→A substitution compared to therapy naïve drug susceptible viral isolates, and iii) if suboptimal APOBEC3G-mediated G→A substitution could be contributing to the development of drug resistance.

Methods

Genotyping viral isolates from subjects failing HAART virologically

Subjects were generally identified for inclusion in this study based on them failing antiretroviral therapy as defined by the measurement of two successive HIV-1 RNA plasma viral loads greater than 1000 copies per millilitre of plasma with intensive adherence counselling given between the successive viral load measurements (Nuclisens v1.2/v2.0; Department of Medical Virology, National Health Laboratory Service, University of Pretoria/Department of Immunology, University of Pretoria). Adult and pediatric subjects who were attending the ARV Clinic in Tshwane (Gauteng Province, South Africa) were included in this study. Ethics approval (46/2011) was obtained for these studies from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (complies with ICH-GCP guidelines).

Total nucleic acids were extracted using 100 – 500 µl plasma with the Nuclisens Lysis Buffer and Magnetic Extraction Reagents (BioMerieux) as per manufacturer's recommendations. Final extracted nucleic acids were eluted in 50 µl elution buffer. In-house amplification began with reverse transcription being performed on 15 µl extracted RNA using the MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (Epicentre Biotechnologies) as per manufacturer's recommendations using gene specific priming (see Table 2 for oligonucleotide information). Samples were then refrigerated or frozen at -20 °C for future use. Up to 7 µl of cDNA was used for the first round of a nested polymerase chain reaction (PCR). Two microlitres of the product from the first PCR round was used in a second round of PCR after the entire first round product had been subjected to decontamination using UNG as per manufacturer's recommendations (Epicentre Biotechnologies) which would reduce possible contamination in second round PCRs by existing second round PCR product. The chemical conditions for each round of PCR were precisely the same, except that dUTP was used in place of dTTP during second round PCRs and the primers were internal to those of the first round. Forty-nine microlitres of each round's master mix was aliquotted per sample, which was made up of 5 µl 10 X Buffer (ExSel, JMR Holdings), 1.75 µl 10 mM dNTPs (Promega), 2 µl of each 10 µM primer, 0.1 µl 5 U/µl Taq (ExSel, JMR Holdings) and 39.15 µl of nuclease-free water per reaction. A negative control was always included. Round 1 cycling consisted of the following steps: 94

°C for 2 minutes, {94 °C for 10 seconds, 50 °C for 30 seconds, 70 °C for 2 minutes} x35 cycles, 70 °C for 10 minutes. Thirty-eight cycles were used for the second round reactions. The region of the polymerase gene that was amplified incorporated 1857 bp of Protease and Reverse Transcriptase. Data was trimmed to 897 bp (PR amino acids 11 - 99 and RT amino acids 38 – 247) so that data generated using the TruGene HIV-1 Genotyping System as recommended (Siemens Healthcare Diagnostics) could also be used. Products from in-house amplification were excised from a 1% agarose gel, purified using the GeneJet Gel Extraction Kit (Fermentas) and sequenced using quarter reaction cycle sequencing. Samples were refrigerated for up to 10 days before being electrophoresed on an ABI3500XL sequencer by an outsourced company (Inqaba Biotec).

Each sequence that was generated was initially checked for quality (peak height, background level and sequence length). For in-house sequences, contiguous sequences were built using CLC DNA Workbench v5.7 (CLC bio) by selecting all individual sequences that had been passed and assembling these to a HIV-1 subtype C *Pol* reference (consensus) sequence (Los Alamos HIV Sequence Database http://www.hiv.lanl.gov). TruGene base-calling was manually checked. A nucleotide alignment was generated, combining both in-house and TruGene data. Gaps, arising from insertions and deletions, were removed from the final alignment. Three additional quality checks ensured that each sequence was unique and of high quality: CPR (Calibrated Population Resistance Tool; http://cpr.stanford.edu/cpr.cgi[23]) and genetic distance (>=2%) was used for quality control of all sequences while SQUAT (World Health Organisation; http://www.who.int/hiv/pub/drugresistance/squat/en/index.html) was also used for quality control of in-house genotypes. Only HIV-1 subtype C sequences were included (REGA HIV-1 Subtyping Tool[24]).

The resistance group consisted of 166 unique sequences, from adult and pediatric subjects failing antiretroviral therapy with evidence of at least one drug resistance mutation. In order to analyse only sub-optimal h3AG response, we excluded sequences that had statistically significant hA3G-mediated hypermutation as determined by automated methods. The dataset that was used for control purposes consisted of 347 sequences from individuals who reported that they were therapy-naïve. No evidence of drug resistance mutations or statistically significant hA3G-mediated hypermutation as determined by automated methods was found.

Automated hypermutation analyses

As it is rare to find evidence of hypermutation in samples obtained from plasma RNA, two different automated methods were first used to analyse each viral isolate of the drug resistance dataset for statistically significant levels of hA3G-mediated hypermutation: HYPERMUTv2.0 (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html[25]) and CPR. Statistically significant hypermutation was not expected for any drug resistant viral isolate due to the RNA nature of the samples. Viral isolates were excluded if there was no evidence of drug resistance or if automated analyses showed statistically significant hypermutation as the primary focus was to determine the contribution of suboptimal hA3G hypermutation to drug resistance. The same automated analyses were carried out on an existing dataset of South African HIV-1 subtype C viral isolates (Free State Province, South Africa) obtained from adult subjects before the initiation of ART[26] and, similar to above, sequences were excluded if there was evidence of statistically significant hypermutation. Sequences were also excluded from this dataset if drug resistance was evident as this would skew the study. Both datasets could suffer from an overestimation of the amount of G→A substitution occurring on any individual viral isolate due to the viral quasispecies population sequencing that was carried out on plasma-derived HIV-1.

Determining the potential for G→A substitution and its effect on drug resistance

Using the drug resistance mutation list given by the Stanford HIV Drug Resistance Database (http://hivdb.stanford.edu[27,28]) those drug resistance-related codons in the consensus HIV-1 subtype C sequence (Los Alamos HIV Sequence Database; http://www.hiv.lanl.gov) which could theoretically be the target of G→A substitution in the hA3G-preferred (5'GG), and non-GG context were identified. The latter group consisted of isolated guanines as well as each guanine in the 3' position of the hA3G-preferred context (i.e.: 5'-GG). For the former group, both 5'-NGG and 5'-GGN codons were considered as potential targets of hA3G mutagenesis. For resistance codons of the 5'-NNG genotype, the adjacent 3'-codon was also taken into consideration since a 5'-GNN sequence at this position would give rise to a hA3G-peferred (5'-GG) site that spanned two sequential codons. In this case, replacement of the 3'-G in the resistance codon (5'-NNG) with an adenine could theoretically lead to drug resistance if G→A resulted in drug resistance for that codon. Since hA3G-mediated mutations can occur at any GG-site along the length of the sequence (including codons that play a compensatory role in the fitness and reproductive capacity of drug resistant viruses),

the analyses were extended to identify all GG- and non-GG guanine sites in the HIV-1 subtype C consensus sequence.

Higher sensitivity analyses

A method for the analysis of G→A substitution in provirus described by Pace *et al* 2006[17] which determines: i) relative G→A substitution preferences; ii) relative G→A substitution burdens; iii) specific APOBEC3G substitution scores; and iv) an overall consolidated APOBEC3G score was initially adopted but could not be used due to the low signal from plasma virus. Since sequences can be hypermutated at levels that do not reach statistical significance, a series of manual analyses were undertaken to increase the chance of detecting hA3G-mediated G→A substitution. Data from both datasets were extracted from the output generated by HYPERMUT v1 (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html[25]). All substitution types and frequencies, versus the consensus C sequence, were described overall by dataset and statistically compared between datasets in terms of the frequency distribution of substitutions per isolate and substitution type. This would give an indication as to whether substitutions were occurring similarly between therapy naïve and drug resistant viral isolates, and at what level.

Overall G→A substitution and hotspot analysis

As G→A substitution was the major focus, further more detailed investigations were undertaken. G→A substitution was identified against the consensus C sequence for each sequence of each dataset following which datasets were compared in terms of the frequency of G→A substitution, after correcting for sample size. This analysis would provide a picture of the level of G→A substitution on a sequential basis, between datasets. The frequency of G→A substitution in PR and RT, relative to their sequenced length, was compared between gene regions and datasets to determine whether there were any G→A hotspots along the sequenced length.

hA3G activity

Datasets were combined and statistically analysed for difference in the number of G→A substitutions occurring at hA3G- versus non-hA3G-preferred sites which would provide an indication if hA3G was active overall. Data were first corrected for differences between the number of sites between the two groups. Additionally, this analysis was

replicated for the therapy naive and resistance datasets respectively to determine if hA3G activity could be identified within each dataset independently. For both the resistance and therapy-naïve dataset, G→A substitution was identified and substitution frequency was determined by comparing sequences to the consensus C sequence.

G→A substitution at different substrates

The resistance and therapy naïve datasets were then compared in terms of G→A substitution occurring at i) non-hA3G-preferred sites and ii) hA3G-preferred sites, independently which would indicate if the resistance dataset showed any evidence that G→A substitution was occurring differently at each type of substrate.

Statistical tests and analyses

Data were non-parametric and therefore the *.ranksum* test (Mann-Whitney two-sample test) was used in all cases in comparisons of datasets using Stata v11.2 (StataCorp).

Results

Few APOBEC3G-preferred substrates within codons sensitive to drug resistance

The majority of Nucleos(t)ide Reverse Transcriptase Inhibitor (NRTI), Non-nucleoside Reverse Transcriptase Inhibitor (NNRTI) and Protease Inhibitor (PI) drug resistance codons are not available to hypermutation in the hA3G-preferred (5'-GG) context when the consensus HIV-1 subtype C reference sequence was analyzed (Table 1). Eight out of 47 POL codons had the hA3G-preferred context and only two could lead to drug resistance if G→A substitution occurred (RT 184 and PR 73). Similarly, a low signal was found for non-hA3G-preferred contexts at drug resistance codons. Nineteen out of 47 drug resistance codons contained guanines which could be potential substrates for context independent APOBEC3G-driven G→A substitution. Only eight of these codons could confer resistance if a guanine was mutated to adenine: Protease (PR) 32, 46 and 71; Reverse Transcriptase (RT) 67, 75, 106, 108 and 118.

Differences between substitution types

As shown in Figure 1, the descending order of substitution frequencies by substitution type was generally consistent between resistant and therapy-naive sequences. Although the same substitution types were observed above 5% for each dataset (resistant: A→G>G→A>T→C>C→T>A→C, therapy naïve: G→A>A→G>T→C=C→T>A→C) it appeared that i) the resistance dataset had a lower frequency of G→A substitutions than the therapy naive dataset and ii) T→C was slightly higher than C→T in the resistance dataset. Statistical comparisons showed that the resistance dataset had a significantly different distribution of the number of substitutions per sequence for each substitution type, compared to the therapy naïve dataset (Figure 2, p=0.0000 for all comparisons).

No G→A hotspots identified in Polymerase (POL), and overall G→A substitution

G→A substitution was observed to affect more sites in the resistance dataset than the therapy-naïve dataset for both Protease (resistance dataset: 45.1%, therapy naïve dataset: 39.4%) and Reverse Transcriptase (resistance dataset: 59.0%, therapy naïve dataset: 47.5%; Figure 3). Over the entire sequenced length, the resistance dataset was affected by G→A substitution at 53.9% of sites while the therapy naïve dataset was affected by G→A substitution at fewer sites (44.6%). Considering only G→A substitution, Figure 4 shows that i) G→A substitution occurred more often per sequence in the resistance dataset and ii) the

resistance dataset had a broader range of the number of G→A substitutions per sequence when compared to the therapy naïve dataset. All statistical comparisons showed that there were more G→A substitutions occurring at hA3G-preferred sites compared to non-hA3G-preferred sites, than expected (all p=0.0000). These results provide evidence of APOBEC3G activity along the regions investigated.

G→A substitution at different substrates

Statistical comparisons at all non-hA3G-preferred substrates and at all hA3G-preferred substrates showed that there were no statistically significant differences in G→A substitution occurring between the resistance and therapy naïve datasets (p=0.1847 and p=0.5357 respectively). These results suggest that resistant isolates are not subject to significantly elevated G→A substitution at either guanine context when compared to therapy naïve isolates.

Discussion

Although Vif appears to be highly conserved, there can be situations where Vif does not block the function of APOBEC3G completely. Vif can be partially genetically deficient due to the presence of key mutations/polymorphisms which affect Vif function, whilst APOBEC3G can have mutations/polymorphisms which confer some level of resistance to Vif[29-31]. In these situations, APOBEC3G can still perform its editing function, albeit to a lesser degree, resulting in suboptimal and therefore sublethal editing of the HIV-1 genome. Sublethally mutated HIV-1 could still replicate, which may result in increased genetic diversity of the resulting viral progeny[32]. Increased viral genetic diversity due to suboptimal APOBEC3G hypermutation has been hypothesised to potentially impact the development of drug resistance in the absence of suboptimal drug pressure[33,34].

The objectives of this study were therefore based on the premise that if provirus had been hypermutated to sub-optimal levels, this would translate to a difference of drug resistance level. This study therefore makes a contribution to what is known about APOBEC3G-mediated G→A substitution, its effect on genetic diversity and its contribution to the development of drug resistance. This is a novel study as samples were used that originated from subjects infected with HIV-1 subtype C, which is the globally dominant HIV-1 subtype. Furthermore, we have compared automated bioinformatics tools with statistical analyses in order to understand the contribution of substrate and mutational pathway in the development of resistance.

All guanine nucleotides in all recognised drug resistance codons were investigated for their theoretical potential to become substrates for 5'-GG preferred APOBEC3G-driven hypermutation as well as for context-independent G→A substitution. This simple analysis has not yet been extensively reported in the literature[33]. Although 8/47 drug resistance positions contain the APOBEC3G-preferred GG context, only two would result in a resistance genotype after G→A substitution. At non-APOBEC3G-preferred drug resistance-related positions (19 out of 47 sites), nine sites could result in drug resistance if G→A occurred.

Broad level drug resistance can however develop from a single mutation and it remains important to consider these few positions. As a case in point, M184V, one of the

most commonly affected resistance-sensitive codons conferring high-level resistance to lamivudine and emtracitabine, has a 5'-GG dinucleotide sequence spread across two codons and can be a potential target for APOBEC3G-mediated hypermutation in the preferred dinucleotide context. Interestingly, M184V is the first NRTI mutation to appear in resistance virus, but it is not associated to any specific subtype[35]. The importance of analysing these applicable drug resistant codons is thus justified. However, determining the specific contribution of APOBEC3G to observed G→A substitutions (rather than being caused by natural variation, suboptimal drug concentrations or other selective pressures), proves extremely difficult for mutations that have a high fitness costs (such as M184V) and revert to wild type after drug pressure is stopped.

The effects of hypermutation on HIV-1 genetic diversity could be lowest, and thus most difficult to detect, in subtype C. HIV-1 subtype C Vif has been shown to have the highest activity against APOBEC3G due to increased binding activity[36]. Gupta and Banerjea (2009)[37] have also shown that an Indian HIV-1 subtype C Vif isolate had a much stronger antagonistic effect on APOBEC3G (degradation) when compared to a subtype B Vif. Interestingly, the subtype C Vif was at least 97% similar to consensus C Vif with the C-terminal domain being responsible for the degradation of 50 – 80% of APOBEC3G in the study while Vif from subtype B degraded less than half (20 – 30%) of that degraded by Vif C[37]. Lower levels of APOBEC3G-driven hypermutation of proviral DNA should thus naturally be found in subtype C sequences when compared to other subtypes due to these differences in Vif binding and APOBEC3G degradation.

Nucleotide substitutions differed significantly between the resistance and therapy naïve datasets indicating that different factors contributed to genetic diversity to different degrees. As A→G substitution is the reverse of G→A substitution, it was noteworthy that A→G substitution was found to occur at a high prevalence similar to that observed for G→A substitution. It has been reported that host adenosine deaminase may contribute to A→G mutations observed in viral sequences although the significance of these substitutions is still unclear[38,39]. Adenosine deaminase acts on RNA and promotes A→G changes in positive-strand HIV-1[38]. The contribution of this enzyme to the elevated levels of A→G substitution is unknown, but clearly warrants further investigation.

APOBEC3G-driven hypermutation of proviral HIV-1 has been found to occur at frequencies between 1.3 and 6.4 substitutions per 100 bp[40] in studies inspecting each viral isolate. In a study carried out by Sadler *et al* (2010)[32], APOBEC3G mutations were found to occur between one and 14 times in the resistance dataset versus between one and three times in therapy naïve sequences. However, these studies were performed using non-subtype C sequences and on pro-viral DNA.

Our study on plasma HIV-1 RNA found that there was significantly more G→A substitution at hA3G-preferred sites compared to other sites, suggesting that hA3G activity may be actively contributing to elevated genetic diversity. However, this result was shown for both datasets indicating that hA3G activity may be active during acute untreated infection and during therapy failure. This is turn allows the conclusion that although hA3G may be contributing to elevated genetic diversity, it is not preferentially occurring in resistant isolates. Analyses of G→A substitution frequency conducted independently for the hA3G-preferred context and other sites showed no statistical evidence that substitution at either of the contexts was occurring differently between resistant and therapy naïve isolates. This provides strong additional evidence that hA3G-activity as well as other G→A substitution activity does not preferentially occur in HIV-1 subtype C resistant viral isolates versus therapy naïve isolates. *In vitro* infectivity studies and full genome sequencing of individual quasispecies (including *Vif*) may be useful in future studies to obtain an even more accurate view of the contribution of suboptimal hA3G hypermutation to genetic variation and drug resistance in HIV-1, as well as to ensure that Vif is fully functional and not contributing to suboptimal hypermutation.

Table 1: Drug resistance codons within POL which contain substrates for G→A substitution.

Guanine context/ Drug Class	PI	NRTI	NNRTI	OVERALL
5'-GG A3G preferred	20, 48, 73	41*, 151, 184	190, 230	8/47
5'-GG: hA3G-preferred where G→A leads to resistance	73	184	None	2/47
Isolated G or 5'-GG	32, 46, 48, 71, 73, 82, 90	62, 65, 67*, 70*, 75, 118, 219*	98, 106(x2), 108, 179, 190	19/47
Isolated G or 5'-GG where G→A leads to resistance	32, 46, 71	67*, 75, 118	106, 108	8/47

*=Thymidine Analogue Mutations (TAMs) codons

Table 2: Oligonucleotides used for in-house amplification and sequencing.

RT / R1 or R2 / CS[*2]	**Primer Name and Orientation (F/R)** [*1]	**Primer Sequence (5'→3')** [26,41]	**Primer position against hxb2**
R1	G25Rev (F)	GCAAGAGTTTTGGCTGAAGCAATGAG	1867 – 1892
RT and R1	IN3 (R)	TCTATVCCATCTAAAAATAGTACTTTCCTGATTCC	4212- 4246
R2 and CS	AV150 (F)	GTGGAAAGGAAGGACACCAAATGAAAG	2036 – 2063
R2 and CS	PolM4 (R)	CTATTAGCTGCCCCATCTACATA	3892 – 3915
CS	SQ5F (F)	AAACAATGGCCATTAACAGAAGAGA	2613 – 2637
CS	SQ6F (F)	CTTTGGATGGGTTATGAACT	3231 – 3250
CS	SQ12R (R)	TGTATGTCATTGACAGTCC	3304 – 3322
CS	SQ13R(2)C(2) (R)	GTGTTATATGGATTTTCAGGCCC	2700 – 2722

[*1]Primers were synthesised by Integrated DNA Technologies (U.S.A.), [*2]RT; reverse transcription, R1; first round of nested PCR, R2; second round of nested PCR, CS; cycle sequencing

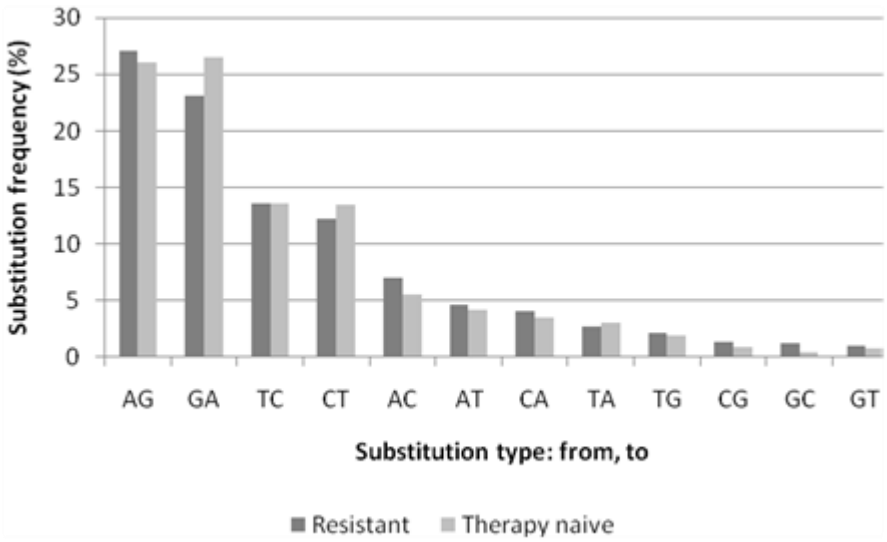


Figure 1: Substitution frequencies by substitution type and dataset.

Each sequence was compared to the HIV-1 Subtype C consensus sequence and substitution frequencies were determined as a function of the relative contribution of each substitution type to overall substitution, for each dataset independently. Over 25% of all substitutions in each dataset were A→G substitutions.

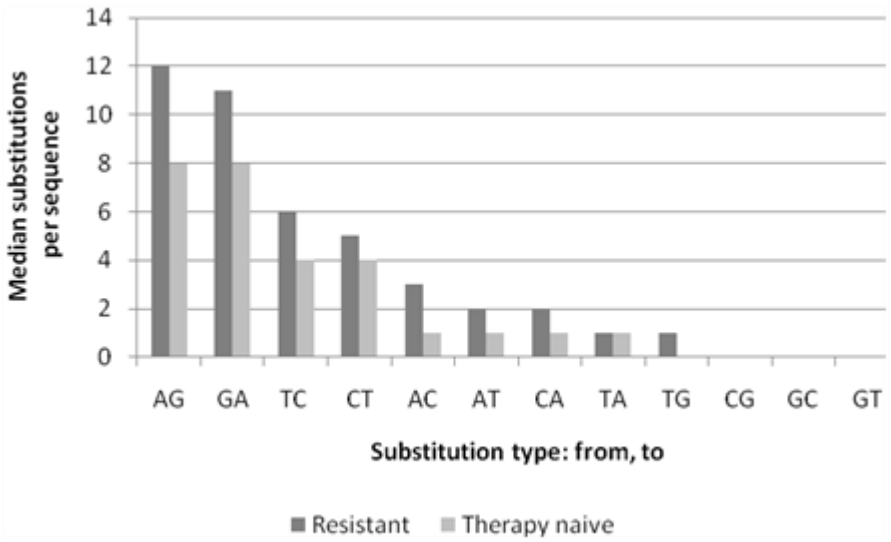
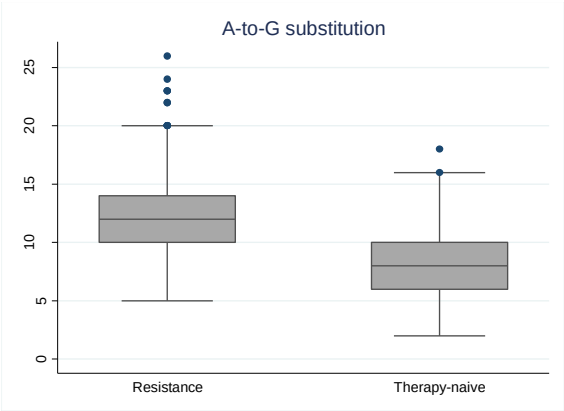
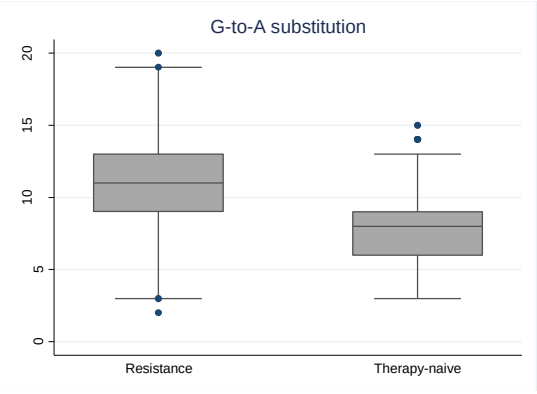
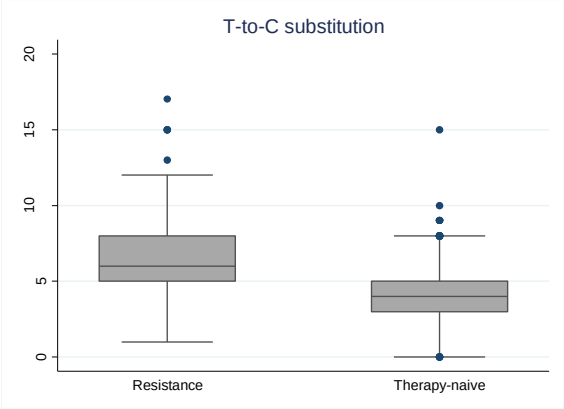


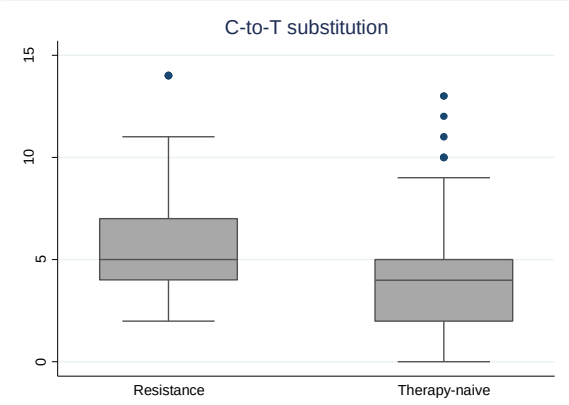
Figure 2: Median substitution frequencies per sequence by substitution type and dataset.

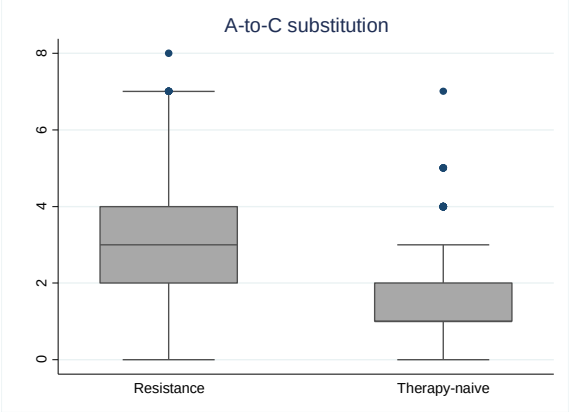
Each sequence was compared to the HIV-1 subtype C consensus sequence and the median number of each substitution type was calculated. The resistance dataset showed a higher median number of G→A and A→G substitutions versus the therapy-naïve dataset, while the therapy-naïve dataset showed equal medians for these two types of substitutions (above). All comparisons of substitutions per sequence type (reflected on the y-axes) between datasets were statistically significantly different which indicates that the distribution of substitutions per sequence (for each substitution type) was different depending on whether isolates were resistant or therapy-naïve. Each of the 12 statistical outcomes were significant (p=0.0000) (below).

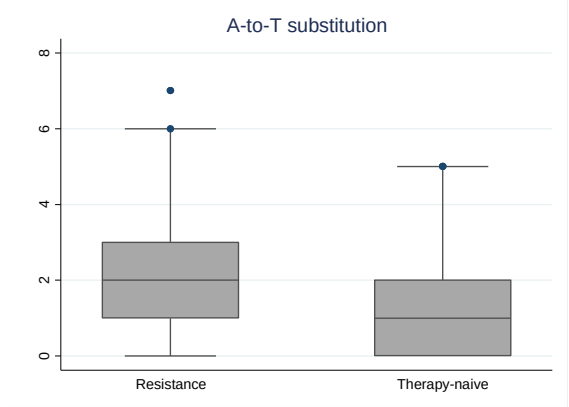


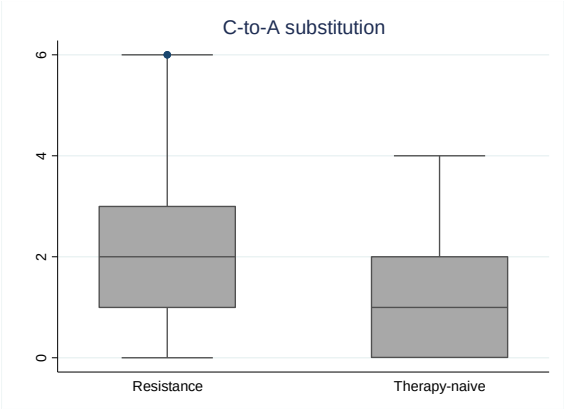


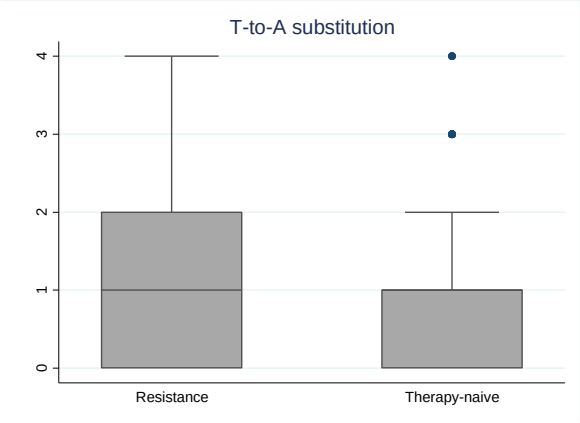


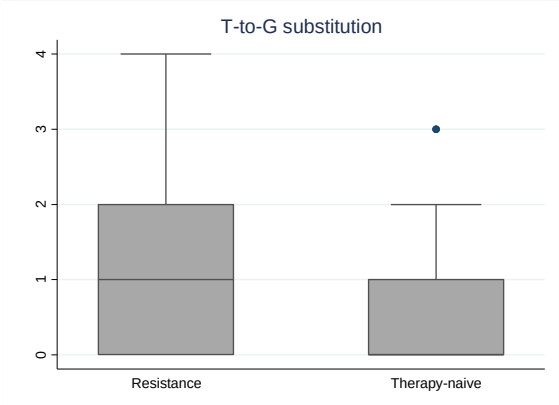


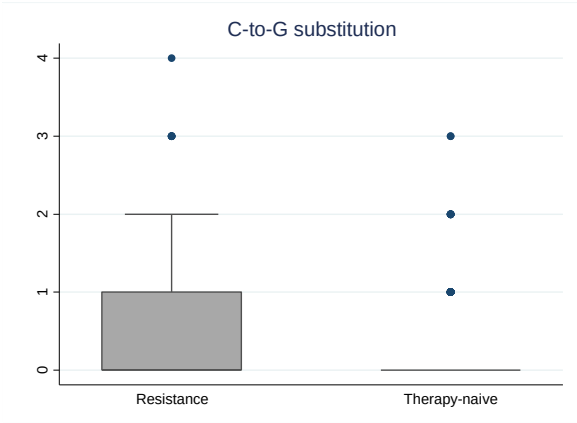


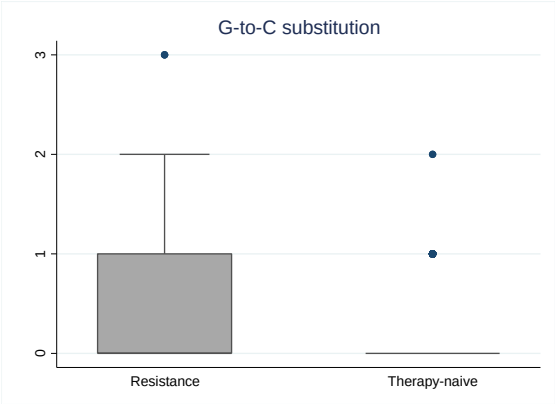


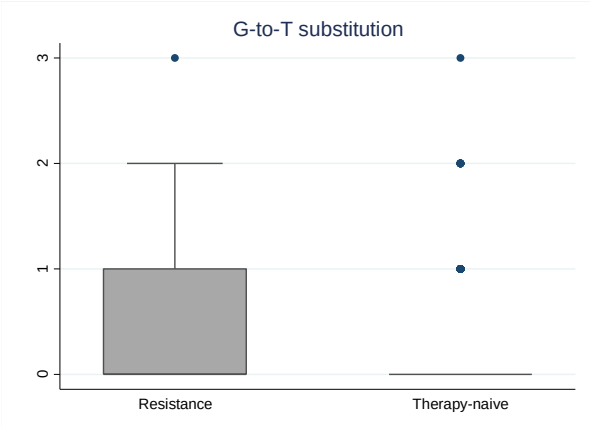












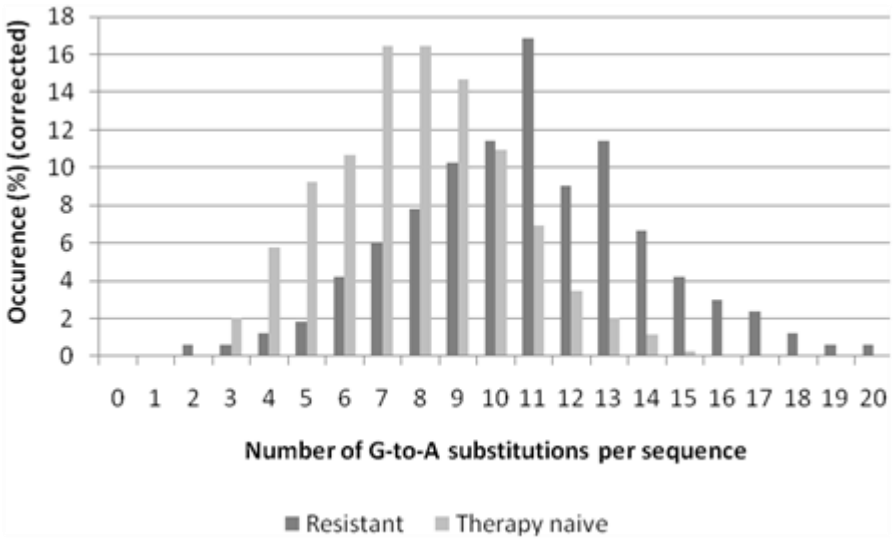
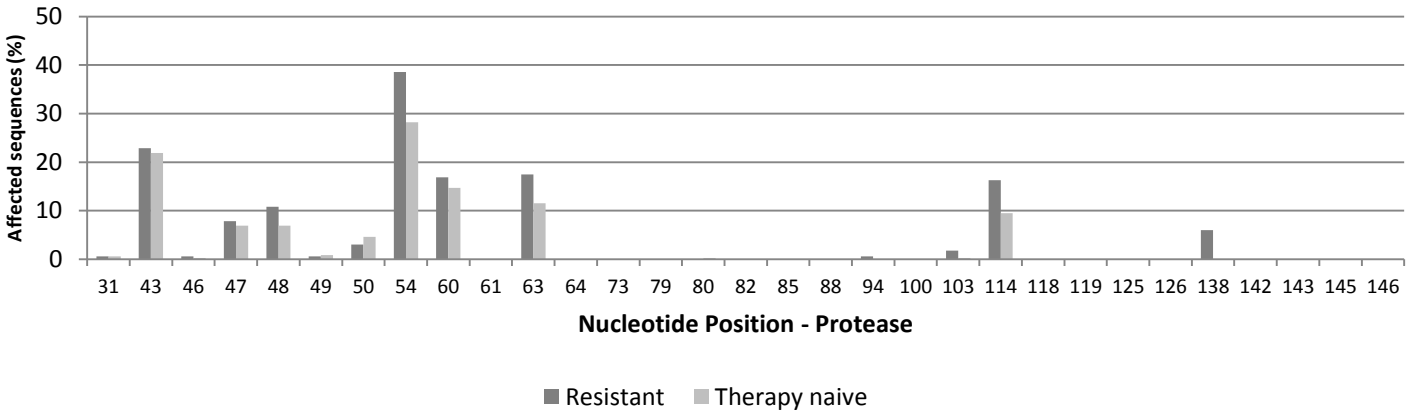
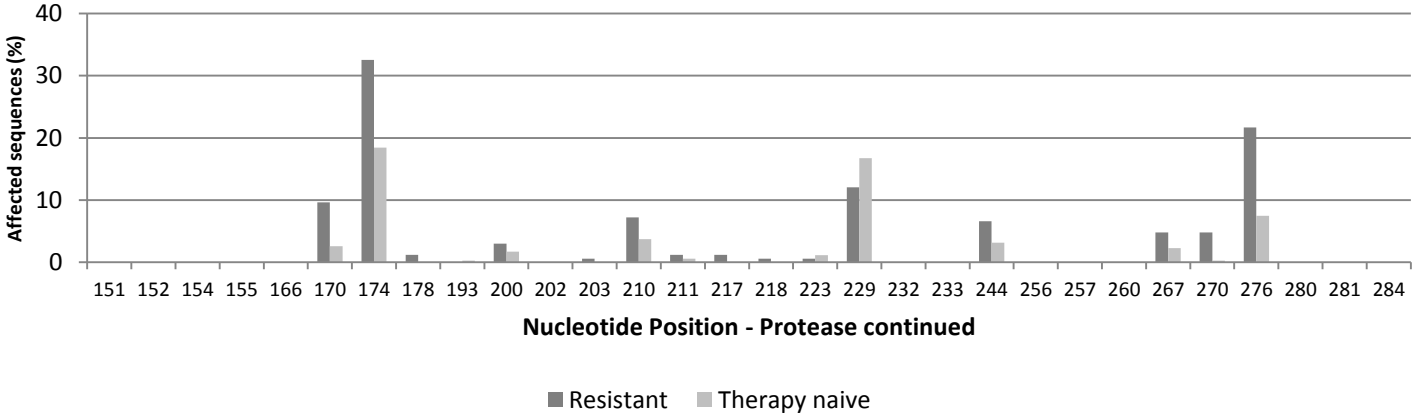
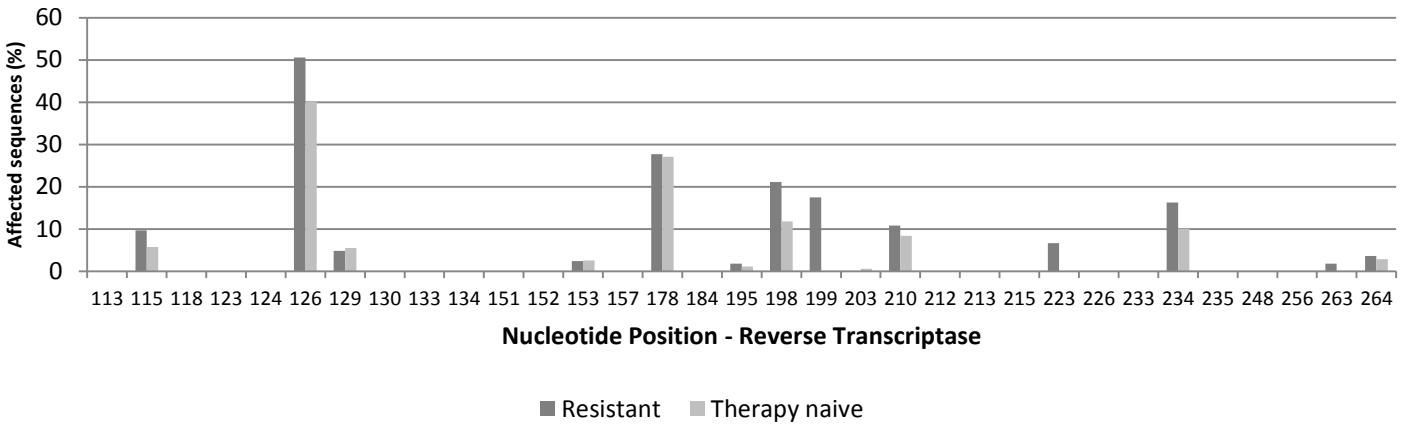


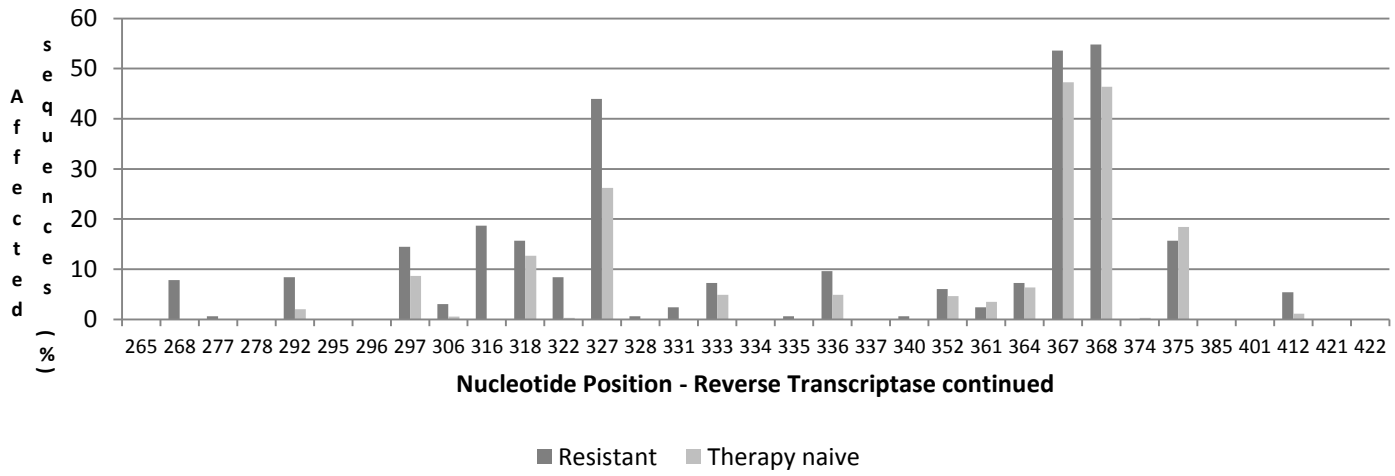
Figure 3: Frequency distribution of G→A substitutions for each dataset when compared to the HIV-1 subtype C consensus sequence.

This analysis shows an elevated frequency and overall higher prevalence of G→A substitution in resistant sequences. For example, over 16% of resistance sequences showed 11 G→A substitutions, while this was prevalent in less than 8% of therapy-naïve sequences.









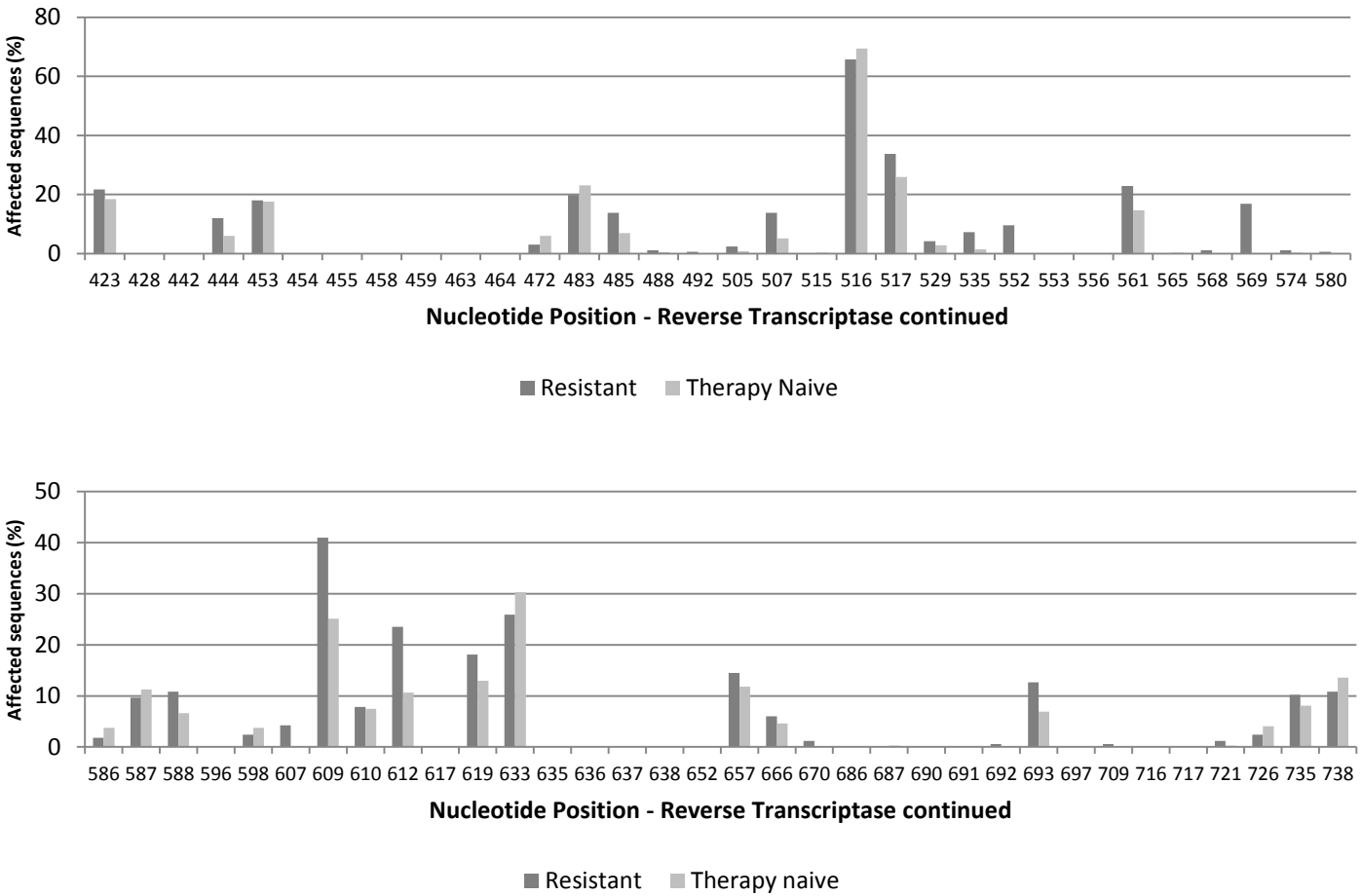


Figure 4: G→A substitution prevalence at each guanine position (n=193) along Protease and Reverse Transcriptase, by dataset.

For example, close to 40% of resistance sequences showed G→A substitution within PR54, while G→A substitution, although still being observed in therapy-naïve sequences, was observed in less than 30% of sequences. No hotspots were observed.

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Conclusions

This body of work investigated HIV-1 subtype C drug resistance profiles among South African subjects in order to contribute to the limited knowledge around HIV-1 subtype C infection, when compared to the knowledge surrounding HIV-1 subtype B. As an overall theme, the studies focused on elucidating aspects which may contribute to the development of drug resistance by investigating differences between urban and rural public treatment programmes, identifying changes in immunological activation profiles between virologically suppressed subjects and those failing treatment with evidence of drug resistance, and determining whether suboptimal APOBEC3G hypermutation is a risk factor for the development of HIV-associated drug resistance.

It was encouraging that although subjects attending rural treatment programmes appeared to have access to a lower treatment programme intensity - as evidenced by less frequent monitoring and longer duration of treatment failure - than those in urban treatment programmes, and subsequently had more severe drug resistance profiles, second line protease inhibitor-based highly active antiretroviral therapy could still be effective. With the large number of subjects on ART in South Africa, it is inevitable that care will become more decentralised and characterised by nurse-managed programs and less intensive monitoring. It is therefore important that the optimal balance between monitoring - which is expensive and resource-intensive - and prevention of the development of severe drug resistance, caused by prolonged, unmanaged treatment failure, is found. This study will contribute to the knowledge base that will inform such deliberations.

For these rural subjects, as well as those in urban treatment programmes, identifying therapy-failure as a result of the development of resistance mutations rather than complete non-adherence to therapy could be beneficial since it limits the time that subjects are on a failing regimen, and can thus reduce the level of resistance. Genotypic drug resistance testing is, however, prohibitively expensive and currently not available in the South African public sector. This study therefore set out to explore whether alternative blood measurements, such as select markers of immune activation, which can be detected by relatively simple and inexpensive ELISA testing, may serve as surrogate markers or early warning indicators for

the development of drug resistance. This study showed that a number of markers were associated with treatment failure and drug resistance, but, the ideal biomarker signature that can be used for this purpose, remains to be determined. This study is limited by the small sample size and sampling at a single time-point but, with further study, the biomarkers that were identified during this work may contribute to a cost-effective solution to determine resistance in subjects far from urban centres and laboratory facilities. Furthermore, a follow-up cohort study with more frequent monitoring of immunological biomarkers over a longer time period against viral load measurements could provide a more accurate depiction of the changes associated with therapy failure as well as virological suppression. In addition, this work highlights the possibility of identifying subjects who may potentially benefit from co-therapy aimed at reducing on-going immunological activation in virologically suppressed subjects.

Finally, the lack of an innate immunological contribution to the development of drug resistance was an important finding that has excluded suboptimal APOBEC3G hypermutation as a risk to the maintenance of virological suppression in these subjects. These data suggest that such hypermutation need not be considered in future treatment and counselling efforts.

As well as contributing to the general knowledge of HIV-1 subtype C infection, these studies could make significant contributions towards how different South African public sector HIV-1 treatment programmes are considered in the future, especially regarding frequency of blood monitoring and timing of genotypic drug resistance testing, the ideal manner of identifying HIV-associated drug resistance as well as subjects at risk of drug resistance, and finally, how subjects may be co-treated to reduce on-going immune activation during highly active antiretroviral therapy. Confirmation that suboptimal hypermutation by APOBEC3G is not a risk factor for the development of drug resistance in South African HIV-1 subtype C-infected subjects was also an important finding that allows efforts to mitigate the development of drug resistance to focus on the importance of adherence to highly active antiretroviral therapy and regular virological and clinical monitoring. Ultimately, it is expected that data from these studies may stimulate further research into ways in which to reduce morbidity and mortality in subjects infected with HIV-1 subtype C.

Availability of supporting data

The relevant nucleotide sequences for this project will be placed in the Genbank repository upon acceptance of the relevant manuscript.

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