THE EPIDEMIOLOGY AND IMPACT OF PRETREATMENT HIV DRUG RESISTANCE IN ADULTS IN SOUTH AFRICA

By

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Thesis submitted in fulfillment of the requirements for the Doctor of Philosophy Degree, in the School of Laboratory Medicine and Medical Sciences

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Dr. Kogieleum Naidoo
Dr. Reshmi Samuel

PREFACE

The study described in this thesis was carried out in the KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP) and the Department of Virology, National Health Laboratory Services (NHLS), in the School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, in Durban, South Africa. It was conducted between February 2016 and November 2018 under the supervision of Prof Tulio de Oliveira, Dr. Kogieleum Naidoo and Dr. Reshmi Samuel.

This thesis is original work done and reported by the author. It has not been used in any form by any person or submitted to any tertiary institution for award of a degree or diploma. Some of the work has already been published in peer-review journals in-line with the thesis guidelines of University of KwaZulu-Natal.

DECLARATION 1: PLAGIARISM

I **Benjamin Chimukangara** declare that:

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Signed:  Date: 24 April 2019

DECLARATION 2: PUBLICATIONS

The publications (published, in print and/or submitted) that constitute this thesis and the contribution I made to each of the manuscripts are presented here;

Publication 1

Chimukangara B, Samuel R, Naidoo K and de Oliveira T. Primary HIV-1 drug resistant minority variants. AIDS Rev. 2017; 19(2): 89-96.

Author contributions:

I conceptualized the review paper, did the literature search and wrote the paper. Co-authors critically reviewed the paper and approved the final version of the manuscript.

Publication 2

Chimukangara B, Kharsany ABM, Lessells RJ, Naidoo K, Rhee S-Y, Manasa J, Gräf T, Lewis L, Cawood C, Khanyile D, Diallo K, Ayalew KA, Shafer RW, Hunt G, Pillay D, Abdool Karim SS, and de Oliveira T. Moderate to high levels of pre-treatment HIV drug resistance in KwaZulu-Natal Province, South Africa. AIDS Res Hum Retro. 2019; 35(2):129-138.

Authors contributions:

I was involved in study conception, study design, data analysis, data interpretation, and manuscript preparation. Co-authors revised the manuscript for important intellectual content and approved the final version of the manuscript.

Publication 3

Chimukangara B, Lessells RJ, Rhee S-Y, Giandhari J, Kharsany ABM, Naidoo K, Samuel R, Hunt G, Vandormael A, Stray-Pedersen B, Gordon M, Makadzange T, Kiepiela P, Ramjee G, Ledwaba J, Kalimashe M, Morris L, Hunt G, Parikh UM, Mellors JW, Shafer RW, Katzenstein D, Moodley P, Gupta R, Pillay D, Abdool Karim SS and de Oliveira T, 2018. Trends in pretreatment HIV-1 drug resistance in antiretroviral therapy-naive adults in South Africa, 2000 – 2016: a pooled sequence analysis. EClinicalMedicine. 2019; 9: 26-34.

Authors contributions:

I was involved in study conception, study design, data curation, data analysis, data interpretation and manuscript preparation. Co-authors critically revised the manuscript for important intellectual content, assisted with data analysis and approved the final version of the manuscript.

Publication 4

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Authors contributions:

I was involved in study conception, study design, laboratory testing, data analysis, data interpretation and manuscript preparation. Co-authors critically revised the manuscript, assisted with data analysis and approved the final version of the manuscript.

Other related publications during the period of study

1. Naidoo K, Dookie N, Naidoo K, Yende-Zuma N, **Chimukangara B**, Bhushan A, Govender D, Gengiah S and Padayatchi N. Recurrent tuberculosis among HIV-coinfected patients: a case series from KwaZulu-Natal. Infect Drug Resist. 2018; 11:1413-1421.
2. **Chimukangara B**, Manasa J, Mitchell R, Nyabadza G, Katzenstein D and Masimirembwa C. Community Based Antiretroviral Treatment in Rural Zimbabwe. AIDS Res Hum Retro. 2017; 33(12): 1185-1191.
3. **Chimukangara B**, Varyani B, Shamu T, et al. HIV drug resistance testing among patients failing second line antiretroviral therapy. Comparison of in-house and commercial sequencing. J Virol Methods. 2017; 243: 151-157.
4. Makadzange A. T, Boyd K. F, **Chimukangara B**, Masimirembwa C, Katzenstein D, Ndhlovu C. E. A simple Phosphate Buffered Saline extraction method improves specificity of HIV viral load monitoring using dried blood spots. J Clin Microbiol. 2017; 55(7): 2172-2179.
5. Musingwini T.V, Zhou D.T, Mhandire D, Duri K, Gomo E, Oktedalen O, **Chimukangara B**, Shamu T, Shawarira-Bote S, Dandara C, Stray-Pedersen B (2017) Use of Proviral DNA to Investigate Virus Resistance Mutations in HIV-Infected Zimbabweans. Open Microbiol J. Bentham Open. 2017; 28(11): 45-52.

Conference Presentations

1. **Chimukangara B,** Lessells RJ, Rhee S-Y, Giandhari J, Naidoo K, Samuel R *et al.*, (2018). High levels of pre-treatment HIV drug resistance in South Africa, 2000 – 2016. 22nd International AIDS Conference, Amsterdam, the Netherlands, Abstract number: THPEC257
2. **Chimukangara B,** Lessells RJ, Kharsany A, Naidoo K, Rhee S-Y, Manasa J *et al.*, (2017). Trends in pre-treatment HIV drug resistance in South Africa, 2000 – 2015. XXVI International Workshop on HIV drug resistance and treatment strategies, Johannesburg, South Africa.
3. **Chimukangara B,** Naidoo K, Kharsany A, Rhee S-Y, Samuel R, Giandhari J, *et al.*, (2017). Increasing levels of pre-treatment HIV drug resistance in South Africa. University of KwaZulu Natal, School of Laboratory Medicine and Medical Sciences Annual Research Day, Durban, South Africa.

Signature: ..... Date: 24 April 2019

As the candidate's supervisor I agree to the submission of this dissertation.

Signature: ..... Date: 10-May 2019

Professor T. de Oliveira

Signature: ..... Date: 3/5/2019

Dr. Kogieleum Naidoo

Signature: ..... Date: 28 April 2019

Dr. Reshmi Samuel

DEDICATION

This thesis is dedicated to my parents Mr. F. Chimukangara and Mrs. R. Chimukangara, my loving wife, Selina Chimukangara, and my first born son, Benoni Chimukangara, for being a great inspiration in my life, and for your support, love and encouragement throughout my studies. May God continue to bless you.

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Praise to God for blessing me with the life, knowledge, guidance and health throughout the years of my studies. To God be all the glory.

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ABBREVIATIONS

3TC	Lamivudine
AHRI	Africa Health Research Institute
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
ARV	Antiretroviral
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CD4	Cluster of differentiation number 4
CRFs	Circulating recombinant forms
DNA	Deoxyribonucleic acid
DTG	Dolutegravir
ECRS	eThekwini Clinical Research site
EFV	Efavirenz
FDC	Fixed-dose combination
FTC	Emtricitabine
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIVDR	HIV drug resistance
HIV/TB	Human immunodeficiency virus and tuberculosis co-infection
DRMVs	Drug resistant minority variants
HIPSS	HIV Incidence Provincial Surveillance System
IC50	50% inhibitory concentration
INSTIs	Integrase strand transfer inhibitors
KRISP	KwaZulu-Natal Research Innovation and Sequencing Platform
KZN	KwaZulu-Natal province
LMICs	Low and middle-income countries
LPV/r	Ritonavir-boosted lopinavir
NGS	Next generation sequencing
NHLS	National Health Laboratory Services
NRTIs	Nucleoside reverse transcriptase inhibitors
NNRTIs	Non-nucleoside reverse transcriptase inhibitors

NVP	Nevirapine
PCR	Polymerase chain reaction
PDR	Pre-treatment drug resistance
PEP	Post-exposure prophylaxis
PIs	Protease inhibitors
PrEP	Pre-exposure prophylaxis
SIV	Simian immunodeficiency virus
STIs	Sexually transmitted infections
TB	Tuberculosis
TDF	Tenofovir
TDR	Transmitted drug resistance
TLD	Combination of tenofovir, lamivudine and dolutegravir
TEE	Combination of tenofovir, emtricitabine and efavirenz
TRAMs	TDF-resistance associated mutations
UTT	Universal test-and-treat
VL	Viral load
UKZN	University of KwaZulu-Natal
vRNA	Viral RNA
WC	Western Cape province
WHO	World Health Organization

ABSTRACT

HIV drug resistance (HIVDR) present prior to initiating or re-initiating antiretroviral therapy (ART), is known as pretreatment drug resistance (PDR). Conventionally, PDR is detected by Sanger sequencing. Drug resistant minority variants (DRMVs) that are not reliably detected by Sanger sequencing can be detected by next generation sequencing. The aims of this research were to assess levels of PDR in HIV hyper-endemic areas (with high HIV incidence and prevalence) in KwaZulu-Natal (KZN) province, trends of PDR in South Africa, and the impact of DRMVs on ART.

To assess PDR in adults from KZN hyper-endemic areas, 1845 sequences were analyzed from two population-based HIV surveillance studies; a longitudinal HIV surveillance programme in northern KZN (2013-2014), and the HIV Incidence Provincial Surveillance System (HIPSS) in central KZN (2014-2015). Overall, 182/1845 (10.0%) had NNRTI-PDR mutations, and when analyzed by study year, NNRTI-PDR was 10.2% (CI:7.5-12.9) for the HIPSS study in 2014. To assess PDR trends in South Africa, 6880 HIV-1 sequences were collated from 38 datasets of ART-naïve adults (2000-2016). Increasing levels of PDR were observed, most marked from 2010. Crude pooled prevalence of NNRTI-PDR reached 10% in 2014, with a 1.18-fold (CI:1.13-1.23) annual increase ($p<0.001$), consistent with findings from the HIPSS data. This provided the first evidence of high-level NNRTI-PDR in KZN and South Africa, supporting the transition to dolutegravir in standard first-line ART, as recommended by the World Health Organization when NNRTI-PDR reaches ≥10%.

A case-control (2:1) study in HIV/TB co-infected adult patients was done to assess the impact of DRMVs at different thresholds. Cases were patients that initiated ART and had viral loads ≥1000 copies/mL after ≥6 months on ART, and controls were those that initiated ART and achieved virologic suppression through 24 months. Pre-ART NNRTI-resistance was associated with ART failure. NGS improved detection of HIVDR at lower thresholds, but reduced the specificity of identifying patients at risk of virologic failure, with the specificity reducing from 97% (CI:92-99) at 20% threshold, to 79% (CI:71-86) at 2% threshold. In all, the findings presented in this thesis provide a broad message about the need to improve quality in HIV prevention and treatment services.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV). HIV is thought to have originated between 1910 and 1930 as a zoonotic transmission through multiple infections from non-human primates infected with Simian Immunodeficiency Virus (SIV) [1,2]. The theory is generally supported by the very close resemblance seen between certain strains of SIVs and the two types of HIV, i.e. HIV type 1 (HIV-1) and type 2 (HIV-2). For example, HIV-1 is highly similar to SIV in chimpanzees (SIV*cpz*) and HIV-2 is similar to SIV in sooty mangabeys (SIV*sm*) [1]. HIV-1 is responsible for most HIV infections globally and has four major groups, i.e. groups M, N, O and P [3]. Of the four groups, group M is the most common accounting for 95% of the pandemic [3], and has nine subtypes; A, B, C, D, F, G, H, J and K, and several circulating recombinant forms (CRFs), unique recombinant forms (URFs) (Figure 1) [4,5], as well as sub-subtypes within some of the subtypes. Most infections are due to the subtype C, which is responsible for approximately 50% of all HIV infections [6].

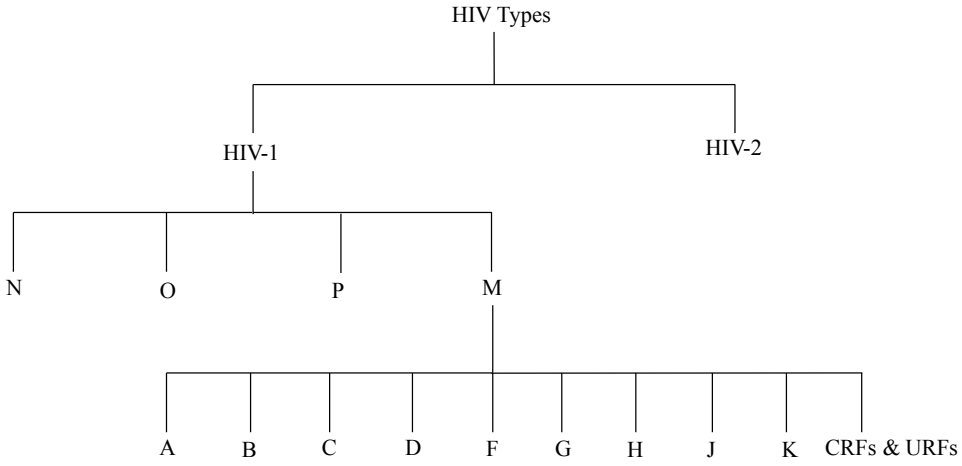

Figure 1 Classification of HIV

Since the beginning of the HIV epidemic, about 77.3 million (uncertainty bounds 59.9 million–100 million) people have been infected by HIV and 35.4 million (25.0 million–49.9 million) have died from AIDS-related illnesses [7]. Approximately 36.9 million (31.1 million–43.9 million)

people were living with HIV in 2017. As the HIV epidemic continues to grow, there have been more new HIV infections than deaths, with about 1.8 million (1.4 million–2.4 million) new infections, and 940 000 (670,000–1.3 million) HIV-related deaths, in 2017 [7]. Despite HIV becoming a global pandemic, East and Southern Africa are the most affected regions. There were approximately 800,000 (650,000–1.0 million) new HIV infections in East and Southern Africa alone in 2017, bringing the number of people living with HIV in the region to approximately 19.6 million (17.5 million–22.0 million) [7], which is about 53% of the global total of people living with HIV. Figure 2 shows the extent of the HIV burden in the African continent in comparison to other continents.

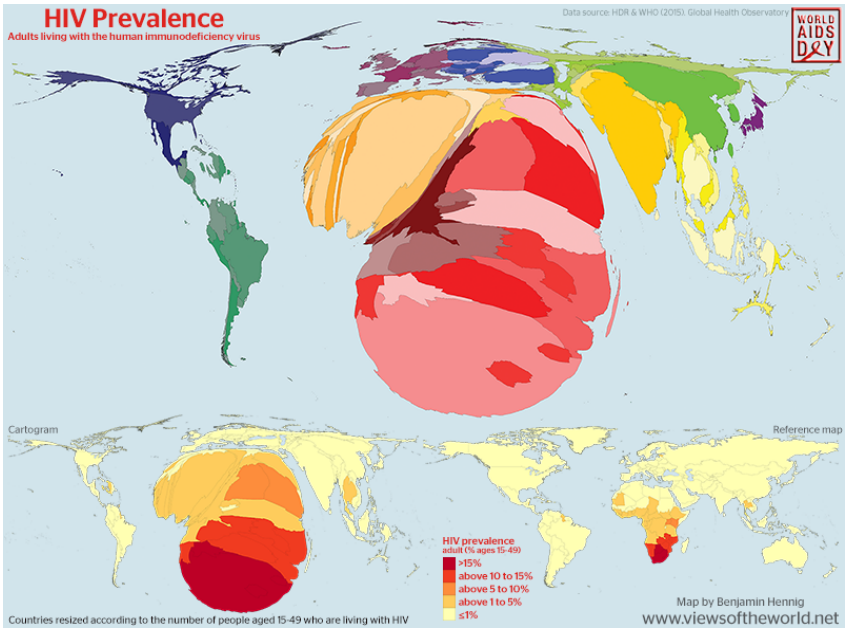

Figure 2 Global distribution of HIV in adults aged between 15 and 49 years with continent sizes relative to HIV burden
(Reproduced as is from Henning B, 2016: http://www.viewsoftheworld.net/wp-content/uploads/2016/12/HIVprevalenceMap.png)

Approximately one in every three new HIV infections in the East and Southern African region in 2017, are from South Africa [8]. The country had approximately 270,000 (240,000–300,000) new HIV infections in 2017 alone, with an estimated 7.2 million (6.6 million – 7.9 million) people living with HIV, and about 110,000 (93,000–140,000) HIV-related deaths [8]. This goes to show

the extent to which the HIV epidemic has affected South Africa, which now has amongst the highest adult HIV prevalence in the world [9] and the largest antiretroviral therapy (ART) programme globally [10]. Of approximately 7.2 million people living with HIV in South Africa by 2017, 90% (82%–>95) knew their HIV status, 61% (56–66) were on ART and 47% (43–52) were virally suppressed [8]. The country is not on track to achieve the 90-90-90 targets in the next couple of years, which means 90% of all people living with HIV knowing their HIV status, 90% of people with diagnosed HIV receiving sustained ART, and 90% of those on ART achieving virologic suppression by 2020 [11–13]. There is need for intensified efforts if South Africa is to achieve the targets to end the HIV epidemic by 2030. KwaZulu-Natal (KZN) is the province with the highest HIV prevalence in South Africa. Despite continued efforts in HIV prevention, treatment, and care, KZN remains the epicenter of the HIV epidemic in South Africa, with a prevalence as high as 27% (23.9-30.4) among adults aged 15 to 49 years, in 2017 [14]. Figure 3 shows a map of South Africa, the overall HIV prevalence distribution, and ART coverage across the provinces [15], by 2015.

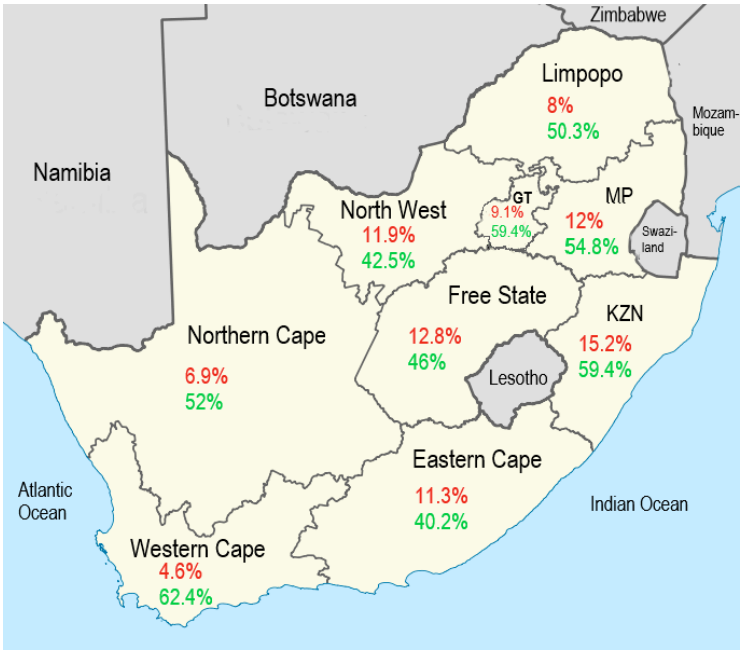

Figure 3 Map of South Africa showing provincial HIV prevalence (in red text) and ART coverage (in green text), in 2015 (Reproduced with modifications from Htonl; https://commons.wikimedia.org/wiki/File:Map_of_South_Africa_with_English_labels.svg)

The introduction of HIV antiretroviral (ARV) drugs has been a major breakthrough in the prevention of new HIV infections [16,17] and in treatment of HIV [18], with the South African national prevalence and incidence survey suggesting reduced incidence rates between 2012 and 2017, though they remained high among women aged 15 to 24 years [14]. Combinations of ARV drugs are used to form treatment regimens, and are referred to as highly active antiretroviral treatment (HAART), or simply as ART [19]. The World Health Organization (WHO) recommends life-long ART in all HIV infected people, from time of diagnosis [20]. This is supported by findings from two large randomized controlled clinical trials, i.e. the Strategic Timing of Antiretroviral Treatment (START) study [21] and the Early Antiretroviral Treatment and/or Early Isoniazid Prophylaxis Against Tuberculosis in HIV-infected Adults (TEMPRANO) study [22]. Both studies showed significant benefits to early treatment initiation compared to delayed ART. South Africa adopted this universal test-and-treat (UTT) approach in 2016 [23–25], with all adults that test positive for HIV being initiated on standard first-line ART, which includes two nucleoside reverse transcriptase inhibitors (NRTIs) and a non-nucleoside reverse transcriptase inhibitor (NNRTI), with protease inhibitors (PIs) and integrase strand transfer inhibitors (INSTIs) reserved for second-line and third-line ART[26].

Despite successful implementation of the UTT strategy in the South African national ART program using standard ART regimens, and the consistent recommendation for treatment monitoring by viral load testing since the introduction of ART, the country still has high numbers of new HIV infections, coupled with concerns of increasing levels of drug resistant virus in ART naïve individuals. This underscores the dire need to strengthen the monitoring arm of the ARV rollout, with timely viral load testing and follow up on results, ensuring that patients are not left on failing ARV treatment. Accumulation of drug resistant mutations occurs in patients left on a failing regimen and increases the risk of transmission of drug resistant virus to ART naïve individuals. This translates into major public health and cost implications where patients with PDR are likely to experience treatment failure on standard regimens. Considering these challenges, the research described in this thesis aimed at assessing the levels of HIV drug resistance (HIVDR) in ART naïve individuals in KZN (the province with the greatest HIV burden) and South Africa in general, as well as exploring whether standard genotypic resistance testing underestimates pretreatment resistance and, if so, what is the impact of low frequency drug resistance mutations on clinical outcomes.

1.2 Literature Review

1.2.1 HIV transmission and ART

There are four major ways by which HIV is transmitted, that is, through sexual intercourse (i.e. horizontal transmission), transmission from mother-to-child (i.e. vertical transmission), through HIV contaminated needles and or syringes, and through transfusion with HIV-infected blood, blood products or organ transplant [27–29]. However, the chances of HIV transmission are greatly reduced when an HIV infected person is on suppressive ART, meaning that their viral load is at undetectable levels, as shown in the HIV Prevention Trials Network 052 (HPTN 052) [16] and the PARTNER study (Partners of People on ART - A New Evaluation of the Risks) [17]. In the HPTN 052 trial, there was a 96% reduction in HIV transmission events among 886 sero-discordant couples that initiated ART early (at CD4 counts between 350-550 cells/mL) compared to those (n=877) that deferred therapy until CD4 counts were at <250 cells/mm^3 (Figure 4). This has resulted in the use of ART as prevention, and prompted the term "U = U", for undetectable equals untransmittable [30].

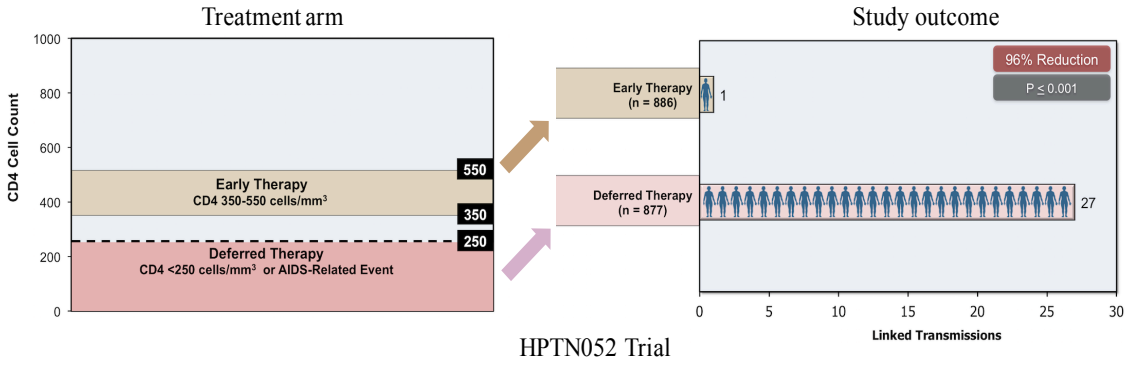

Figure 4 Summary of HPTN052 trial for use of ART as prevention of HIV transmission (Reproduced with modifications from Kinney RG, Spach, DH (2017, August 25). Preventing HIV Transmission in Persons with HIV. National HIV Curriculum. (University of Washington). Retrieved November 2018, from https://www.hiv.uw.edu/go/prevention/prevention-positives/core-concept/all#antiretroviral-treatment-as-prevention)

1.2.2 Antiretroviral therapy and South Africa

ART was rolled-out in the public health sector in South Africa, in 2004 [31]. Since then, ART initiation guidelines have changed gradually from initiating patients with CD4 counts ≤200 cells/µl or WHO stage IV [32], to the current UTT approach [23–25]. The preferred first-line regimen for late adolescents (≥15 years) and adults is a combination of tenofovir (TDF) with emtricitabine (FTC) (or lamivudine (3TC)) and efavirenz (EFV) [33]. The South African HIV treatment guidelines recommend that patients on first-line ART have viral load (VL) testing done at ART initiation, at 6 and 12 months, and thereafter annually, if the VL remains at <1000 copies/mL [26]. In the case of insufficient viral suppression (i.e. VL >1000 copies/mL), intensive adherence counselling with repeat VL testing after 2 months is recommended. If the VL remains unsuppressed after intensive adherence counselling, the patient is considered as failing treatment and a switch to second-line ART is recommended [26]. The preferred second-line regimen for late adolescents and adults failing a TDF-based regimen is a combination of zidovudine, 3TC, and ritonavir-boosted lopinavir (LPV/r) [33]. As with first-line ART, patients with persistent viremia while receiving second-line ART for at least 6 months, are considered to be failing treatment, and only then is genotypic drug resistance testing done to select drugs for third-line treatment [33]. Currently, third-line ART is only administered with expert advice, is managed centrally by the National Department of Health third-line committee, and is based on the patient resistance profile and ART history [33].

Besides use of ARV drugs in life-long ART, they are also used to prevent infection in key populations, such as those at substantial or high risk of HIV exposure [20]. The WHO defines key populations as people who face social or legal challenges that make them vulnerable to HIV, such as adolescent girls and young women, prisoners, men who have sex with men, injection drug users, transgender people and sex workers [34]. Substantial risk means living in a population where HIV incidence is high, defined as higher than 3 per 100 person-years [20]. In such key populations ARV drugs are used for pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP). PrEP is when ARV drugs are used before HIV exposure to lower chances of HIV infection, whilst PEP is when ARV drugs are used to prevent infection after potential HIV exposure, such as in cases of rape or percutaneous needle-stick injuries in healthcare workers [20]. The optimal use of PrEP, PEP and ART is imperative for the continued efficacy of a standardized regimen approach in South Africa. However, despite the significant benefits of ART

(as shown in the HPTN052, the START and TEMPERANO studies), its success is greatly threatened by development of HIVDR.

1.2.3 Pre-treatment drug resistance

HIVDR is a phenomenon where the virus mutates such that ARV drugs can not optimally inhibit the virus from replicating. Acquired drug resistance is the most common type of resistance, which occurs when the virus continues to replicate in the presence of ARV drugs. This occurs with sub-optimal drug levels, insufficient to completely suppress viral replication, but high enough to exert a positive selection pressure on the virus [35]. Low drug concentrations can be caused by factors such as poor treatment adherence, sub-optimal dosage and by genetic factors associated with drug metabolism, i.e. the cytochrome P450 genes [36,37]. HIVDR can also be a result of transmission of a resistant strain at primary infection, known as transmitted drug resistance (TDR), or could result from ART interruption and intermittent use of ART for PrEP, and PEP [38–40]. Such HIVDR in individuals that have not yet initiated ART, or that have prior use of ART and are re-initiating first-line treatment is now commonly termed pre-treatment drug resistance (PDR) [41,42].

HIVDR in ART naïve patients can develop spontaneously by de novo mutagenesis. This is because the reverse transcriptase enzyme that converts the viral ribonucleic acid (vRNA) to deoxyribonucleic acid (DNA) during viral replication is error prone and lacks proof-reading activity [43,44]. Therefore, several viral variants are generated each day, some of which have drug resistant mutations. This creates a viral pool known as “quasispecies” that has drug sensitive and resistant virus. The drug sensitive virus (also known as wild-type virus) has greater replicative capacity compared to drug resistant virus [45,46]. However, once treatment is initiated, drug resistant virus has the ability to outcompete the wild-type virus due to drug selective pressure, with the ability to revert back to wild-type virus once treatment is stopped [35]. Figure 5 shows selection of drug resistant virus under drug pressure, and re-emergence of wild-type virus after stopping ART.

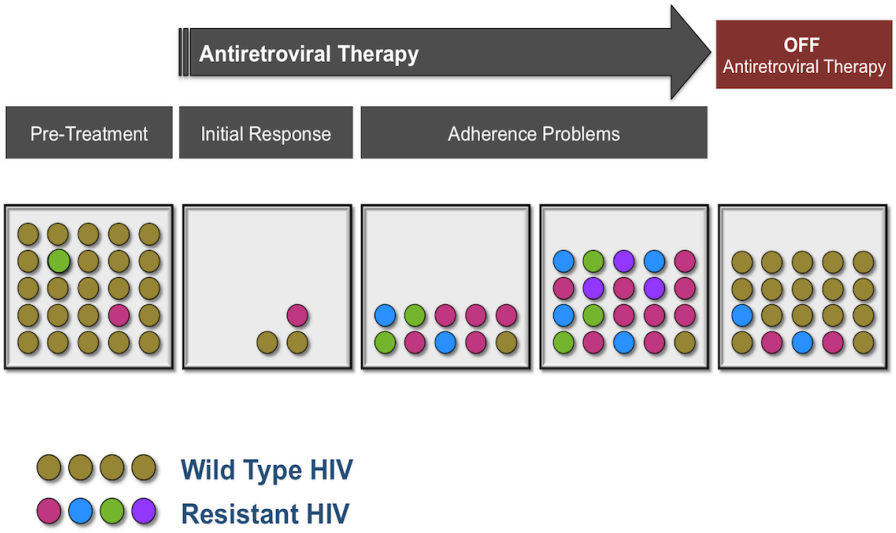

Figure 5 Selection of drug resistant virus under drug pressure, with re-emergence of wild-type virus after stopping ART

(Reproduced as is from Spach, DH, Kinney RG, (2018, November 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved November 2018, from https://www.hiv.uw.edu/go/antiretroviral-therapy/evalation-management-virologic-failure/core-concept/all#hiv-drug-resistance-assays)

Therefore, drug resistance mutations in individuals that have not yet initiated ART can result in inadequate viral suppression following ART initiation [47,48]. This is of great concern especially for drugs that have a low genetic barrier to resistance, meaning only one mutation can cause high-level resistance to a drug, as is seen with NNRTIs [49]. Over the years, drug resistance surveys focused on assessing TDR in selected treatment naïve individuals, since it can be challenging to identify recently infected people. The WHO in the past recommended drug resistance threshold surveys for TDR among people who are newly diagnosed and are likely to be recently infected, such as people <25 years at HIV diagnosis, primigravid women, or people with known recent infection, who have no known exposure to ART [50,51]. With this strategy, there is a higher chance of sampling a high proportion of recently infected individuals among the newly diagnosed in countries scaling up ART.

However, the WHO now recommends monitoring the prevalence of PDR to NNRTIs, and urgently switching to first-line regimens that do not contain NNRTIs, when NNRTI-PDR has reached ≥10%. This is due to evidence showing increasing levels of NNRTI PDR, that are associated with virologic failure on first-line ART [52]. Alternatively, drug resistance testing should be implemented before ART initiation [41,52,53], the latter being limited by costs and access to the specialized test in most low and middle-income countries (LMICs) such as South Africa. Evidence from South Africa suggested relatively low-levels of PDR in the first decade of ART roll-out [54]. However, following the extensive scale-up of ART in 2010, increasing levels of PDR are expected, considering the increased strain on health care systems, which has resulted in lack of timely viral load monitoring, ART switching, and retention of patients in care [55,56]. Vigilant surveillance of PDR and of the evolution of HIV virulence is required, as more people are exposed ART [57].

1.2.4 HIV drug resistance testing

There are two main types of HIVDR testing; phenotypic testing and genotypic testing. Phenotypic testing is when the virus is grown in a medium with increasing strengths of ARV drugs [49]. The replication of the virus is then monitored at different drug concentrations and compared to its replication capacity in the absence of the drug. The drug concentration required to inhibit viral replication by 50% (IC50) is calculated and compared to the IC50 of a reference virus as a ratio [49,58], and is reported as fold-resistance. An IC50 at the right of the reference virus means drug resistance and to the left means hyper-susceptibility of the virus to the drug (Figure 6).

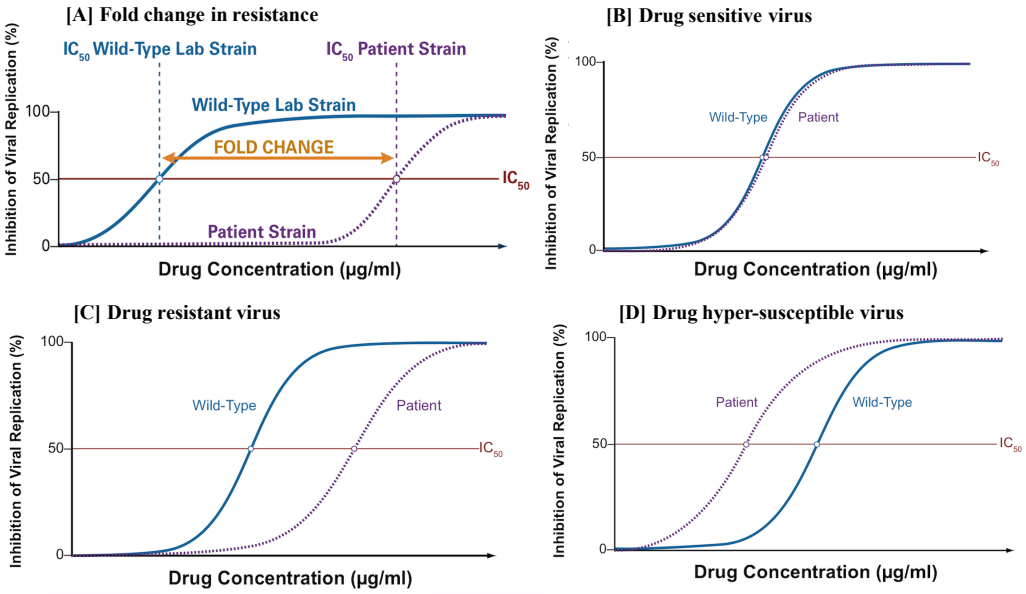

Figure 6 Graphs showing phenotypic drug susceptibility curves

Graph A, shows a measure of fold resistance between wild-type/ reference virus and the patient viral strain; Graph B, shows a plot with the patient IC50 similar to the wild-type virus for drug sensitive virus; Graph C, shows a plot with the patient IC50 curve on the right for a drug resistant virus; Graph D, shows a plot with patient IC50 curve on the left for a virus that is hyper-susceptible to a drug.

(Reproduced with modifications from Spach, DH, Kinney RG, (2018, November 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved November 2018, from https://www.hiv.uw.edu/go/antiretroviral-therapy/evalation-management-virologic-failure/core-concept/all#hiv-drug-resistance-assays)

Genotypic drug resistance testing is often done using Sanger sequencing, to detect viral variants in relevant viral genes, such as the *protease* and *reverse transcriptase* genes. In summary, conventional genotypic testing involves vRNA extraction, reverse transcription to DNA, polymerase chain reaction (PCR) amplification and detection, PCR product purification, sequencing reaction, sequence reaction purification, capillary electrophoresis, sequence editing and mutation detection. A single nucleotide change can result in an amino acid change, which causes drug resistance (Figure 7). Databases such as the Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu) are used to determine these mutations, and provide an estimate of

susceptibility as a genotypic score for each drug, based on synthesis of published data [59]. This is also guided by the the International AIDS Society mutation list, which is an annual updated list of all known HIVDR mutations that affect particular ARV drugs [60].

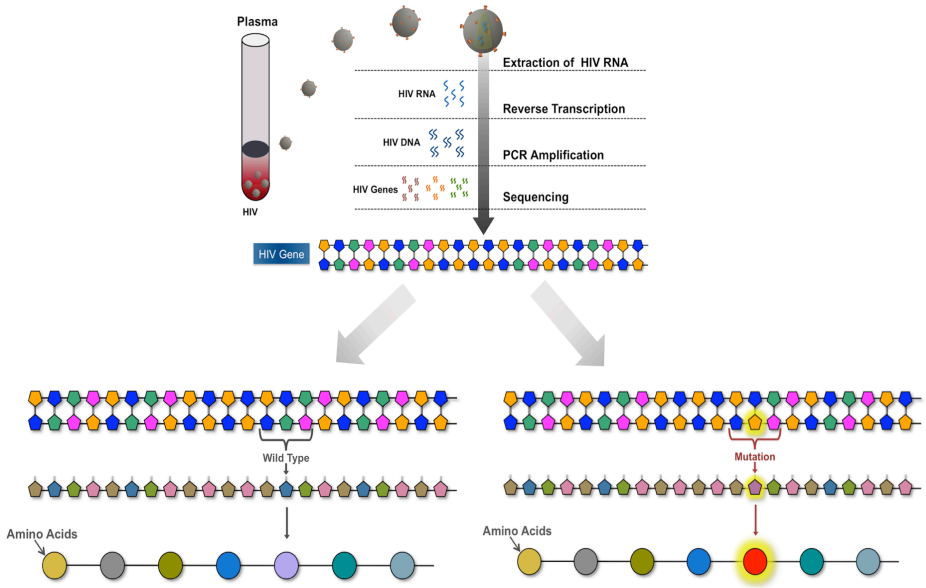

Figure 7 Summary of genotypic HIV drug resistance testing
(Reproduced with modifications from Spach, DH, Kinney RG, (2018, November 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved November 2018, from https://www.hiv.uw.edu/go/antiretroviral-therapy/evalation-management-virologic-failure/core-concept/all#hiv-drug-resistance-assays)

Genotypic drug resistance testing by Sanger sequencing can detect mixtures of wild-type and resistant HIV, and it is relatively cheaper, and has faster turnaround times compared to phenotypic testing [61,62]. However, the Sanger sequencing (conventional sequencing) technique relies on sequencing the dominant viral quasispecies. Thus, Sanger sequencing detects viral variants that a well represented in the viral quasispecies and does not reliably detect minor viral variants that occur below 20% [49], i.e. variants that are not well represented in the viral pool, as shown in Figure 8.

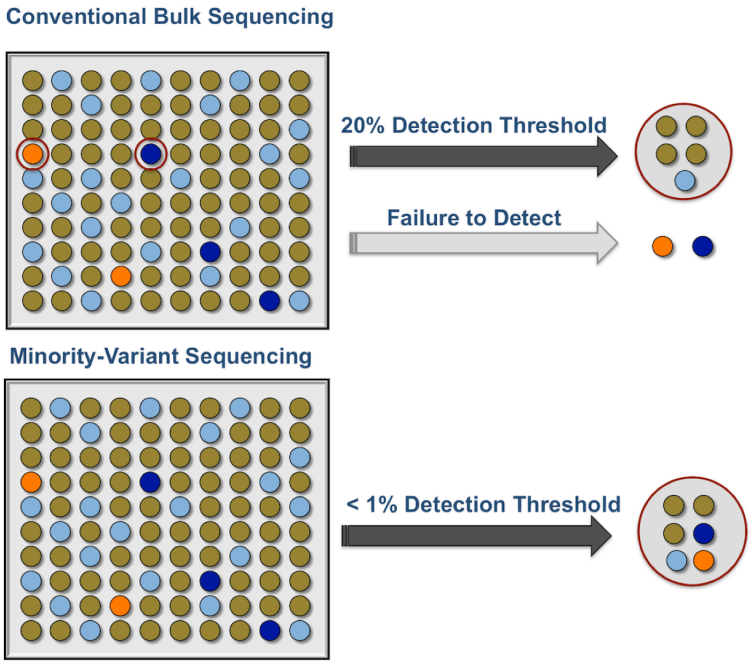

Figure 8 Selective detection of well represented viral strains by conventional sequencing (Reproduced with modifications from Spach, DH, Kinney RG, (2018, November 21). Evaluation and Management of Virologic Failure. National HIV Curriculum. (University of Washington). Retrieved November 2018, from https://www.hiv.uw.edu/go/antiretroviral-therapy/evalation-management-virologic-failure/core-concept/all#hiv-drug-resistance-assays)

1.2.5 HIV drug resistant minority variants

HIVDR mutations occurring at low frequencies (<20%) are called drug resistant minority variants (DRMVs) [63]. Although these DRMVs can not be reliably detected by Sanger sequencing, more sensitive point mutation technologies such as allele-specific PCR [62], oligonucleotide ligation assay [64], and Pan Degenerate Amplification and Adaptation [65] have the ability to detect these viral variants, even at 1% frequencies [62] (Figure 8). However, point-mutation assays are limited in the number of mutations they can detect concurrently [62]. Despite this, they have the potential to become point of care testing assays, as they can be used to detect signature mutations that are known to impact specific drugs used in ART regimens [66]. For instance, detection of three mutations; the K65R mutation that causes high-level resistance to TDF, the M184V mutation that

causes high level resistance to 3TC and increased susceptibility to TDF, and the K103N mutation that causes high-level resistance to nevirapine (NVP) and EFV [59], can help to assess the effectiveness of a typical first-line regimen that contains TDF + 3TC (or FTC) + EFV [26]. Therefore, point mutation assays could be relevant for use in LMICs where costs of drug resistance testing are a limiting factor [67,68], and at a time when point of care testing has become imperative [69].

With advancements in technology, ultra-deep sequencing, also known as next generation sequencing (NGS) is becoming more popular and is slowly replacing Sanger sequencing [63]. NGS is highly sensitive and can sequence the whole viral genome in a single run, but the quantitative and qualitative reliability of the sequencing reads is directly proportional to the initial plasma viral load copies [70], as well as the sequencing chemistry used [63]. The cost of acquiring the necessary infrastructure, equipment and expertise for NGS testing remains a major limiting factor for the extensive use of NGS in LMICs. Also, the amount of data generated from NGS is huge and creates challenges in storage and access, with data analysis pipelines not well defined for diagnostic testing [63]. Despite the ability to detect and quantify DRMVs, the clinical relevance of the low frequency mutations in guiding the use of ART is not well understood. For instance, the effect of a mutation at a certain frequency may vary based on the drug class barrier to resistance, and it might be more relevant to quantify the viral load of drug resistant virus (i.e. the mutational load) rather than the entire viral pool, as there is evidence suggesting a dose-dependent effect of mutational load on virologic outcomes [71]. The mutational load can be estimated by multiplying the frequency of the DRMV by viral load copies/mL [70]. Therefore, there remains a lot to be understood on the significance of DRMVs, which could be critical in predicting treatment response and in determining how to incorporate fast developing NGS technologies into current HIV treatment and monitoring practices. Details of the different NGS platforms, chemistries, implementation and knowledge gaps have been presented as a manuscript in Chapter 2 of this thesis.

1.3 Problem Statement

South Africa has had access to ARV drugs and routine viral load monitoring since public roll-out of ART. However, due to several weaknesses in the ART program and largely in the viral load

cascade, levels of PDR mutations are becoming a cause of concern, as they affect the success of standard ART regimens. Moreover, the impact of DRMVs on ART is not well understood.

1.4 Research Questions

1. What are the levels of PDR in HIV hyper-endemic settings in KZN?
2. What are the trends of PDR in South Africa, and which mutations are responsible for these trends in PDR?
3. What is the effect of lowering the PDR detection threshold on prediction of treatment outcome?

1.5 Justification

These research questions are important because inadequate treatment and monitoring of HIVDR can result in treatment failure. One of the five strategic objectives of the Global Action plan on HIVDR, is to have continual monitoring and surveillance of levels of drug resistance [53]. In the first national PDR survey in South Africa, the overall level of surveillance PDR was 9.0% (95% confidence interval (CI): 6.1–13.0) [72], which would be classified as moderate resistance according to previous WHO guidelines [50]. A closer look at drug-class mutations showed that resistance was mainly driven by NNRTIs (8.3%, CI: 5.6–12.2), with NRTI (2.5% CI: 1.1-5.2) and PI (0.7%, CI: 0.0-2.8) resistance remaining at low-levels [72]. Another recent study in the Western Cape province (WC) reported moderate levels of PDR at 10%. Drug-class specific resistance was also higher in the NNRTIs (8.3%) and lower in the NRTIs (1.7%) and PIs (0%) [73]. This suggests that the increase in PDR is mainly being driven by NNRTIs, and this has been consistent with other regional findings from Uganda (7.5%) [74], Botswana (8%) [75] and Angola (14%) [76]. A continual increase in levels of PDR could have several consequences on HIV-related deaths, new infections and on ART program costs, with predicted costs of up to $6.5 billion that could be incurred in sub-Saharan Africa alone, between 2016 and 2030, if no changes are made in ART programs once PDR levels reach ≥10% [77].

PDR mutations at low frequencies could also pose a risk of ART failure with a few studies from high-income counties showing that pre-treatment NNRTI-DRMVs have an impact on NNRTI-based ART outcomes [71,78–81]. On the other hand, the CASTLE study done across 5 continents, showed that transmitted DRMVs had no significant effect on PI-based regimens (i.e.

atazanavir/ritonavir or LPV/r), even in patients with NNRTI-DRMVs [81]. Such conflicting findings warrant the need for further research into DRMVs, and considering most studies on DRMVs have been done in participants predominantly infected with HIV-1 subtype B virus (or non-C subtypes), understanding the role of DRMVs on ART in subtype C-dominant populations is required. In addition, understanding these dynamics in patients with HIV and tuberculosis (TB) co-infection (HIV/TB) is of great importance since HIV infected individuals are 10 times more likely to develop TB, with it being the leading cause of death in people living with HIV [82]. Ultimately, the goal will be to understand how NGS results can be interpreted for use in routine clinical practice, to inform treatment decisions that help improve the quality of patient care.

1.6 Main objective

To assess levels of PDR in HIV hyper-endemic settings and South Africa in general, and to understand the impact of PDR on treatment response, including DRMVs.

1.6.1. Specific objectives

1. To describe levels of PDR in two-population based studies in HIV hyper-endemic settings, in northern and central KZN
2. To describe the trends in the levels of PDR and the impact of PDR on treatment response, in South Africa
3. To describe the impact of pretreatment DRMVs on ART in individuals receiving concurrent HIV/TB treatment

1.7 Research methods

1.7.1 Study area

1.7.1.1 Africa Health Research Institute longitudinal HIV surveillance programme

This study used sequence data from both longitudinal and cross-sectional studies. It involved analysis of sequence data from two HIV hyper-endemic settings in KZN and across South Africa. One of the studies from which we harnessed sequence data to assess levels of PDR is a

longitudinal HIV surveillance programme in the uMkhanyakude district in KZN. The longitudinal household HIV surveillance programme was started in January 2003, following setup of a demographic surveillance system in 2000, by the Africa Health Research Institute (AHRI) [83]. In summary, the demographic surveillance area is in the southern part of the Umkhanyakude district in northern KZN. Its is approximately 440 km^2 in size and home to about 11 000 households and about 90 000 people [84]. The study population includes all household members in the area. Through the household survey, demographic and clinical information is collected from all registered households every year in a two-phased approach. Firstly, a household survey is administered every four months to a senior household member to report on all resident and non-resident individuals in the household. Secondly, each year trained workers collect information from individuals 15 years and older through interviews, and HIV testing is offered to each individual [85]. A dried blood spot specimen is collected for HIV related tests, such as viral load and drug resistance testing.

From 2003 to 2007 the eligibility criteria for testing as an adult in the surveillance programme was 15 – 54 years for men and 15 – 49 years for women. After 2007, all individuals >15 years of age who reside in the area are eligible for HIV testing. There are six primary health care clinics in the surveillance area where ART can be accessed for free [85,86]. Based on all HIV positive adults, ART coverage was estimated at 30.7% (29.3 – 32.1) and the HIV prevalence in the area was as high as 29.0% (27.9–30.1), in 2011 [87]. The HIV incidence rates have been recorded as 6.6 per 100 person-years in women aged 24 years and at 4.1 per 100 person-years in men aged 29 years [88]. Details of the AHRI health surveillance area have been published previously [83,85–88]. Figure 9 shows a map of the AHRI surveillance area.

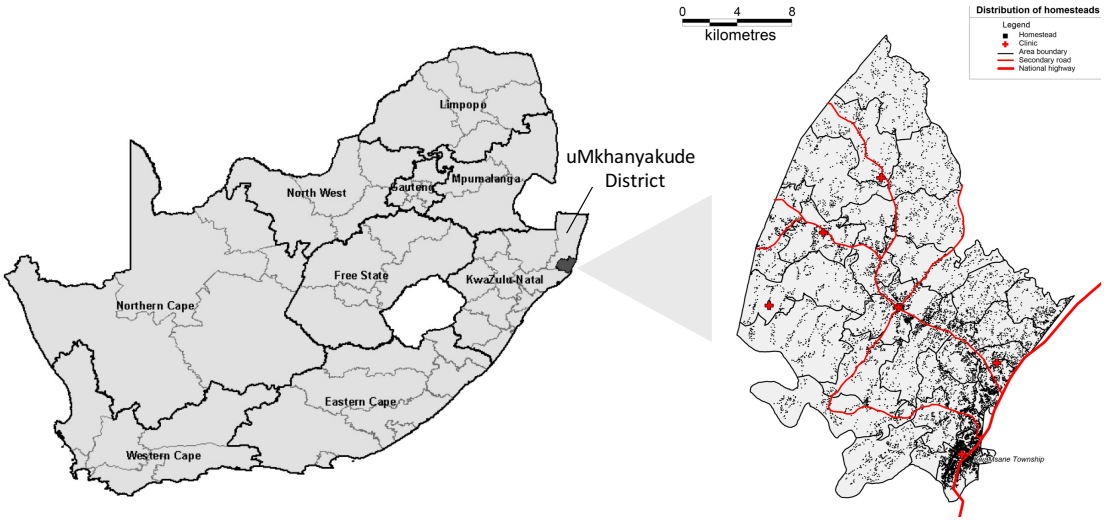

Figure 9 Geographic location of the AHRI study area in uMkhanyakude district, KZN (Namosha E et al., PLOS One, 2013)

On the left is a map of South Africa, showing the location of the uMkhanyakude district (shaded) in KZN province. On the right is a map showing the AHRI health surveillance area in the uMkhanyakude district.

1.7.1.2 The HIV Incidence Provincial Surveillance System

The HIV Incidence Provincial Surveillance System (HIPSS) is coordinated by the Epicentre AIDS Risk Management, the Centre for the AIDS Programme of Research in South Africa (CAPRISA), and the United States Centers for Disease Control and Prevention. It is a HIV household survey that is conducted in two neighboring sub-districts of the uMgungundlovu district in central KZN [89–91]. In summary, HIPSS is a HIV cross-sectional survey of randomly selected individuals between the ages of 15–49 years living in the Vulindlela and Greater Edendale sub-districts of the uMgungundlovu district. The survey has broad objectives that aim at assessing the effectiveness of programmatic HIV intervention efforts in a non-trial setting. This includes (but is not limited to) estimating the proportion of people on ART in the surveillance area, the prevalence of HIV, measuring temporal trends of HIV incidence, community viral loads, the levels of CD4 counts in

HIV infected individuals, and the proportion that are treatment naive with detectable and undetectable viral loads. It also includes estimating the prevalence of pulmonary TB, sexually transmitted infections (STIs), hepatitis B and C infections, prevalence of ART resistance, and to identify HIV transmission networks and risk factors for HIV incidence at the individual, household and community levels [91].

The study area represents peri-urban, rural traditional settlements, farmlands and informal settlements in the district. Vulindlela is largely a rural community dominated by Zulu speaking people, with a population of around 150,000 people in 2015 and the Greater Edendale area is the second largest peri-urban area in KZN, with a population of approximately 210,000 people in 2015, and has been the main economic hub of the district [91]. The study area has 16 primary health care facilities supported by three district hospitals, and 60 community-based organizations that provide HIV prevention and home-based care services. The are approximately 95,000 households in which approximately 368,000 people reside, 176,000 (48%) being males and 192,000 (52%) females, with the majority (50%) of people being those between the ages of 15–49 years [91].

The HIPPS study used a two-stage cluster-based sampling method of enumeration areas to randomly select households. Households were then identified using geographic coordinates from a global positioning system receiver [91]. Only one individual per household was enrolled and if the individual refused to participate in the survey, the next randomly selected individual was enrolled in the study, and subsequent refusal was followed by replacing the household [91]. During the study the head of household was identified and data of the age, gender, and basic socio-demographic profile of all usual household members was recorded. The individual who meets the eligibility criteria, aged between 15 - 49 years, was enrolled and biological specimens (i.e. blood, sputum, urine (in males) and vaginal swabs (in females)) were collected for testing [91]. Longitudinal follow-ups were done in all enrolled HIV negative individuals aged between 15 - 35 years. Details of the HIPSS study have been published previously [91]. Figure 10 shows a map of the study area.

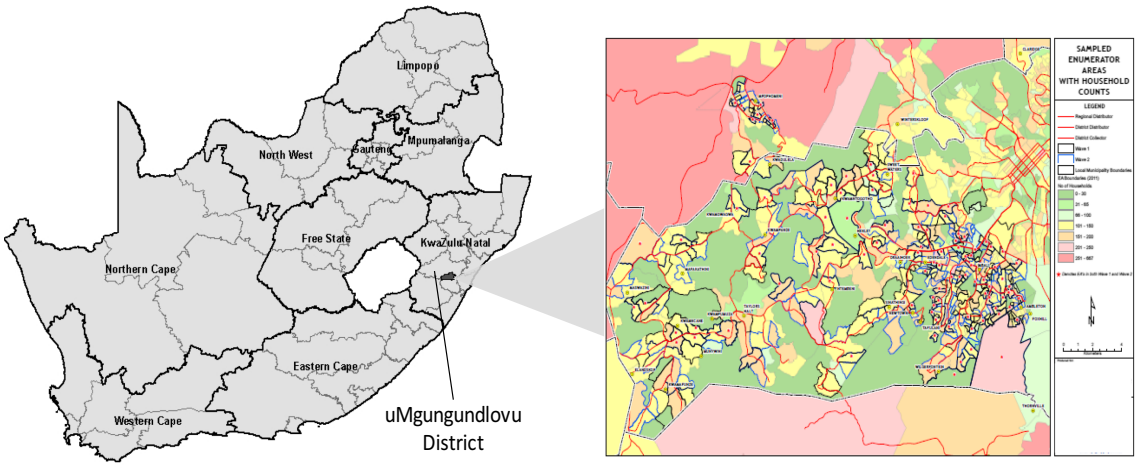

Figure 10 Geographic location of the HIPSS study area in uMgungundlovu district, KZN (Kharsany et al. BMC Public Health, 2015)

On the left is a map of South Africa, showing the location of the uMgungundlovu district (shaded) in KZN province. On the right is a map showing the HIPSS study area in the uMgungundlovu district.

Levels of PDR were analyzed using these two cohorts (AHRI and HIPPS), as well as by combining the sequences with national PDR data.

1.7.1.3 Starting ART at three points in tuberculosis treatment (SAPIT trial)

Starting antiretroviral therapy at three points in tuberculosis treatment (known as the SAPiT trial) was an open-label, randomized, controlled trial in Durban, South Africa, conducted by CAPRISA between June 2005 and July 2008, that aimed at assessing the optimal timing for ART initiation in patients with HIV/TB co-infection. In summary, the study was conducted at the eThekwini Clinical Research site (ECRS) which is next to the Prince Cyril Zulu Communicable Disease Centre; one of the largest TB facilities for outpatients in South Africa [92,93]. The ECRS is located in Durban's central business district close to commuters from local townships and outlying areas, and it has a HIV/TB treatment clinic and a STI prevention clinic, which offer free diagnosis and treatment for TB and STIs [93]. Patients with confirmed HIV/TB co-infection were recruited upon consent and they were randomly assigned to one of three study arms in a 1:1:1 ratio [92]. In the first arm, patients were initiated on ART within a month of initiating TB treatment and were known as the

integrated-therapy group. In the second arm, ART was initiated within a month of completing the intensive TB treatment phase, and these were the late integrated-therapy group. The third arm had patients who initiated ART within a month of completing the continuation phase of TB treatment, and was called the sequential-therapy group [92].

During the recruitment period (2005-2008) consenting TB patients ≥18 years of age with confirmed HIV infection (using two rapid HIV tests) were enrolled. All patients received adherence counseling, prophylaxis against HIV-related opportunistic infections, and the same once-daily ART regimen of didanosine (ddI) + 3TC + EFV [92,94]. The primary end point was death from any cause and secondary end points were treatment discontinuation due to adverse drug reactions, poor HIV suppression and TB outcomes, and the occurrence of the immune reconstitution inflammatory syndrome [92]. Results of the SAPiT trial helped determine the optimal time for initiating ART in HIV/TB co-infected patients, and the findings resulted in changes in treatment policies by the WHO and South African government. A continuation study of the SAPiT trial, known as "TB Recurrence upon Treatment with HAART" (TRuTH), was done to assess if TB recurrence in HIV/TB treated patients is due to relapse or re-infection. Each case of TB recurrence on ART was investigated to assess whether the infecting *Mycobacterium tuberculosis* is similar to the one from the previous infection and whether immune responses differ when there is relapse and re-infection [93]. Further details of the SAPiT study have been published previously [92,94]. Samples collected from this cohort were used to assess the impact of DRMVs on ART.

1.7.2 Ethical approval

Ethical approval for the AHRI and HIPSS studies were obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN) (reference numbers BF233/09 and BF269/13) the KZN Provincial Department of Health (HRKM 08/14) and the Centre for Global Health, CDC. Ethical approval for the SAPiT (reference number: E107/05) and TRuTH (reference number: BF051/09) studies, were obtained from the Biomedical Research Ethics Committee of the UKZN. Ethical approval to investigate the impact of DRMVs on ART (reference number: BF340/17) was obtained from the Biomedical Research Ethics Committee of the UKZN (Appendix 1). Participants gave informed consent for sample storage and sample use for future studies.

1.8 Thesis outline

This thesis comprises a background, literature review and justification in chapter 1, manuscripts in chapters 2 to 5 and a synthesis consolidating the findings of the thesis in chapter 6. Manuscript formats and referencing styles used in chapters 2 to 5 are according to specific journal requirements, and published papers have been presented in their current publication format.

Chapter 1: Introduction, literature review and justification
This chapter introduces the work that was conducted and provides an overall literature review on HIV drug resistance.

Chapter 2: Manuscript: "Primary HIV-1 drug-resistant minority variants"
This chapter reviewed the topic of pretreatment DRMVs, the challenges in NGS implementation, and the knowledge gaps in HIV resistance.

Chapter 3: Manuscript: "Moderate to high levels of pre-treatment HIV drug resistance in KwaZulu-Natal Province, South Africa"
This chapter is based on a study that was done to assess PDR in adult ART naïve patients from two population-based studies, in HIV hyper-endemic, in KZN.

Chapter 4: Manuscript: "Trends in pretreatment HIV-1 drug resistance in antiretroviral therapy-naive adults in South Africa, 2000 – 2016: a pooled sequence analysis."
This chapter is based on a meta-analysis study that was done to assess trends of PDR in adult ART naïve patients across South Africa, before and after scale-up of ART in 2010.

Chapter 5: Manuscript: "Impact of HIV pre-treatment drug resistant minority variants on antiretroviral therapy in HIV/TB co-infected patients."
This chapter is based on a study that was done to assess if standard genotypic resistance testing underestimates pretreatment resistance and, if so, the impact that DRMVs have on clinical outcomes.

Chapter 6: Synthesis of the thesis
The final chapter presents an overall synthesis of the thesis, consolidating the key findings of data presented as manuscripts in chapters 3 to 5, as well as recommendations for policy, and for future research, based on research findings.

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CHAPTER 2: PRIMARY HIV-1 DRUG-RESISTANT MINORITY VARIANTS

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Primary HIV-1 Drug-Resistant Minority Variants

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Abstract

Primary HIV drug-resistant mutations are mutations that occur in an HIV-infected individual prior to the initiation of antiretroviral therapy. These mutations may arise by de novo mutagenesis or result from transmission. Drug-resistant mutations may reduce the effectiveness of antiretroviral therapy, leading to inadequate virological outcomes. Currently, Sanger sequencing is the standard method for detection of drug-resistant mutations to inform treatment decisions, but it does not detect minor variant mutations. Drug-resistant minority variants can be detected by next generation sequencing. However, several challenges, including cost of infrastructure and the need for complex data analysis bioinformatics tools, remain major setbacks for next generation sequencing use. More importantly, the clinical impact of drug-resistant minority variants on antiretroviral therapy is not well understood, underscoring the importance for understanding whether the levels of primary drug-resistant minority variants for different mutations impact on the effectiveness of antiretroviral therapy and the rationale for inclusion in routine diagnostics. Understanding the impact of primary drug-resistant minority variants will help inform how next generation sequencing may be utilized in the future for pre-emptive clinical antiretroviral therapy decision making.

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Key words

Primary drug resistance. Minority variant. Next generation sequencing. Antiretroviral therapy.

Introduction

Drug-resistant mutations (DRM) are a major obstacle to effective suppression of HIV by antiretroviral therapy (ART), which is the life-long use of at least three antiretroviral (ARV) drugs from two drug classes in HIV treatment[1]. Drug resistance mutations in ART-naive patients, defined as primary drug resistance, can develop spontaneously by *de novo* mutagenesis or may result from transmitted resistance[2]. International guidelines recommend genotypic drug resistance testing before initiating ART to optimize treatment outcomes[3]. Genotyping relevant viral genes, such as the HIV pol gene or the HIV-1 envelope V3 loop, offers a cost-effective strategy[4] to customize effective, individualized drug regimens for use at ART initiation[5-7]. Curbing adverse consequences conferred by resistance, such as high programmatic costs, complex ART regimens, and multiple variable dosing strategies, will be major milestones toward achieving part of the 90-90-90 goals set

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by the World Health Organization (WHO), i.e. virological suppression in 90% of patients on ART[8].

Standard genotyping using Sanger sequencing does not reliably detect mutations at less than 15-25% of the viral quasispecies, and will therefore miss virus present at lower frequencies (i.e. < 20%)[9]. Recent technologies such as next generation sequencing (NGS) and point mutation assays such as allele-specific PCR (AS-PCR) are faster, cheaper, and can detect drug-resistant minority variants (DRMV). While point mutation assays detect specific targeted mutations at any given time, the inability of the assays to simultaneously detect multiple mutations limits their use in clinical practice.

On the other hand, NGS technologies, such as the 454-Pyrosequencing (GS FLX and GS Junior, Roche), MiSeq HiScan SQ system (Illumina), PacBio RS II (Pacific Biosciences), and Ion Torrent PGM (Life Technologies)[10], are effective in detecting multiple mutations in as low as 1% of the viral population[11]. The NGS also produces high-throughput data at relatively low costs per base[12]. However, the effect of primary HIV DRMVs in achieving virological suppression or informing patient clinical outcomes has not yet been fully elucidated. This review focuses on primary DRMVs, their clinical impact, challenges in their detection, and inclusion in routine diagnostics.

Drug-resistant minority variants in antiretroviral-naive patients

Primary DRMVs are mutations that occur as low-frequency viral variants in pre-ART patients that are not detected by Sanger sequencing[9]. In the absence of drug pressure, resistant viral variants are often outcompeted by wild-type viruses, which have better replicative capacity, making detection of resistant viral variants in treatment-naive patients less likely using Sanger sequencing[13]. However, NGS platforms are able to detect very low frequency viral variants, permitting the detection of these DRMVs in the absence of drug pressure and making these platforms an appealing tool for this application. Despite the enhanced sensitivity, the more important question pertains to the clinical impact of DRMVs and hence the clinical utility of their detection.

Clinical impact of primary drug-resistant minority variants

There is currently limited and varying evidence on the clinical impact of primary DRMVs on treatment outcomes. Much of what we understand about the clinical impact of DRMVs has come from nonnucleoside reverse transcriptase inhibitor (NNRTI)-based studies[7,14-22]. A pooled analysis by Li, et al. showed that there is more than double the risk of developing early virological failure on NNRTI-based regimens in patients with underlying NNRTI DRMVs. Additionally, a dose-dependent effect for the increased risk of treatment failure was also found in patients with higher DRMV copies per milliliter of plasma[22].

A cross-sectional study done in Spain amongst newly diagnosed HIV infected patients categorized DRMVs by frequencies at 1, 5, and 10% thresholds[23]. Using the GS-Junior (Roche-454), NGS showed a predictive increase in resistance to all drug classes (nucleoside reverse transcriptase inhibitors [NRTI], NNRTIs and protease inhibitors [PI]) when mutations were analyzed at 1% threshold[23]. In a case-control study by Johnson, et al., 2008, using AS-PCR to detect protease (PR) and reverse transcriptase (RT) mutations in treatment-naive patients, an increased risk of virological failure was seen when patients initiated efavirenz (EFV)-based treatment in the presence of primary DRMVs, compared to patients with no primary DRMVs ($p = 0.0038$)[17]. On the contrary, the STaR study did not show an association between mutations emerging during EFV and rilpivirine (RPV)-based treatment with DRMVs at baseline[24].

There is a paucity of data on rates of primary DRMVs in sub-Saharan Africa. In a clinical research cohort of treatment-naive patients in Ethiopia, NNRTI DRMVs were reported at 5.4% using AS-PCR that targeted K103N and Y181C mutations[25]. A cross-sectional study to determine DRMVs in Zambia reported a higher frequency of NRTI DRMVs (7/10) than NNRTI DRMVs (1/10) using 454 Pyrosequencing[26] in patients with no prior exposure to ART. However, the clinical impact of these DRMVs was not assessed.

A prospective study done in Cameroon in patients on first-line treatment showed incomplete adherence as a strong predictor of virological failure rather than primary DRMVs[27]. Interestingly, an ART cohort study in Malawi, investigating the effects of DRMVs in proviral DNA, reported 2/5 patients having DRMVs prior to treatment initiation[28]. However, the primary proviral DNA DRMVs were not associated with early emergence of DRMs on ART. Such conflicting findings warrant the need for continual research on the clinical impact of primary DRMVs in different settings.

Primary drug-resistant minority variants and prior antiretroviral drug exposure

Use of single and dual ARV drugs for prevention of mother-to-child transmission (PMTCT) of HIV poses a risk of ART drug resistance acquisition, thereby compromising future ART options[29,30]. The WHO PMTCT strategies of Option A, Option B, and Option B+ have been widely used for PMTCT in resource-limited settings over recent years[10]. Options A and B recommend the mother discontinue ARV drugs *post partum* until eligible to initiate ART, whilst Option B+ involves initiating life-long ART in recently diagnosed patients[10].

Exposure to ARV drugs for PMTCT (Option A and B) could select for mutations that persist for long periods of time[31]. This includes mutations such as K103N and Y181C, which are often associated with inadequate treatment outcomes and virological failure on first-line NNRTI-based regimens[14,32]. A study by Boltz, et al. following the OCTANE Trial 1 showed a higher risk of developing virological failure in women with baseline K103N and Y181C minority variants when initiated on a nevirapine (NVP)-based regimen with prior single dose (sd) NVP exposure[19]. High levels of primary NRTI and NNRTI DRMVs (70%) were also detected using AS-PCR in Tanzanian women who received a complex PMTCT prophylaxis[33].

A study conducted in Soweto, South Africa, using AS-PCR (K103N and Y181C) showed persistence of NVP resistance following exposure to sd NVP for PMTCT[31]. The study demonstrated presence of NNRTI DRMVs in 16 of 21 (76%) women, a year after sd NVP exposure. Similarly, a study in Johannesburg amongst women with and without prior exposure to sd NVP showed persistence of the K103N minority variant, and suggested the mutation could be a strong predictor of inadequate viral suppression and viral rebound[14]. With the maturing ARV program in sub-Saharan countries, there is need for more studies pertaining to the clinical relevance of these minority NNRTI-resistant variants, especially when patients initiate NNRTI-containing ART.

Post-exposure prophylaxis (PEP) involves use of ARV drugs following potential exposure to HIV to prevent infection. Pre-exposure prophylaxis (PrEP) involves use of ARV drugs before HIV exposure to lower the chances of infection. The WHO 2015 guidelines recommend offering PrEP not only to high-risk groups, but also to people at substantial risk of HIV infection (i.e. HIV incidence > 3/100 person-years) as part of HIV prevention strategies[34]. Both PrEP and PEP could result in the development of DRMs[35,36], but the evidence for the benefits of PrEP and PEP outweigh the risk of pre-ART resistance[36-38]. Mutations due to PrEP and PEP often decay rapidly[39] and could be missed by Sanger sequencing, underscoring the need for genotyping patients with a history of intermittent ARV exposure using NGS before initiating life-long ART.

Antiretroviral treatment and primary drug-resistant minority variants

The WHO recommends the use of two NRTIs and an NNRTI, as first-line regimens in adults[34]. Pre-ART DRMs can reduce the effectiveness of these ARVs in achieving viral suppression. However, there is little known about the frequencies of primary DRMVs in each ARV class.

Nucleoside reverse transcriptase inhibitors and primary drug-resistant minority variants

Nucleoside reverse transcriptase inhibitors inhibit viral replication by viral reverse transcription chain termination[40]. Most recommended first-line regimens and PrEP contain tenofovir (TDF)[34]. Minority TDF DRMs may negatively affect TDF-based ART. The most common mutation causing resistance to TDF is K65R[41]. Kozal, et al. in 2011 showed 4/411 (0.97%) treatment-naive patients had the K65R minority variants detected at > 1% by ultra-deep sequencing, with two of the four experiencing virological failure[42].

The prevalence of primary K65R minority variants in South Africa is 4%. A study amongst patients failing first-line TDF-based ART in South Africa found a high rate of TDF resistance (~ 60%)[43]. Thus, the potential for transmission of TDF resistance does exist, which may impact negatively on the effectiveness of PrEP. More studies that investigate the clinical impact of minority K65R variants are required.

In a German ART-naive cohort, the K65R minority variants occurred in 2.7% of chronically infected patients, but did not affect treatment outcome[44]. However, a case of an Eritrean immigrant showed the risk of early treatment failure associated with the K65R minority variants[45]. Determining prevalence of the K65R mutation in ART-naive patients may help inform the choice of first-line NRTI-based treatment[46]. This warrants the need for surveillance of TDF resistance in ART-naive patients, as TDF-associated mutations, most commonly the K65R mutation, could reduce the effectiveness of TDF in first-line ART and/or in its use for PrEP.

M184V is the main mutation that confers resistance to lamivudine and emtricitabine[47]. The mutation is known to reduce viral replicative capacity and increase viral susceptibility to stavudine, zidovudine, and TDF[48]. M184V is rarely detected in treatment-naive patients by Sanger sequencing, but is rapidly selected for under drug pressure[49], suggesting presence of M184V minority variants in ART-naive patients before initiating treatment[50]. The M184V minority variants are more common in recently and acutely infected patients than in chronically infected patients prior to ART initiation[2], suggesting rapid decay of the M184V minority variants due to the lower replicative capacity associated with the mutation[2]. There is, however, need for more evidence-based studies on the frequencies of such mutations in ART-naive patients and their implications on the currently recommended NRTIs.

Nonnucleoside reverse transcriptase inhibitors and primary drug-resistant minority variants

Nonnucleoside reverse transcriptase inhibitors inhibit viral replication by interacting with a non-active site of the viral reverse transcriptase[40]. The NNRTIs have a low genetic barrier to resistance[51]. This increases the chances of virological failure in patients with primary NNRTI DRMVs who initiate EFV- and NVP-based treatment[22]. The most common mutations associated with NNRTI resistance are K103N and Y181C[52].

High levels of NNRTI DRMVs in ART-naive patients have also been reported following use of sd NVP for PMTCT[14,31], with K103N and Y181C minority variants increasing the risk of virological failure on NNRTI-based treatment[16,20,21,53]. Coovadia, et al. in 2009 showed that the minority K103N mutation was associated with reduced virological response on NNRTI-based treatment[14]. The continued use of NNRTIs for PMTCT, the high level of transmitted NNRTI drug resistance[54], and the increasing evidence for the negative clinical impact of NNRTI DRMVs underscore the need for further studies describing primary NNRTI DRMVs.

Next generation sequencing platforms

Various NGS platforms are being used for detecting DRMVs. The NGS platforms vary in the sequencing coverage generated. Sequencing coverage is the average number of times a base is read from individual high-quality fragments across a genome during a sequencing run. It is calculated as C = LN/G where C is the coverage, L is the length of the reads, N is the number of reads, and G is the length of the genome[55]. The more times a base is read from individual fragments, the higher the sensitivity and confidence in calling minor variant mutations. Illumina platforms generate relatively more sequence reads, which increases the sequencing depth obtained, with PacBio generating the least number of reads per unit[56]. Table 1 compares NGS platforms, showing the advantages and disadvantages of each. Instrument costs, data throughput, and run times have been shown as ranges to represent the different generations of equipment under each product line, which cater for different user preferences.

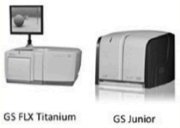
Challenges in next generation sequencing implementation

The NGS platforms have revolutionized genomic medicine by producing large amounts of data, with high depths of coverage, at lower sequencing costs, and in relatively shorter periods of time[57]. However, there are several challenges limiting use of NGS in routine clinical diagnostics. The analysis of NGS data is challenging and time consuming, partly due to the large amounts of data generated and high error rates, complicating its use in clinical decision-making compared to the current gold standard, i.e. Sanger sequencing (Table 2).

Automation of the complex steps is making NGS relatively simpler and faster to perform. However, such automated equipment is costly to acquire. Although batching and multiplexing of samples has helped reduce the cost of NGS, the technical expertise and bioinformatics tools required for data analysis, such as alignment and phylogenetics of multiple shorts reads, remain a challenge. To produce high-quality data there is need to separate actual sequence variants from background noise or contamination[58]. The ability to detect the low-level variants increases the chances of detecting carryover contamination, affecting data accuracy, and complicating the interpretation of data for patient management[12,59].

The large amounts of data ("data deluge") generated through NGS create challenges for data storage and ease of accessibility on public databases. There is need for uninterrupted high-performance computing systems that have the ability to store and process large data sets in a timely manner[60]. In resource-limited settings, NGS

Table 1. Comparison of next generation sequencing platforms

Platform (manufacturer)	 **454, GS FLX and GS Junior (Roche)**	 **SOLID (LifeTechnologies)**	 **MiSeq HiScan SQ system (Illumina)**	 **Ion Torrent PGM (LifeTechnologies)**	 **PacBio RS II (PacificBiosciences)**
First introduced[64]	2005	2006	2007	2010	2011
Chemistry[64]	Pyrosequencing	Ligation-based sequencing	Sequencing by synthesis	Ion semiconductor sequencing	Real-time sequencing
Output/day[65]	0.04-0.7 Gb	8-24 Gb	5.5-600 Gb	0.2-64 Gb	2-20 Gb
Cost/Mb	$10[66]	$0.13[66]	$0.05-0.15[66]	$1.00[66]	$7[67]
Average accuracy	99.997%[68]	99.99%[68]	99.9%[67]	> 99.0%[68]	99.999%[65,69]
Quality score	> Q30[66]	> Q30[66]	> Q30[66,70]	> Q20[70]	> 50[69]
Most frequent error[71]	Deletions	A-T bias	Single nucleotide substitutions	Short deletions	CG deletions
Cost per instrument[65]	$125,000-500,000	$125,000-500,000	$125,000-1,000,000	$50,000-149,000	$350,000-700,000
Reads per unit[56]	20,000-700,000	81,500,000-266, 666,667	1,000,000-400,000,000	400,000-60,000, 000	22,000-47,000
Advantages	Long read length (400-700 bp)[64,68] Short run time[68]	Low error rate[68] Low reagent costs[68] High throughput[68] High sensitivity for detecting MVs	Low error rate[68] Low reagent costs[68] High throughput[68] High sensitivity for detecting MVs	Medium read lengths (200 bp) Short run time[68] Low instrument cost[66]	Long read lengths[68] Short run time[64] Simple sample preparation[68]
Disadvantages	Homopolymer errors[68] High costs of reagents[68] High cost per base and low throughput[66]	Short reads[68] Long run time[68] Palindromic sequence errors[66]	Short read lengths[68] Long run times	Longer hands-on time[68] High costs of reagents[68] Homopolymer errors[66,68]	High instrument cost[68] No paired end reads[68] Low sensitivity for detecting MVs

Gb: gigabases; Mb: megabases (equivalent to a million bases); MV: minority variants.

Table 2. Comparison of Sanger sequencing to next generation sequencing

	Advantages	Disadvantages
Sanger sequencing[63]	Higher accuracy (99.999%) Long read lengths (400-900 bp) Simpler data analysis Low instrument costs	High sequencing cost ($2,400/Mb) Not ideal for large genome sequencing Does not reliably detect minority variants Low throughput and low depth of coverage
Next generation sequencing	Lower sequencing costs (< $10/Mb) Ideal for whole genome sequencing[72] Detects minority variants High throughput and depth of coverage Multiplexing in a single run[73]	Higher error rates[70] Short read lengths Many complex procedures required Complex data analysis[74] High instrument costs

bp: base pairs; Mb: million bases.

is limited by instrument costs (Table 1), infrastructural requirements, expertise, and sophisticated data analysis tools required, resulting in most studies using AS-PCR to detect low-frequency mutations in ART-naive patients. Also, generation of high-quality genotypic results using a point-of-care approach remains a major challenge in HIV drug resistance testing (including Sanger sequencing). With the WHO recommendation on initiating ART in all recently diagnosed patients, irrespective of their CD4 counts[34], shorter NGS turn-around times are required if it is to become a useful tool for making decisions at ART initiation.

Current knowledge gaps in HIV resistance

A good starting point would be to adequately estimate the prevalence and patterns of primary resistance in resource-limited settings. Few prospective studies have determined the clinical impact of primary DRMVs. Moreover, use of point mutation assays, such as AS-PCR and oligonucleotide ligation assay, detects targeted mutations, thus missing several other DRMVs that could impact treatment. It would be important to determine the cumulative effect of the primary DRMVs detected by NGS and the clinical impact of these on ART rather than analyzing the mutations in isolation.

Sanger sequencing has been used for the past two decades to make clinical decisions on use of ART[12]. However, NGS is being used for routine clinical work in some settings[61,62], slowly replacing Sanger sequencing, which has higher sequencing costs (Table 2)[63]. Also, NGS has the advantage of whole genome sequencing of HIV in single runs, allowing for more informed analyses of virus evolution. Unlike Sanger sequencing, NGS sequencing can detect HIV mutations at 1% and lower[12].

However, the effect of these low-level resistant mutations is still not well understood, compounded by the glaring gap that exists in the bioinformatics tools available to analyze such results. There is need to develop algorithms that are consistent and can be standardized in providing useful clinical information, such as the Stanford HIVdb mutation scoring algorithm (http://hivdb.stanford.edu) used in analyzing Sanger sequencing results. This entails determining cutoff values for each DRMV that might subsequently reduce the effectiveness of ART, as well as the cumulative effect of the mutations when they occur simultaneously. It is also important to understand whether the differences in the mutation frequencies, treatment adherence, as well as mutational loads result in different treatment outcomes.

Conclusion

Despite evidence for the presence of DRMVs detected in ART-naive patients for about a decade now, there still remains a knowledge gap on the clinical impact of DRMVs. It is plausible that primary DRMVs would outgrow wild-type virus under drug pressure to become the dominant viral population, leading to virological failure, as shown with NNRTIs[18]. Due to the complexities with DRMVs, viral evolution, the dynamic field of NGS, and the limited studies, there is a need for further in-depth research on primary DRMVs.

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Declaration of interest

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The authors confirm they do not have any conflict of interest.

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The manuscript in chapter 2 gave an overview of PDR, challenges with NGS implementation, and the current knowledge gaps that exist in the filed of HIVDR, in relation to NGS. It also emphasizes on the importance of understanding the impact of DRMVs and adequately estimating the prevalence and patterns of PDR in settings where genotypic testing assays are not feasible at ART initiation. This motivated the following chapters; assessing levels of PDR as detected by Sanger sequencing (chapter 3 and 4), and the impact of DRMVs on ART (chapter 5). The following chapter presents a study that was conducted to assess levels of PDR in adult ART naïve patients in two HIV hyper-endemic settings in KZN, South Africa. The manuscript supplementary material is provided in Appendix 2.

CHAPTER 3: MODERATE TO HIGH LEVELS OF PRE-TREATMENT HIV DRUG RESISTANCE IN KWAZULU-NATAL PROVINCE, SOUTH AFRICA

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Moderate to high levels of pre-treatment HIV drug resistance in KwaZulu-Natal Province, South Africa

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Abstract

Introduction: There is evidence of increasing levels of pre-treatment HIV drug resistance (PDR) in Southern Africa. We used data from two large population-based HIV surveillance studies to estimate prevalence of PDR in KwaZulu-Natal, the province with the highest HIV prevalence in South Africa.

Methods: Sanger sequencing was performed on samples obtained from a longitudinal HIV surveillance programme (study A, 2013-2014) and the HIV Incidence Provincial Surveillance System (study B, 2014-2015). Sequences were included for adult HIV positive participants (age ≥15 years for study A, age 15-49 years for study B) with no documented prior exposure to ART. Overall and drug class-specific PDR was estimated using the World Health Organization 2009 surveillance drug resistance mutation (SDRM) list and phylogenetic analysis was performed to establish evidence of drug resistance transmission linkage.

Results: One thousand eight hundred and forty-five (1845) sequences were analysed (611 study A; 1234 study B). An overall PDR prevalence of 9.2% (95% confidence interval (CI): 7.0-11.7) was observed for study A and 11.0% (95% CI 8.9-13.2) for study B. In study B, the prevalence of non-nucleoside reverse-transcriptase inhibitor (NNRTI) PDR exceeded 10% for sequences collected in 2014 (10.2%, 95% CI 7.5-12.9). The most prevalent SDRMs were K103NS (7.5%), M184VI (2.4%) and V106AM (1.4%). There was no evidence of large transmission chains of drug-resistant virus.

Conclusion: High level NNRTI-PDR (>10%) suggests a need to modify the standard first-line ART regimen and to focus attention on improving the quality of HIV prevention, treatment and care.

Introduction

After approximately two decades of combination antiretroviral therapy (ART), the global response to the human immunodeficiency virus (HIV) is threatened by the development of HIV drug resistance (HIVDR).[1] Pre-treatment drug resistance (PDR) refers to the presence of drug resistance in a person initiating or re-initiating ART, and can therefore be a combination of transmitted and acquired drug resistance (ADR). Such resistance is considered the best indicator to guide the selection of effective first-line ART regimens.[2–4] While the levels of PDR in low- and middle-income countries have been low to moderate historically, there are concerns over increasing levels, given the rapid expansion in ART access and the persistent high incidence of new HIV infections.[5] Once PDR exceeds 10%, modelling suggests that in Africa, HIVDR could account for almost half a million new infections, and $6.5 billion in additional ART costs between 2016 and 2030.[6]

As part of its coordinated approach to prevent, monitor and respond to the emergence of HIVDR, the World Health Organization (WHO) recommends surveys of PDR.[4] As more people receive ART and develop ADR, the risk of transmission of drug-resistant HIV increases.[7] The presence of PDR can lead to inadequate virologic suppression on ART and further accumulation of drug resistance mutations,[8,9] and as a result, the levels of PDR have to be continually monitored to ensure the effective use of ART. At this critical juncture in the global response to HIV, with scale-up of universal test-and-treat and pre-exposure prophylaxis (PrEP) for HIV prevention,[10] it is important to understand the current epidemiology of PDR in high-prevalence settings.

South Africa has the largest ART programme in the world, with approximately 3.9 million people on treatment as of August 2017.[11] Generally, low levels of PDR have been documented in the country,[12,13] but there is recent evidence of higher levels, which raises concern over the continued effectiveness of first-line non-nucleoside reverse-transcriptase inhibitor (NNRTI)-based ART regimens.[14–16] The evidence of increasing PDR is in the context of a growing number of people with virological failure on ART and delayed switching to second-line ART, creating an expanding pool of those with ADR.[17] In this paper, we present estimates of PDR from two population-based studies in KwaZulu-Natal Province (KZN), South Africa.

Materials and Methods

Setting

The Africa Health Research Institute (AHRI) has conducted longitudinal population-based HIV surveillance in the uMkhanyakude District Municipality, northern KZN since 2003 (Study A, Figure 1).[18] All individuals 15 years and older in a population of approximately 65,000 resident members are invited to provide dried blood spot (DBS) specimens on an annual basis. For this study, viral reverse transcription PCR was performed on the DBS specimens of the participants with a positive HIV enzyme-linked immunosorbent assay (ELISA) in 2013 or 2014, who had a DBS HIV ribonucleic acid (RNA) ≥10,000 copies/mL. PCR and sequencing were also attempted on some DBS specimens with HIV RNA <10,000 copies/mL, but this was not pursued as the rate of successful amplification was low. We excluded sequences obtained from participants with documented ART initiation prior to the date of specimen collection. Information about ART use was obtained through linkage of the population surveillance data with routine HIV programme data.[19] This did not include

information about prior use of antiretrovirals for preventing mother-to-child transmission (pMTCT). We estimated the date of HIV infection using the midpoint between the last negative test date and the first positive test date, and estimated the duration of infection in months, by calculating the time between the estimated date of infection and the sample collection date, as described previously.[14]

Epicentre AIDS Risk Management, The Centre for the AIDS Programme of Research in South Africa (CAPRISA), and the United States Centers for Disease Control and Prevention (CDC) coordinate the HIV Incidence Provincial Surveillance System (HIPSS) in two sub-districts of uMgungundlovu District Municipality in central KZN (Study B, Figure 1).[20–22] In 2014-2015, a representative cross-sectional household survey enrolled 9812 individuals aged 15-49 years. A multistage cluster sampling technique was used to randomly select the households and individuals included in the study, as described previously.[23] One individual was selected per household. From the 14618 households found and occupied, 9812 individuals were eligible and consented to participate. HIV ELISA was performed on the peripheral blood specimens of the participants,[22] and sequencing was performed on the plasma specimens from participants with positive HIV serology and plasma HIV RNA ≥1000 copies/mL.[21] For the analysis, we excluded sequences from those who self-reported any prior ART use (for treatment or pMTCT). Details on the timing of recruitment and ascertainment of ART status for the two population-based studies, are provided in Supplementary data.

Laboratory methods and data analysis

Genotypic drug resistance testing of the HIV-1 *reverse transcriptase (RT)* and *protease (PR)* genes was done on stored specimens by Sanger sequencing on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) using previously described methods.[24] Sequence quality and coverage were assessed using the Calibrated Population Resistance (CPR) tool (http://cpr.stanford.edu/cpr.cgi). Sequences that had quality concerns, such as stop codons, or that did not cover all possible surveillance drug resistance mutation (SDRM) positions, were excluded. We included participants with complete *RT* sequences, with or without the *PR* sequence.

PDR was determined by detecting SDRMs with the CPR tool using the WHO 2009 SDRM list.[25,26] The results were used to estimate the levels of overall and drug class-specific resistance for each study, with the data being analyzed using STATA version 15.1 (StataCorp, College Station, TX) and SAS version 9.4 (SAS Institute, Cary, NC). The chi-square test was used to establish any difference in PDR prevalence across the years, within each study, with a Rao-Scott chi-square test being used for study B to adjust for the survey design. Logistic regression analysis was performed to explore associations between PDR and individual participant characteristics for each study (i.e. sex, age, HIV RNA, and for study A; the estimated duration of infection), and accounted for the survey sample design in study B. Where appropriate, analyses for study B were conducted by applying sampling weights and using survey procedures. The sampling weights adjusted for non-equal probabilities of selection associated with the complex survey design, and for non-response across age and gender categories.[20] The confidence intervals were calculated using Wald

confidence limits, and the Taylor series linearization method was used to estimate standard errors of proportions.

To establish evidence of SDRM transmission, we performed phylogenetic analysis to identify HIV transmission clusters. Sequences with drug resistance mutations identified in this study were aligned with a background dataset of 15,313 HIV-1 subtype C *pol* sequences. This consisted of publicly available sequences from the Los Alamos HIV Database (http://www.hiv.lanl.gov), isolated from Southern African countries, and sequences generated previously from the AHRI surveillance population.[14,17,27] To avoid cluster formation due to convergent evolution under ART pressure, codon positions associated with drug resistance mutations were removed from the alignment.[28]

A maximum likelihood (ML) phylogenetic tree was constructed using FastTree2,[29] and cluster support was assessed with Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) with 1000 pseudo-replicates. HIV-1 transmission clusters were identified from the ML tree using the ClusterPicker software version 1.2.3,[30] where the definitions of a transmission cluster were set to a minimum clade support of 90 SH-aLRT and a maximum within-cluster genetic distance of 4.5%. To identify if sequences clustering together had similar SDRMs, we further submitted the full-length sequences (with all codon positions) to the CPR tool.

Ethics Statement

Approval for the two studies was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (UKZN) (reference numbers BF233/09

and BF269/13) the KZN Provincial Department of Health (HRKM 08/14) and the Centre for Global Health, CDC. Written informed consent for use of stored specimens was obtained from all study participants.

Results

A total of 1845 HIV-1 sequences were included in the analysis, and consisted of 611 sequences for study A, with 254 from 2013 and 357 from 2014. From study B, 1234 sequences were included, with 737 from 2014 and 497 from 2015 (Figure 2).

Overall, 1841 had complete *RT* and *PR* sequences, and four had only the complete *RT* sequence. The characteristics of the participants included in the analysis are summarised in Table 1.

The estimated prevalence of PDR was 9.2% (95% confidence interval (CI) 7.0-11.7) for study A and 11.0% (95% CI 8.9-13.2) for study B. The estimated prevalence of NNRTI PDR was 7.5% (95% CI 5.6-9.9) for study A and 9.2% (95% CI 7.2-11.3) for study B. There was no evidence of an increase in overall PDR or NNRTI PDR across the two years in either study (Figure 3). The estimated prevalence of PDR was higher for women than men in both studies: 9.9% vs. 7.1% for study A (odds ratio (OR) 1.45, 95% CI 0.73-2.87); and 13.6% vs. 8.3% for study B (OR 1.73, 95% CI 1.06-2.81) (Table 3 and Supplementary Figure S1).

The prevalence of PDR peaked at 17.0% (95% CI 11.9-22.1) in women aged 25-34 years (Supplementary Table S1). There was no strong evidence of an association between PDR and age or HIV RNA in either study (see Table 3). In study A, the

prevalence of PDR was lower in those with an estimated duration of infection ≤24 months than in those with estimated duration >24 months (3.0% vs. 8.6%), but the analysis was limited by small numbers with recent infection (n=66) (see Table 3).

Of all 1845 sequences across the two studies, 212 (11.5%) had at least one SDRM. The frequency of individual SDRMs by study year is displayed in Table 2. Overall, 182/1845 (10.0%) had NNRTI mutations, 59/1845 (3.2%) had nucleoside reverse-transcriptase inhibitor (NRTI) mutations, and 23/1841 (1.2%) had protease inhibitor (PI) mutations (Table 2). Of those with SDRMs, 162 (76.4%) had single class resistance, 48 (22.6%) dual class resistance, and two (1.0%) triple class resistance. The most frequently observed SDRM was the NNRTI mutation K103NS, occurring in 139 participants (7.5% of all participants, or 65.6% of those with SDRMs). In 100 participants (47.2% of those with drug resistance mutations), the K103NS mutation was the only SDRM detected (Supplementary Table S2), which lists the most frequently observed patterns of mutations).

The most common NRTI mutation was M184VI (2.4%), and in almost all cases (43 of 44) it was detected in combination with at least one NNRTI mutation, while in half the cases (22 of 44) with other NRTI mutations. The K65R mutation associated with tenofovir (TDF) resistance was detected in 11 participants overall (0.6%), with no evidence of an increase across the two years in either study. Of the 23 with PI mutations, 20 had a single PI mutation. The most common PI mutation was the M46IL mutation, occurring in 18 participants. Two participants with four or more PI mutations had similar patterns of PI resistance (M46I, I54V, L76V, V82A), with one participant having in addition the L90M mutation. In both cases, there was triple

class resistance with NRTI mutations (M184V, L74V) and an NNRTI mutation (K103S).

From the phylogenetic analysis, we identified 25 transmission clusters with individuals harbouring at least one PDR mutation in common (Supplementary Figure S2). In total, 57 individuals were grouped in these transmission clusters, 56% (32/57) were from studies A and B, and 44% (25/57) were South African individuals whose sequences were present in the background dataset. Individuals from studies A and B (in the transmission linkages) comprised 15% (32/212) of the sequences with any PDR mutations identified here. From the background dataset, we had information about ART exposure for 18 of the 25 individuals. Sixteen of the 18 (89%) were ART experienced, and were linked to 14 ART-naïve individuals with PDR (Supplementary Figure S2). K103NS was the most common PDR mutation, observed in 72% (41/57) of individuals involved in linked transmissions.

Discussion

Surveillance of HIVDR is a key component of the public health approach to sustainable use of ART. In this analysis, we found moderate to high levels of PDR in two KZN districts between 2013 and 2015, at approximately the turn of the second decade of ART rollout. The results from the AHRI longitudinal population surveillance suggest a continued trend of steadily increasing PDR since 2010.[14] In the HIPSS cross-sectional survey, the level of NNRTI PDR was close to 10%, the current threshold at which the WHO recommends urgent public health action.[2,3] The attempt to exclude people with prior ART exposure means that our findings are likely to reflect predominantly transmitted resistance. The levels of resistance documented

and the phylogenetic analysis therefore suggest increasing transmission of HIVDR from people treated with ART. This raises concerns about the quality of HIV prevention, treatment and care, and should prompt consideration of appropriate public health measures to ensure the long-term sustainability of ART.

The timing of this increase in levels of PDR is consistent with other findings from sub-Saharan Africa, where PDR rose to moderate levels about ten years into ART scale-up.[31,32] To some extent, our findings are consistent with a nationally-representative survey conducted in South Africa in 2013-2014, which estimated the prevalence of PDR at 9.0% and NNRTI PDR at 8.3% nationally.[15] The two sites are similar in terms of demographics, HIV epidemiology and HIV care cascades.[22,33] However, there were some differences in the study populations, particularly the higher HIV RNA levels in study A due to the use of DBS samples for sequencing, as the amplification success rate reduces at lower HIV RNA levels (<10,000 copies/mL) in DBS samples.[34] Using DBS samples which have a higher HIV RNA requirement for genotyping, could have resulted in an underestimation of the levels of resistance for study A, as drug-resistant viruses have a lower replicative capacity than the wild-type virus, which could result in lower HIV RNA levels, although this may depend on the specific profile of mutations.[35,36]

Another difference between the two studies was the method used to determine prior ART use. It is possible that the self-report of ART use in the HIPSS (study B) was less reliable than linkage to health service records as a method for uncovering current or prior use of ART. Significant undisclosed ART use has been documented in other population-based surveys,[37,38] and if people on ART were inadvertently

included in the sample for this analysis, then the levels of PDR from the HIPSS (study B) may be overestimated. Given these difficulties, it is possible that future studies should include testing for antiretroviral drug levels to determine the true ART status. Although the PDR prevalence was somewhat higher in women in both studies, the difference was not as marked as that reported for a number of recent national PDR surveys.[5] In addition, we did not observe a clear gradient in PDR across age groups, unlike a recent study in Kenya,[39] although it is notable that PDR levels were particularly high in young women, the group with the highest HIV incidence in these populations.[21]

The rapid expansion of ART coverage has been a considerable achievement in South Africa, having resulted in substantial gains in life expectancy.[40,41] Routine viral load (VL) monitoring to guide ART switches has been incorporated into the treatment programme since the start of ART roll-out. Despite this, implementation remains inconsistent, and the results are often not appropriately acted upon.[42–45] With the resulting delays in switching to second-line ART regimens, people spend more time viraemic and at risk of accumulating drug resistance.[17] With the growing caseloads of people on first-line ART, this suggests that there will be an expanding pool of people with ADR, creating conditions for an increase in the transmission of HIVDR.[46] Our findings lend support to calls for increased focus on quality improvement within the HIV treatment programme, particularly with respect to adherence support, routine virologic monitoring and timely ART switching.[44,45]

Overall, around one in ten participants had at least one NNRTI SDRM, the most frequent mutation being K103NS, which is consistent with the national PDR survey,[15]

and is the most common NNRTI mutation documented in the context of ADR on first-line ART.[47] The persistence of this mutation in the absence of drug pressure [48] may increase the chance of onward transmission, and the levels documented here raise concern about the continued effectiveness of efavirenz (EFV) and nevirapine in first-line regimens. The levels of NRTI resistance were relatively low, and the estimated prevalence of key SDRMs associated with TDF resistance (K65R and K70E) was below 1% in both studies. This was also consistent with the national survey that estimated the prevalence of K65R at 1.4%.[15] At the time of these studies, TDF had still only been in widespread use in first-line ART regimens for under five years, so continued vigilance is required, especially as high levels of K65R have been documented in those with ADR on first-line ART, and with evidence supporting the transmissibility of K65R variants.[47,49] However, these findings provide reassurance for now about the place of TDF in first-line ART and PrEP regimens,[50] and although the levels of PI resistance were low, the two instances of multiple major PI mutations and triple class resistance raise some concern. The information available from the public sector health records suggested that neither had been exposed to ART. However, multiple major PI mutations and triple class resistance suggest the two individuals had prior use of ART. Therefore, these findings should be interpreted with caution, given the potential for data linkage problems, access to ART in a public sector programme outside the study area, or access to ART in the private sector.

From the phylogenetic analysis, approximately 15% of the individuals with SDRM from study A and B were linked in a transmission cluster with at least one other person with an identical SDRM. Fourteen of 32 ART-naïve individuals were linked with ART-experienced individuals whose sequences were in the background dataset.

In these linkages, we can infer that drug-resistance mutations were most likely transmitted from ART-experienced individuals to ART-naïve individuals in the same cluster. For the other 18 ART-naïve individuals with PDR, the source of drug resistance is most likely to be from ART-experienced individuals that were not part of our study sample, although we cannot exclude onward transmission from ART-naïve individuals, and undisclosed ART exposure. K103N was the most common mutation observed in the linked cases, consistent with a study from Aruba, a highly HIV endemic area in the Caribbean.[51] However, unlike the Aruba cluster, where onward transmission of K103N was observed among ART-naive individuals in a large transmission chain, the pattern of small and independent transmission clusters observed here is more suggestive of multiple transmission events from people with ADR on ART.

Our findings suggest that additional public health interventions may now be required in South Africa, as recommended by the WHO.[2,3] One option would be to introduce genotypic resistance testing prior to ART initiation, but this would have substantial cost implications and present considerable operational challenges. The more likely option would be to change the standard first-line ART regimen, probably to an integrase inhibitor-based regimen. Dolutegravir (DTG) has already replaced EFV in first-line ART in neighbouring Botswana and there are plans for it to be introduced for first-line ART in South Africa in 2018.[11,52] While there are some concerns about introducing DTG in the South African context (e.g. with paucity of studies on the use of DTG in pregnancy and dosing in people with TB on rifampicin), cost savings are an additional driver to adopt this for first-line therapy.[52]

Our findings should be interpreted with certain limitations, as the two studies were not conducted as formal drug resistance surveys for pre-treatment or transmitted drug resistance. Although population-based studies present challenges in accurately determining current and prior ART use, they might have some advantages over facility-based HIVDR surveys in that they include a more broadly representative sample of HIV-positive people in the population, including people not accessing health care. However, this representativeness is diminished if there are low levels of consent in population-based surveys, as seen in the AHRI surveillance. There was substantial attrition in the laboratory processes, particularly with the DBS specimens, and we cannot be certain that the participants whose virus was successfully sequenced were representative of all eligible ART-naïve people in the study populations. Our capacity to uncover linked drug resistant transmissions was limited by the relatively low coverage of people living with HIV in the study areas, particularly for study B. Finally, as these studies were in geographically-restricted populations, these results should not be taken to be representative of the entire province of KZN, or of South Africa more generally.

In conclusion, the high levels of PDR documented here highlight the need for renewed focus on improving the quality in HIV prevention, treatment and care. In particular, the systems for routine VL monitoring for people on ART and switching to second-line ART should be strengthened. These findings should be interpreted together with results of the national drug resistance survey to inform the need for modification of the standard first-line ART regimen or the introduction of other public health measures to prevent the spread of drug resistance.

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Author Disclosure Statement

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Figure Legends

Figure 1. Location of the two population-based studies

Figure 2. Summary of the specimens and HIV-1 sequences from the two studies in KZN, South Africa

[a] Excluded due to incomplete reverse transcriptase (*RT*) sequences

Figure 3. Levels of pre-treatment drug resistance (A) and NNRTI-specific pre-treatment drug resistance (B)

Marker line in Figure 3B corresponds to 10% threshold for NNRTI PDR

Prevalence estimates and confidence intervals for study B were weighted to adjust for the survey design, and for non-response across age and gender categories

Supplemental Data

Figure S1. Estimated prevalence (with 95% confidence intervals) of pre-treatment HIV drug resistance by sex and age in the AHRI population-based surveillance study (A) and the CAPRISA HIPSS (B)

Prevalence estimates and confidence intervals for study B have been weighted to adjust for the survey design and for non-response across age and gender categories

Figure S2. Maximum likelihood phylogenetic tree of clusters involving drug-resistance

Figure 1

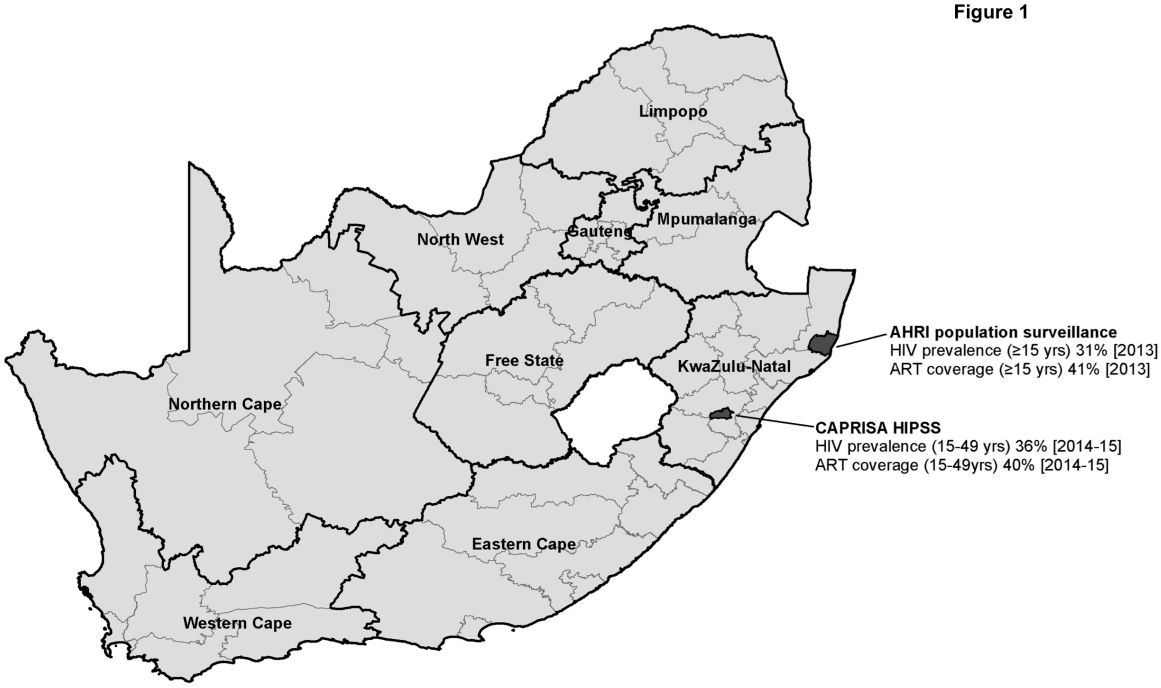
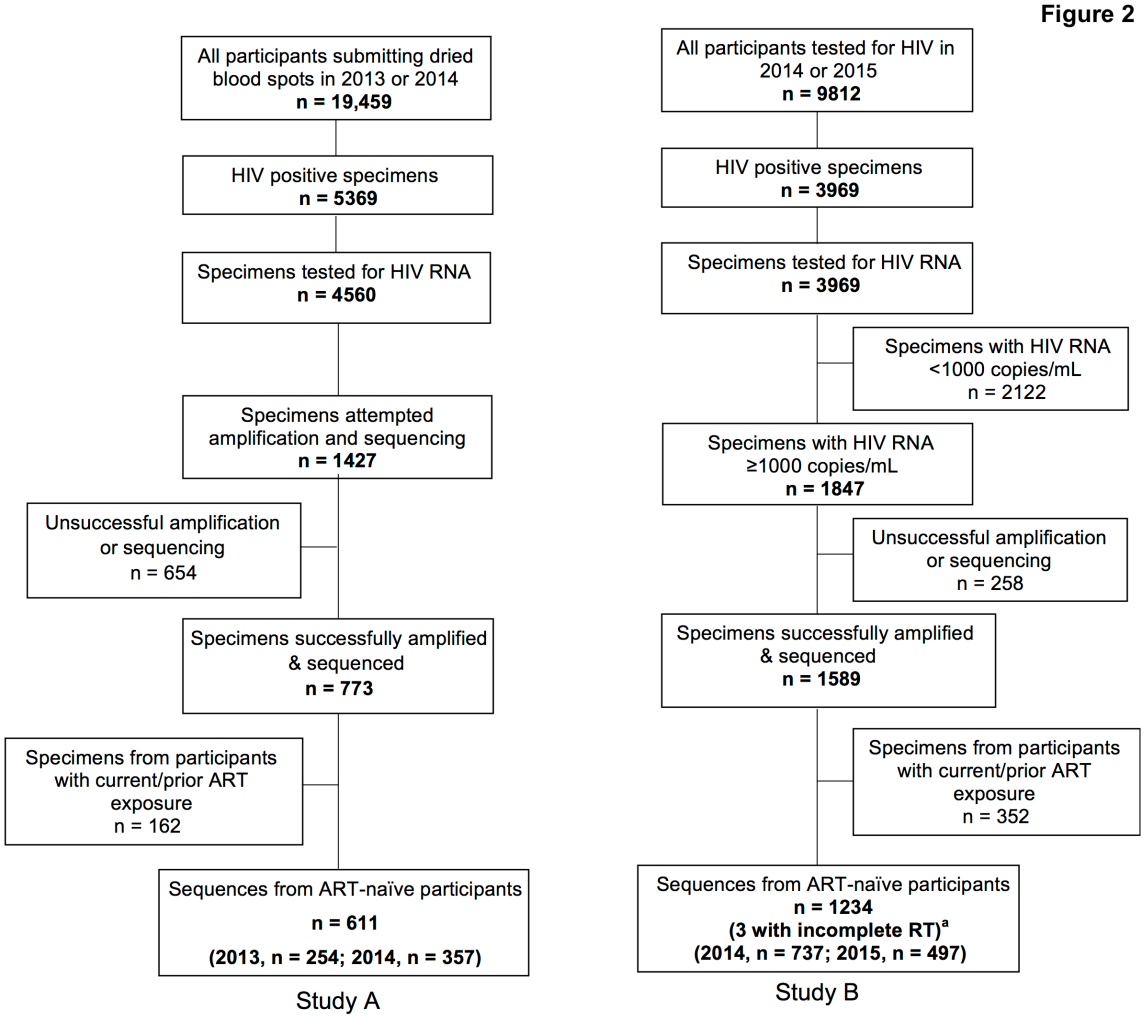
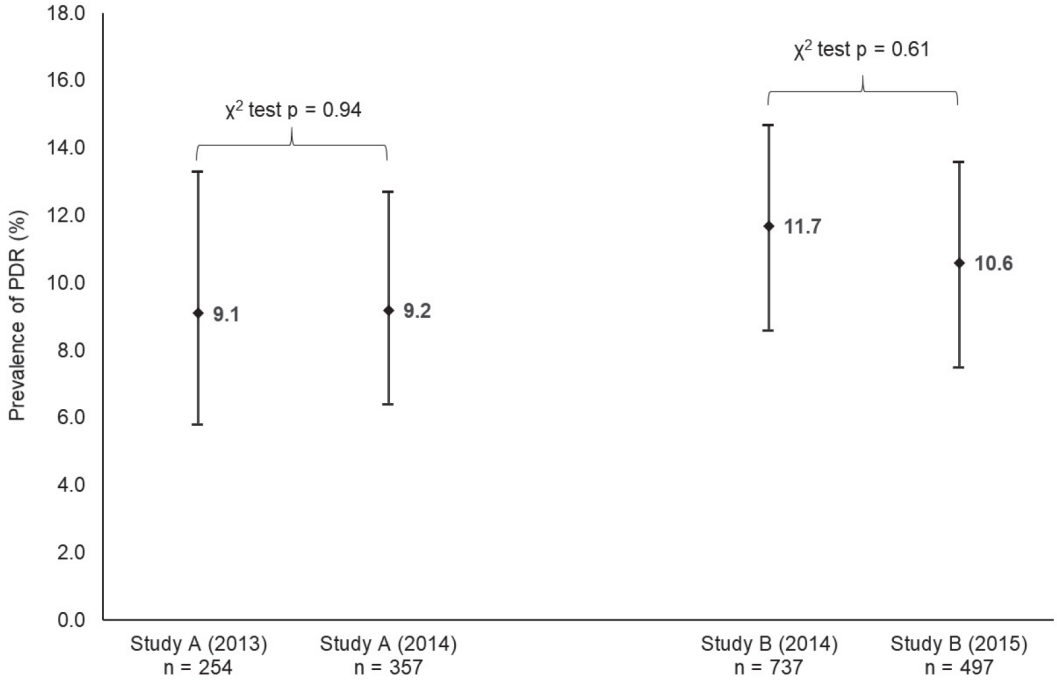

Limpopo
Mpumalanga
Gauteng
North West
Free State
KwaZulu-Natal
Northern Cape
Eastern Cape
Western Cape
AHRI population surveillance
HIV prevalence (≥15 yrs) 31% [2013]
ART coverage (≥15 yrs) 41% [2013]
CAPRISA HIPSS
HIV prevalence (15-49 yrs) 36% [2014-15]
ART coverage (15-49yrs) 40% [2014-15]

Figure 2


All participants submitting dried blood spots in 2013 or 2014
n = 19,459
HIV positive specimens
n = 5369
Specimens tested for HIV RNA
n = 4560
Specimens attempted amplification and sequencing
n = 1427
Unsuccessful amplification or sequencing
n = 654
Specimens successfully amplified & sequenced
n = 773
Specimens from participants with current/prior ART exposure
n = 162
Sequences from ART-naïve participants
n = 611
(2013, n = 254; 2014, n = 357)
Study A
All participants tested for HIV in 2014 or 2015
n = 9812
HIV positive specimens
n = 3969
Specimens tested for HIV RNA
n = 3969
Specimens with HIV RNA <1000 copies/mL
n = 2122
Specimens with HIV RNA ≥1000 copies/mL
n = 1847
Unsuccessful amplification or sequencing
n = 258
Specimens successfully amplified & sequenced
n = 1589
Specimens from participants with current/prior ART exposure
n = 352
Sequences from ART-naïve participants
n = 1234
(3 with incomplete RT)[a]
(2014, n = 737; 2015, n = 497)
Study B

A

Figure 3


18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Prevalence of PDR (%)
χ2 test p = 0.94
χ2 test p = 0.61
9.1
9.2
11.7
10.6
Study A (2013)
n = 254
Study A (2014)
n = 357
Study B (2014)
n = 737
Study B (2015)
n = 497

B

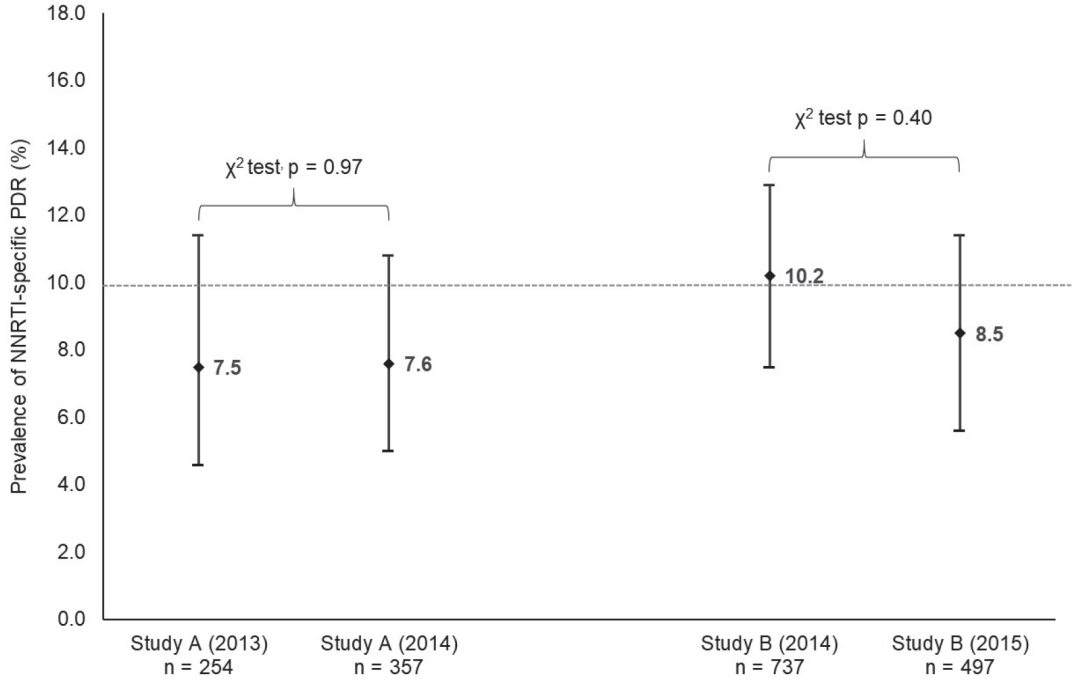

18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Prevalence of NNRTI-specific PDR (%)
χ2 test p = 0.97
χ2 test p = 0.40
7.5
7.6
10.2
8.5
Study A (2013)
n = 254
Study A (2014)
n = 357
Study B (2014)
n = 737
Study B (2015)
n = 497

Table 1. Study participants' demographic and clinical characteristics

	Total	AHRI surveillance (Study A)		HIPSS (Study B)	
		2013	2014	2014	2015
Participants	1845	254	357	737	497
Sex, female, n (%)	1269 (69)	186 (73)	269 (75)	507 (69)	307 (62)
Age, years, median (IQR)	30 (25-39)	30 (24-40)	33 (26-42)	30 (24-38)	30 (24-36)
HIV RNA, log_{10} copies/mL, median (IQR)[a]	4.50 (4.02-4.96)	4.77 (4.35-5.18)	4.70 (4.36-5.10)	4.39 (3.80-4.87)	4.41 (3.91-4.83)

AHRI, Africa Health Research Institute; HIPSS, HIV Incidence Provincial Surveillance System; IQR, interquartile range; RNA, ribonucleic acid

[a] HIV RNA missing for 84 participants (77 study A, 7 study B)

Table 2. Surveillance drug resistance mutations detected in two population-based studies in KZN, South Africa

	AHRI surveillance (Study A)		HIPSS (Study B)		Overall
	2013	**2014**	**2014**	**2015**	
NNRTI Mutations	n=254	n=357	n=737	n=497	n=1845
L100I	0	0	0.4	0.2	0.2
K101EP	0.8	0.3	1.1	0.6	0.8
K103NS	**6.3**	**6.2**	**9.2**	**6.6**	**7.5**
V106AM	0.8	0.8	2.0	1.0	1.4
Y181C	0.4	0	0.1	0.6	0.3
Y188LC	0.4	0	0.3	0.6	0.3
G190AS	0	0.8	0.7	0.2	0.5
P225H	1.2	0.8	1.1	0.8	1.0
M230L	0	0.3	0.4	0.2	0.3
Overall NNRTI resistance	**7.5**	**7.6**	**11.9**	**9.7**	**9.9**
NRTI Mutations	n=254	n=357	n=737	n=497	n=1845
M41L	0	0.3	0.3	0.2	0.2
K65R	1.2	0.3	0.7	0.4	0.6
D67N	0.8	0	0.1	0.4	0.3
T69D	0	0.3	0	0	0.1
K70R	0.8	0	0	0.4	0.2
K70E	0.0	0.3	0.0	0.6	0.2
L74VI	0.4	0.3	0.1	0.2	0.2
Y115F	0	0	0.3	0.2	0.2
M184VI	3.1	2.0	2.6	2.0	2.4
L210W	0	0	0.1	0	0.1

T215DEV	0.4	0	0.1	0	0.1
T215Y	0.4	0	0	0	0.1
K219ENR	0.8	0.6	0.3	0.2	0.4
Overall NRTI resistance	4.7	3.4	3.1	2.4	3.2
PI Mutations[a]	n=254	n=356	n=736	n=495	n=1841
L24I	0	0	0	0.2	0.1
M46IL	0.4	0.6	0.7	2.0	1.0
F53Y	0	0	0	0.2	0.1
I54V	0.4	0.3	0	0	0.1
L76V	0.4	0.3	0	0	0.1
V82A	0.4	0.3	0	0	0.1
I85V	0	0.3	0	0.4	0.2
L90M	0.8	0	0	0	0.1
Overall PI resistance	0.8	0.8	0.7	2.6	1.2

AHRI, Africa Health Research Institute; HIPSS, HIV Incidence Provincial Surveillance System; NNRTI, non-nucleoside reverse-transcriptase inhibitor; NRTI, nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor

All figures are percentages; figures in bold and underlined are levels of resistance ≥5%

The following surveillance drug resistance mutations were not detected in either study and are therefore not listed: L23I, D30N, V32I, I47VA, G48VM, I50LV, G73STCA, N83D, I84VAC, N88DS, T69ins, V75MTAS, F77L, F116Y, Q151M, V179F

[a] Denominator for PI mutations based on number of complete *PR* sequences

Table 3. Association between pre-treatment drug resistance and participant characteristics

		Study A			Study B		
	Category	N	n (%)	OR (95% CI)	N	n (%)	OR (95% CI)
Sex	Male	156	11 (7.1)	1	420	39 (9.3)	1
	Female	455	45 (9.9)	1.45 (0.73-2.87)	814	117 (14.4)	1.64 (1.12-2.41)
Age, yrs	15-24	145	15 (10.3)	1.28 (0.62-2.62)	314	40 (12.7)	0.88 (0.58-1.34)
	25-34	217	18 (8.3)	1	507	72 (14.2)	1
	35-44	125	10 (8.0)	0.96 (0.43-2.15)	319	30 (9.4)	0.63 (0.40-0.98)
	45+	124	13 (10.5)	1.29 (0.61-2.74)	94	14 (14.9)	1.06 (0.57-1.97)
HIV RNA, log_{10} copies/mL	<4	51	4 (7.8)	1	372	61 (16.4)	1
	4-5	309	33 (10.7)	1.40 (0.48-4.15)	619	61 (9.9)	0.56 (0.38-0.82)
	>5	174	14 (8.0)	1.03 (0.32-3.27)	236	33 (14.0)	0.83 (0.52-1.31)
	Missing	77	5 (6.5)	0.82 (0.21-3.20)	7	1 (14.3)	0.85 (0.10-7.18)
Duration of infection[a]	≤24 months	66	2 (3.0)	0.33 (0.08-1.42)	-	-	-
	>24 months	385	33 (8.6)	1	-	-	-
	Unknown	160	21 (13.1)	1.61 (0.90-2.88)	-	-	-

CI, confidence interval; OR, odds ratio; RNA, ribonucleic acid

[a] For those with a prior negative HIV ELISA in the population surveillance, estimated date of infection was calculated as mid-point between last negative HIV ELISA and first positive HIV ELISA. Estimated duration of infection was then calculated from that date of infection to the date the sample processed for sequencing was collected. For those with only prior positive HIV ELISA tests, duration of infection was taken to be >24 months if there was a positive HIV ELISA more than 24 months prior to the sample date. Unknown duration of infection implies no prior negative HIV ELISA and no prior positive HIV ELISA beyond 24 months

The manuscript in chapter 3 showed important results that suggested HIV PDR has increased in KZN, with NNRTI-PDR exceeding 10% in sequences collected in 2014 in the HIPSS study. This provided the first evidence of NNRTI-PDR ≥10% in South Africa. Considering that the WHO guidelines now recommend switching to DTG-based ART when NNRTI-PDR levels reach levels of ≥10%, this study was done at a timely moment, supporting the transition to DTG-based first-line ART. Further to the findings of high levels of NNRTI-PDR in KZN, we conducted a meta-analysis of sequence data from all pre-ART drug resistance studies in South Africa. This included all studies on adult ART naïve individuals, conducted between January 2000 and September 2016, including sequences from the two population-based studies described in chapter 3. The following chapter presents a meta-analysis that was done on assessing the trends of PDR in South Africa. The manuscript supplementary material is provided in Appendix 3.

CHAPTER 4: TRENDS IN PRETREATMENT HIV-1 DRUG RESISTANCE IN ANTIRETROVIRAL THERAPY-NAIVE ADULTS IN SOUTH AFRICA, 2000 – 2016: A POOLED SEQUENCE ANALYSIS

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Trends in pretreatment HIV-1 drug resistance in antiretroviral therapy-naive adults in South Africa, 2000 – 2016: a pooled sequence analysis

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References: 73

Summary (244 words)

Background South Africa has the largest public antiretroviral therapy (ART) programme in the world. We assessed temporal trends in pretreatment HIV-1 drug resistance (PDR) in ART-naïve adults from South Africa.

Methods We included datasets from studies conducted between 2000 and 2016, with HIV-1 *pol* sequences from more than ten ART-naïve adults. We analysed sequences for the presence of 101 surveillance drug resistance mutations (SDRMs). We pooled sequences by sampling year and performed a sequence-level analysis using a generalized linear mixed model, including the dataset as a random effect.

Findings We identified 38 datasets, and retrieved 6880 HIV-1 *pol* sequences for analysis. The pooled annual prevalence of PDR remained below 5% until 2009, then increased to a peak of 11·9% (95% CI 9·2-15·0) in 2015. The pooled annual prevalence of non-nucleoside reverse-transcriptase inhibitor (NNRTI) PDR remained below 5% until 2011, then increased to 10·0% (95% CI 8·4-11·8) by 2014. Between 2000 and 2016, there was a 1·18-fold (95% CI 1·13-1·23) annual increase in NNRTI PDR ($p<0·001$), and a 1·10-fold (95% CI 1·05 – 1·16) annual increase in nucleoside reverse-transcriptase inhibitor PDR ($p=0·001$).

Interpretation Increasing PDR in South Africa presents a threat to the efforts to end the HIV/AIDS epidemic. These findings support the recent decision to modify the standard first-line ART regimen, but also highlight the need for broader public health action to prevent the further emergence and transmission of drug-resistant HIV.

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Disclaimer: The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of CDC.

Research in context

Evidence before this study

We searched PubMed for systematic reviews and meta-analyses of pretreatment or transmitted HIV drug resistance in South Africa. We used the search terms HIV AND South Africa AND drug resistance AND (systematic review OR meta-analysis). We found two meta-analyses exploring regional prevalence of pretreatment or transmitted HIV drug resistance, where data from South Africa were combined with data from other countries in a regional analysis (southern Africa or sub-Saharan Africa). We found a meta-analysis of pretreatment HIV drug resistance in children younger than 12 years, which included data from South Africa. We also found a systematic review from our own group which analysed transmitted drug resistance up to 2010. We did not identify any studies that focused on South Africa and incorporated sequences collected since 2010, when scale-up of antiretroviral therapy accelerated.

Added value of this study

In this pooled analysis of 6880 HIV-1 sequences from 38 datasets, we provide up-to-date estimates of the prevalence of pretreatment HIV drug resistance (PDR) in South Africa. We present evidence of increasing PDR, particularly since the acceleration of ART scale-up in 2010. We demonstrate that the increase is largely driven by non-nucleoside reverse-transcriptase inhibitor (NNRTI) PDR, but that levels of nucleoside reverse-transcriptase inhibitor (NRTI) PDR are also rising. In particular, we note a concerning increase in the prevalence of tenofovir resistance-associated mutations (TRAMs), which could have important implications for current treatment and prevention strategies.

Implications of all the available evidence

Our findings provide clear evidence that PDR in South Africa has reached the threshold at which the World Health Organization recommends urgent public health action (NNRTI PDR >10%). Whilst our data provide support for the decision to move to a new dolutegravir-based first-line regimen, they also highlight the broader need to improve quality of HIV treatment and prevention if South Africa is to achieve the UNAIDS goal of ending AIDS by 2030.

Introduction

The roll-out of antiretroviral therapy (ART) has been a major breakthrough in the global response to HIV, helping to reduce HIV-related deaths by 48% between 2005 and 2016, and new HIV infections by 11% between 2010 and 2016.[1] Despite these impressive public health gains, substantial expansion of access to ART will be required to achieve the target of ending the HIV epidemic by 2030. The emergence and transmission of HIV drug resistance (HIVDR) poses a threat to the successful treatment and prevention of HIV, and there is now strong evidence that levels of HIVDR are increasing substantially in southern Africa, the region that faces the greatest challenges to ending the HIV epidemic.

Pretreatment HIV drug resistance (PDR) is drug resistance in a person initiating or re-initiating ART (i.e. with or without prior ART exposure).[2,3] PDR can arise in one of three ways: transmission of drug-resistant HIV from a person with acquired drug resistance (ADR); transmission of primary drug-resistant HIV from another ART-naïve person; or ADR resulting from prior exposure to antiretroviral drugs for treatment or prevention. The presence of PDR is associated with poorer virological outcomes on first-line ART.[4,5]

South Africa, with over seven million people living with HIV (PLHIV) in 2016, accounts for almost one in five PLHIV globally.[1] The country has the largest public ART program in the world, with more than four million people on ART by early 2018.[6] In the first few years of ART rollout, the levels of PDR were low (<5%).[7] More recent studies, conducted since the accelerated expansion of ART coverage in 2010, have suggested higher levels of PDR[8,9].

Given this evidence of rising levels of PDR in the country and the wider region, and the continued expansion of ART for treatment and prevention, we performed a pooled analysis of HIV sequence data from South Africa, firstly to determine the annual trends in PDR and secondly to explore in detail the patterns of observed drug resistance mutations.

Methods

Search strategy and selection criteria

This study was a systematic review and pooled analysis aimed at determining trends in PDR amongst ART-naïve adults in South Africa. We conducted and reported this in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement

(checklist included in appendix, p1).[10] To identify relevant studies we first searched for published articles in MEDLINE using the OvidSP interface on 12 September 2017 (appendix, p 3). We then scanned the reference lists of all articles selected for inclusion and conducted forward citation searches using Google Scholar. Finally, we searched South African HIV-1 sequence datasets not linked to a published article, using the PopSet database on the National Center for Biotechnology Information (NCBI) website.[11]

We included studies involving adults (defined for the purpose of this analysis as 15 years or older) in South Africa with recent or chronic HIV infection and no documented prior ART exposure. We obtained information about prior ART exposure from either the article or the sequence annotation in GenBank. We excluded studies that enrolled women with documented exposure to antiretrovirals for prevention of mother-to-child transmission (pMTCT). We excluded studies with fewer than ten HIV-1 *pol* sequences; and studies where the sequences were generated from samples collected prior to 2000. Where articles reported on multiple separate cross-sectional studies (for example a series of annual antenatal surveys), we separated the sequences into individual datasets according to the sampling year. If results from the same study were presented in more than one publication, we pooled the sequences into a single dataset. We included sequences from one multi-national study,[12] as South African sequences could be identified through the sequence annotation in GenBank.

From the articles, we retrieved a core set of information, including the year(s) of sample collection, province, study type, study population, proportion of participants that were female, and method for determining prior ART exposure.

Sequence analysis

We downloaded publicly available sequences for the included studies from GenBank.[11] Where sequences were not publicly accessible, we contacted the study authors to request the sequences. We aligned and visually inspected the sequences in AliView v1.18 (http://ormbunkar.se/aliview/).[13] We manually edited the sequences until perfect codon-based alignments were produced. We assessed sequences for their completeness and quality using the Calibrated Population Resistance (CPR) tool (http://cpr.stanford.edu/cpr.cgi).[14] Stop codons, frame-shift mutations, APOBEC3G/F hyper-mutations, highly unusual mutations and highly ambiguous nucleotides (B, D, H, V and N), were all used as indicators of poor sequence quality. We excluded from the analysis any sequence that did not meet the sequence inclusion criteria of the CPR tool. We included all sequences that

had complete reverse transcriptase gene (*RT*) sequences (codons 40 to 240), with or without complete protease (*PR*) sequences. Where multiple sequences were identified from the same study participant (for example in cohort studies), we only included the sequence from the earliest time point. Most sequences were not annotated with information about participant sex or age, so we did not include this information in the datasets.

We defined PDR as the presence of any of 101 drug resistance mutations. The mutation list included the 93 mutations from the WHO 2009 list of surveillance drug-resistance mutations (SDRMs);[15] and eight additional tenofovir resistance-associated mutations (TRAMs) characterised in a recent international collaborative analysis (A62V, K65N, S68GDN, K70QT, and V75L),[16] (appendix, p 4). Overall, the mutation list encompassed 42 nucleoside reverse-transcriptase inhibitor (NRTI) resistance mutations at 17 *RT* positions, 19 non-nucleoside reverse-transcriptase inhibitor (NNRTI) resistance mutations at ten *RT* positions, and 40 protease inhibitor (PI) resistance mutations at 18 *PR* positions. We used the CPR tool to calculate the proportion of sequences with overall and drug class-specific PDR.[14]

Trends in pretreatment drug resistance

To assess the annual increase in overall and drug class-specific PDR, we pooled sequences from different studies by year of sample collection and performed a generalized linear mixed regression model using the R package (v3.3.1) lme4. We used the presence or absence of PDR (or drug class-specific PDR) as the binary outcome variable and the sampling year as the explanatory variable. Where samples from the same study had been collected over more than one year and where the sequence annotation did not include year of sample collection, we allocated the sequences to the median sampling year. To account for heterogeneity between studies, we included the dataset as a random effect in the model. Given the relatively small number of sequences with specific mutations, we also pooled the sequences into three periods (2000-2008, 2009-2012, and 2013-2016) and checked for any trend in prevalence of specific NRTI and NNRTI resistance mutations using the chi-squared test for trend.

Results

We initially identified 856 articles through our database search and nine articles through other sources. After removing duplicate publications, we screened 790 abstracts and assessed 46 full-text articles for eligibility. We excluded 14 articles on the basis of our eligibility criteria: eight contained

fewer than 10 HIV-1 *pol* sequences; two had only PR sequences with no RT sequences; one reported on a duplicate sequence dataset; one contained only sequences generated from samples collected prior to 2000; one was based on targeted sequencing for a single mutation (K65R); and sequences were unavailable for one study (appendix, p 5). From the 32 articles, we identified 38 datasets with at least ten HIV-1 *pol* sequences from ART-naïve adults (Figure 1, Table 1, appendix, pp 6-9).[7,8,23–32,9,33–42,12,43,17–22] Seventeen datasets were from formal surveys of pretreatment drug resistance or transmitted drug resistance.

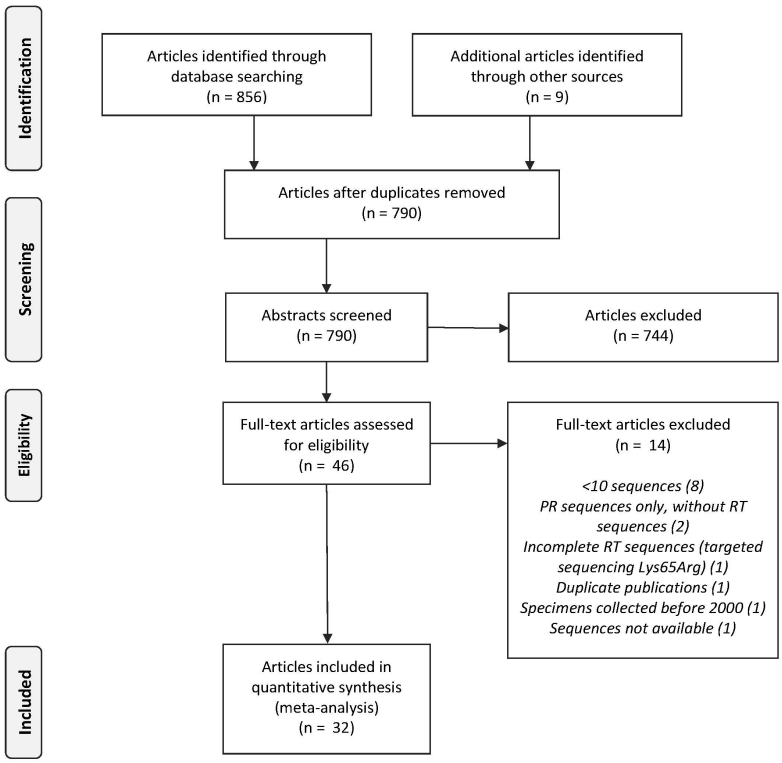

Figure 1 Flow diagram of articles and datasets identified and selected

Table 1 Characteristics of included datasets with ten or more RT sequences from ART-naïve adults

Dataset ID	Source	Sampling years	Province(s)	Study type	Study population	Proportion females	Method for determining prior ART use	Met criteria for WHO TDR/PDR survey
1	Bessong	2001	LP	Genetic diversity	ART-naïve adults	79%	NS	No
2	Bessong	2001-2004	GT, LP	TDR	ART-naïve adults	68%	NS	Yes
3	Chimukangara	2013	KZN	Population HIV surveillance	HIV-positive adults >15 years	73%	Linkage to public sector records	No
4	Chimukangara	2014	KZN	Population HIV surveillance	HIV-positive adults >15 years	75%	Linkage to public sector records	No
5	Chimukangara	2014-2015	KZN	Population HIV surveillance	HIV-positive adults 15-49 years	66%	Self-report	No
6	Gordon	2001-2002	KZN	Genetic diversity	ART-naïve adults	66%	NS	No
7	Hamers	2007-2008	GT, MP	PDR	Adults eligible for ART	62%	Self-report	Yes
8	Huang	2006	FS	TDR	ART-naïve adults	NS	Self-report	Yes
9	Hunt	2005	GT, KZN	ANC survey	Primigravid female <25 years	100%	NS	Yes
10	Hunt	2006	GT, KZN	ANC survey	Primigravid female <25 years	100%	NS	Yes
11	Hunt	2007	GT, KZN	ANC survey	Primigravid female <25 years	100%	NS	Yes
12	Hunt	2008	GT, KZN	ANC survey	Primigravid female <25 years	100%	NS	Yes
13	Hunt	2009	GT, KZN	ANC survey	Primigravid female <25 years	100%	NS	Yes
14	Hunt	2010	GT, KZN	ANC survey	Primigravid females ≤21	100%	NS	Yes

					years			
15	Hunt	2011	EC, FS, GT, KZN, WC	ANC survey	Primigravid females ≤25 years	100%	NS	Yes
16	Hunt	2012	EC, FS, GT, KZN, LP, MP, NC, NW, WC	ANC survey	Primigravid females ≤21 years	100%	NS	Yes
17	Iweriebor	2007-2008	LP	Genetic diversity	ART-naïve adults	90%	Self-report	No
18	Jacobs	2002-2004	WC	Genetic diversity	ART-naïve adults	66%	Self-report	No
19	Jacobs	2008-2010	WC	Neurocognitive study	ART-naïve females	100%	NS	No
20	Manasa	2010	KZN	Population HIV surveillance	HIV-positive adults >15 years	85%	NS	No
21	Manasa	2011	KZN	Population HIV surveillance	HIV-positive adults >15 years	76%	Linkage to public sector records	No
22	Manasa	2012	KZN	Population HIV surveillance	HIV-positive adults >15 years	71%	Linkage to public sector records	No
23	Matthews	2000-2004	KZN	Chronic infection cohort	ART-naïve adults	92%	Self-report	No
24	Msimanga	2009	MP	Genetic diversity	ART-naïve adults	95%	Self-report	No
25	Musyoki	2007	GT	Genetic diversity	Adults initiating ART	NS	Self-report	No
26	Nwobegahay	2008	LP	TDR	ART-naïve adults	70-73%	Self-report	Yes
27	Papathanasopoulos	2006-2007	GT	Genetic diversity	ART-naïve adults	74%	Self-report	No
28	Parboosing	2009	KZN	TDR	Primigravid female <22	100%	NS	Yes

					years			
29	Parikh	2010-2011	KZN	Trial screening (HIV prevention)	Females 18-40 years first positive test	100%	NS	No
30	Pillay	2000	GT	Trial screening (pMTCT)	ART-naïve pregnant females	100%	NS	No
31	Pillay	2002	GT	ANC survey	Primigravid females <22 years	100%	NS	Yes
32	Pillay	2004	GT	ANC survey	Primigravid females <22 years	100%	NS	Yes
33	Seoighe	2003-2005	GT, KZN	Trial baseline (pMTCT)	Pregnant females	100%	NS	No
34	Steegen	2013-2014	EC, FS, GT, KZN, LP, MP, NC, NW, WC	PDR	Adults initiating ART or in pre-ART care	59%	Self-report	Yes
35	Treurnicht	2004-2005	KZN	Acute infection study	Females with documented acute infection	100%	NS	No
36	van Zyl	2016-2017	WC	PDR	ART-naïve adults initiating ART	52%	Self-report	Yes
37	Wilkinson	2000	WC	Phylogenetic study	ART-naïve patients	NS	NS	No
38	Wilkinson	2004	WC	Phylogenetic study	ART-naïve patients	NS	NS	No

ANC, antenatal care; ART, antiretroviral therapy; EC, Eastern Cape; FS, Free State; GT, Gauteng; KZN, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NS, not stated; NW, North West; PDR, pretreatment drug resistance; pMTCT, prevention of mother-to-child transmission; TDR, transmitted drug resistance; WC, Western Cape

We retrieved 7025 *RT* sequences and 6501 *PR* sequences. We excluded 145 RT sequences and 207 PR sequences that did not meet sequence quality criteria. Therefore, we included 6880 *RT* sequences and 6294 *PR* sequences in the analysis (i.e. 6294 sequences with combined *PR* and *RT* and 586 with *RT* only) (appendix, pp 10, 11). The majority of sequences were subtype C (99·2%). Overall, 478 of 6880 sequences (6·9%) had at least one drug resistance mutation. The majority of these sequences had only NNRTI resistance mutations (289/478, 60·5%); dual class NRTI and NNRTI PDR was present in 79/478 (16·5%) (appendix, p 12). The prevalence of overall and drug class-specific PDR in each dataset is displayed in Figure 2, and the crude pooled prevalence of overall and drug class-specific PDR by year is shown in Table 2.

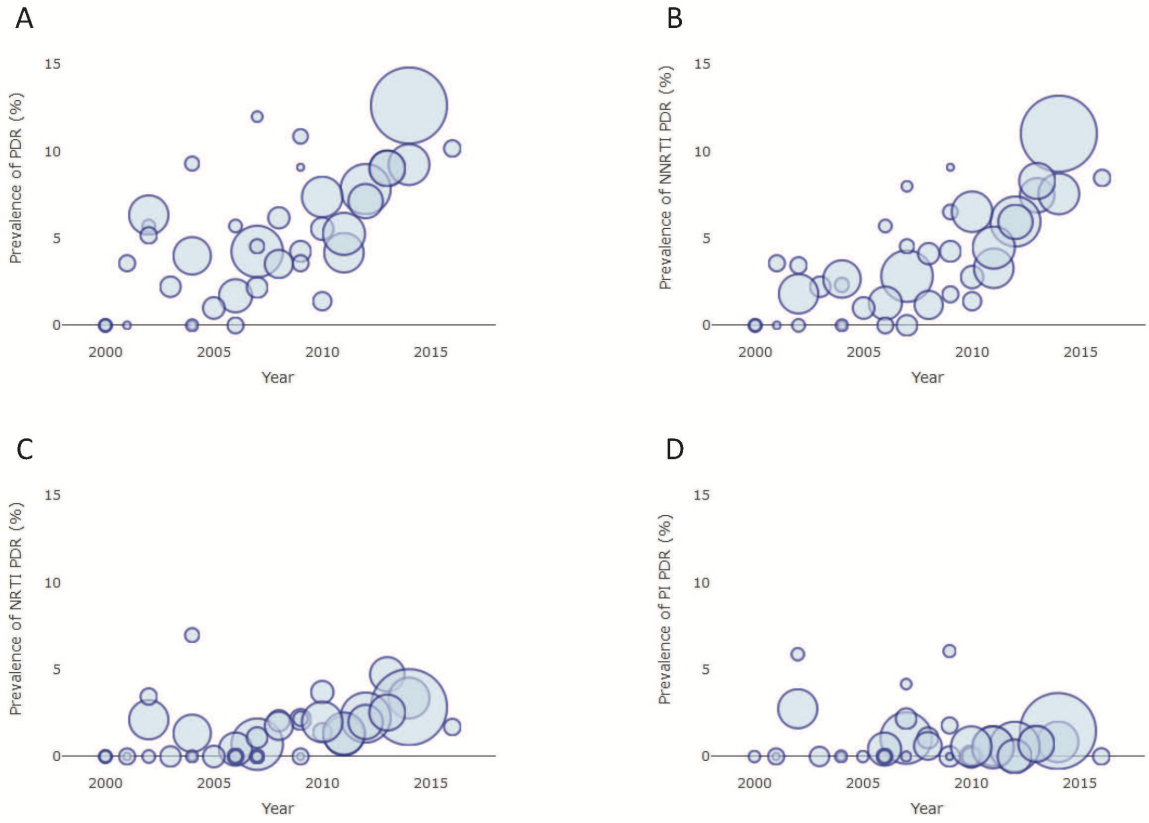

Figure 2 Prevalence of pretreatment HIV drug resistance by year of sampling

A) Overall B) Non-nucleoside reverse-transcriptase inhibitor C) Nucleoside reverse-transcriptase inhibitor D) Protease inhibitor. Each bubble represents a dataset and the size of the bubble is proportional to the number of sequences in the dataset

Table 2 Pooled prevalence of pretreatment HIV drug resistance (PDR), NNRTI PDR, and NRTI PDR, by year

Year	Number of RT sequences	Any DRM	Any PDR (95% CI)	NNRTI DRM	NNRTI PDR (95% CI)	NRTI DRM	NRTI PDR (95% CI)
2000	66	0	-	0	-	0	-
2001	69	2	2·9 (0·4 - 10·1)	2	2·9 (0·4 - 10·1)	0	-
2002	424	26	6·1 (4·0 - 8·9)	8	1·9 (0·8 - 3·7)	9	2·1 (1·0 - 4·0)
2003	90	2	2·2 (0·3 - 7·8)	2	2·2 (0·3 - 7·8)	0	-
2004	377	16	4·2 (2·4 - 6·8)	9	2·4 (1·1 - 4·5)	7	1·9 (0·7 - 3·8)
2005	113	1	0·9 (0 - 4·8)	1	0·9 (0 - 4·8)	0	-
2006	303	5	1·7 (0·5 - 3·8)	4	1·3 (0·4 - 3·3)	1	0·3 (0 - 1·8)
2007	748	32	4·3 (2·9 - 6·0)	21	2·8 (1·7 - 4·3)	5	0·7 (0·2 - 1·6)
2008	290	13	4·5 (2·4 - 7·5)	7	2·4 (1·0 - 4·9)	5	1·7 (0·6 - 4·0)
2009	172	7	4·1 (1·7 - 8·2)	6	3·5 (1·3 - 7·4)	2	1·2 (0·1 - 4·1)
2010	306	17	5·6 (3·3 - 8·7)	12	3·9 (2·0 - 6·7)	6	2·0 (0·7 - 4·2)
2011	953	54	5·7 (4·3 - 7·3)	45	4·7 (3·5 - 6·3)	16	1·7 (1·0 - 2·7)
2012	788	60	7·6 (5·9 - 9·7)	47	6·0 (4·4 - 7·9)	17	2·2 (1·3 - 3·4)
2013	370	36	9·7 (6·9 - 13·2)	31	8·4 (5·8 - 11·7)	16	4·3 (2·5 - 6·9)
2014	1255	142	11·3 (9·6 - 13·2)	126	10·0 (8·4 - 11·8)	38	3·0 (2·2 - 4·1)
2015	497	59	11·9 (9·2 - 15·0)	48	9·7 (7·2 - 12·6)	12	2·4 (1·3 - 4·2)
2016	59	6	10·2 (3·8 - 20·8)	5	8·5 (2·8 - 18·7)	1	1·7 (0 - 9·1)

CI, confidence interval; DRM, drug resistance mutation; NRTI, nucleoside reverse-transcriptase inhibitor; NNRTI, non-nucleoside reverse-transcriptase inhibitor; PDR, pretreatment drug resistance; RT, reverse transcriptase

The prevalence of NNRTI PDR remained below 5% until 2011 and then increased rapidly to above 10% by 2014. The pooled prevalence of NRTI PDR and PI PDR remained below 5% across all years. Over the entire study period (2000-2016), there was a 1·10-fold yearly increase in the odds of PDR (95% confidence interval (CI) 1·06-1·15), which was driven by increasing NNRTI PDR (odds ratio (OR) 1·18, 95% CI 1·13-1·23) and NRTI PDR (OR 1·10, 95% CI 1·05-1·16) (Table 3).

Table 3 Annual change in odds of pretreatment HIV drug resistance, 2000 - 2016

Drug Class	Odds ratio (95% CI)	p value
NRTI	1·10 (1·05 – 1·16)	0·0001
NNRTI	1·18 (1·13 – 1·23)	<0·0001
PI	0·96 (0·89 – 1·04)	0·3650
Overall	1·10 (1·06 – 1·15)	<0·0001

CI, confidence interval; NRTI, nucleoside reverse-transcriptase inhibitor; NNRTI, non-nucleoside reverse-transcriptase inhibitor; PI, protease inhibitor

Overall, 374 sequences (5·4%) had at least one NNRTI DRM (appendix, p 13). The most prevalent mutation was K103NS, occurring in 278 sequences (58·2% of sequences with any DRM; 4·0% of all sequences) (Figure 3). In the majority of these sequences (218/278), K103NS was the only DRM. Other common NNRTI resistance mutations included V106AM (n=47), Y181C (n=34), K101EP (n=29) and G190AS (n=27). Overall, 77/374 (20·6%) had more than one NNRTI DRM, most commonly K103N + P225H (n=16) and K103N + V106M (n=12). The prevalence of some specific NNRTI resistance mutations increased over time. This trend was most marked for the K103NS and V106AM mutations, and less so for the K101EP mutations. There was no evidence of changing prevalence of Y181C or G190ASE (appendix, p 14).

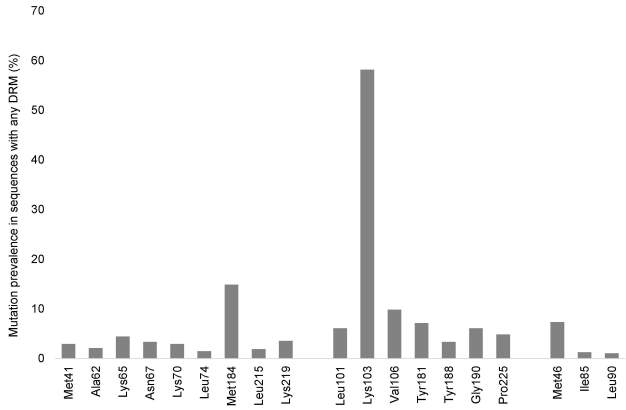

Figure 3 Prevalence of specific mutations in HIV-1 sequences with any drug resistance mutation. This includes all mutations observed in >1% of the sequences with any drug resistance mutation

M184VI was the most common NRTI resistance mutation, present in 71 sequences (14·9% of sequences with any DRM; 1·0% of all sequences) (appendix, p 15). Most of the sequences with M184VI had at least one NNRTI DRM (66/71) and just under half had additional NRTI DRMs (31/71). The other NRTI DRMs accompanying M184VI included thymidine analogue mutations (TAMs, n=11), TRAMs (n=11), L74VI and/or Y115F (n=7), and other multi-NRTI mutations (n=2). Classical TAMs (M41L, D67N, K70R, L210W, T215FY, and K219EQ) were detected in 36 sequences (7·5% of sequences with any DRM; 0·5% of all sequences). The majority of these (30/36) had a single TAM; and eleven sequences had the M41L mutation alone without other DRMs. Overall, TRAMs were detected in 37 sequences (7·7% of sequences with any DRM; 0·5% of all sequences). The TRAM most frequently detected was K65R (n=21). Twelve sequences had a TRAM not on the WHO SDRM list (A62V, n=10; K70T, n=2), although in four of these sequences the mutation was present with the K65R mutation. The prevalence of TRAMs increased in later time periods: 0·1% (3/2480) in 2000-2008, 0·5% (11/2219) in 2009-2012, and 1·1% (23/2181) in 2013-2016, and for the M184VI mutation: 0·2% (4/2480) in 2000-2008, 0·9% (20/2219) in 2009-2012, and 2·2% (47/2181) in 2013-2016 ($p<0·001$, χ^2 test for trend) (appendix, p 14).

Fifty-six sequences (0·9%) had at least one PI DRM. The most frequently observed mutation was the relatively non-polymorphic M46IL mutation, which was detected in 35 sequences (0·6%) (appendix, p 16).

Discussion

In this pooled analysis with more than 6000 HIV-1 sequences from ART-naïve adults in South Africa, we observed a sustained increase in pretreatment HIV drug resistance between 2000 and 2016, driven primarily by NNRTI resistance. The increase in PDR seems to have accelerated since 2010, which coincides with the rapid expansion of ART coverage in the country from just 20% in 2010 to 56% in 2016.[44] By 2014, the pooled prevalence of NNRTI PDR had reached 10%, the threshold at which the WHO now recommends urgent public health action.[45] There was also some evidence of increasing NRTI PDR, particularly tenofovir-associated resistance and the M184VI mutation associated with lamivudine and emtricitabine resistance. However, the pooled prevalence of NRTI resistance remained low (<5%) in each sampling year.

These findings are consistent with those from recent meta-analyses exploring drug resistance across Africa, which showed levels of resistance rising to moderate levels about ten years into the scale-up of ART in the region.[46,47] The overall 11% annual increase in odds of PDR between 2000 and 2016 in

South Africa is comparable to the 12% increase in odds of transmitted drug resistance across sub-Saharan Africa between 2000 and 2013.[46] The 18% annual increase in odds of NNRTI PDR is somewhat lower than the 24% reported for the southern Africa region in a more recent meta-analysis.[47] That could be explained by the fact that we only included ART-naïve adults, whereas the regional meta-analysis included a small number of sequences from people with prior ART exposure. Alternatively, it could be that the higher rate of increase in PDR in the regional meta-analysis was reflective of higher levels of PDR in other southern African countries.

Our analysis was restricted to ART-naïve individuals and our assumption is therefore that transmitted drug resistance is the primary driver of the increasing PDR prevalence. There are limitations to this assumption, best illustrated by the most prevalent DRM, the K103NS mutation. This mutation, selected by efavirenz (EFV) and nevirapine (NVP), is the most common acquired NNRTI DRM in people with virological failure on standard first-line ART regimens in South Africa.[48] Viruses with the K103NS mutation have transmission fitness similar to wild-type virus,[49,50] and can persist for years in the infected host.[51] It's therefore entirely plausible that the high prevalence of this mutation is a consequence of frequent transmission. However, K103NS is also the most common mutation to emerge in women who receive single-dose NVP for the prevention of mother-to-child transmission and, in this context too, the mutation can persist for years in the absence of antiretroviral therapy.[52,53] Although we restricted the analysis to ART-naïve individuals, we could not be certain that participants in the individual studies were truly ART naïve. Most studies relied on self-report of antiretroviral use, which can be unreliable.[54–59] Given that the majority of sequences were from women, it is possible that some of the NNRTI resistance arose from prior exposure to NVP for pMTCT rather than from transmitted drug resistance.

We also revealed evidence of increasing NRTI resistance, at a rate similar to that observed in the larger regional meta-analyses.[46,47] We specifically demonstrated increasing prevalence of TRAMs and the M184VI mutation, which is of some concern as tenofovir and emtricitabine/lamivudine remains the NRTI backbone of choice for first-line ART regimens. In the latter years (2013-2016), the pooled prevalence of the M184VI mutation was approximately 2% and the prevalence of TRAMs was 1%. Tenofovir and emtricitabine/lamivudine have been part of the standard first-line ART regimen in South Africa since 2010. The national drug resistance survey in 2013-14 showed that most people with virological failure on first-line NNRTI-based ART harboured the Met184Val/Ile mutation and about half had TRAMs.[48] Whilst our findings could be a signal of increasing transmission of NRTI-resistant virus, we urge some caution in interpretation. Viruses with the M184VI and K65R mutations are thought to be infrequently transmitted due to low transmission fitness.[49,50] If they are transmitted, the mutations revert rapidly in the absence of drug pressure.[51,60] It is possible that some of the sequences with NRTI resistance were obtained from people who reported themselves to be ART naïve

but who had previously been exposed to NRTIs. This is certainly plausible as there is an increasing frequency of cyclical engagement in care as ART programmes have matured.[61] Somewhat against that was the observation that the prevalence of TAMs did not change and remained very low (<1%) throughout the study period, although this may be a reflection of the diminished use of stavudine and zidovudine in first-line regimens.

We included a number of TRAMs that are not currently in the WHO SDRM list, but that are associated with TDF selection pressure.[16] We did identify sequences with these TRAMs, in particular the A62V mutation, which was present both with and without the signature K65R mutation. Further work is required to understand the significance of these mutations and their effect on response to TDF-based regimens.

Without appropriate action, PDR at the levels we have documented would be likely to have a significant impact on the HIV epidemic in South Africa. One mathematical model suggested that with PDR prevalence ≥10% and no change in the rates of resistance acquisition and transmission, 16% more AIDS deaths each year, 9% higher HIV incidence, and 8% higher ART costs would be attributable to drug resistance in Africa between 2016 and 2030.[62] Once prevalence of NNRTI PDR exceeds 10%, the WHO recommends that national programmes consider switching to an alternative non-NNRTI first-line ART regimen.[45] Many countries, including South Africa, have taken the decision to transition to a new first-line regimen of co-formulated generic tenofovir, lamivudine and dolutegravir (DTG).[63] This is the option that mathematical models have predicted will mitigate the effects of HIVDR, will produce the most health benefits and a reduction in overall programme cost.[64,65] However, there remain unanswered questions around DTG in the South African context, and strengthening of HIVDR surveillance and response systems will still be important to maximise the impact of the new regimen.[66,67]

An alternative approach to the modified first-line ART regimen would be to introduce pretreatment HIVDR testing and shift towards individualised drug regimens.[45] Whilst there is some evidence that HIVDR testing can be implemented in a research setting in South Africa,[68] there is no evidence that it can be delivered cost-effectively through the public health system. The shift towards more rapid initiation of ART (including same-day initiation) would make it particularly challenging to deliver pretreatment HIVDR testing. We still lack simple, rapid and inexpensive HIVDR assays, although there are promising technologies in development.[69] Given the increasing complexity of HIV care and the uncertainty about the long-term effectiveness of DTG-based regimens, there is still a need to develop and evaluate HIVDR assays and pretreatment HIVDR testing strategies.

We believe it would be a mistake to think that modifying the first-line ART regimen is an adequate response on its own to the rising levels of PDR. Whilst there will clearly be a reduced risk of drug resistance emergence with DTG-based regimens, the public health approach to ART creates scenarios where the risk may be higher, particularly where DTG is the only fully active agent in the regimen.[66,67] The increasing prevalence of PDR reflects weaknesses in prevention, treatment and care. Although South Africa implements routine viral load monitoring for people on ART, there are critical gaps in the viral load testing cascade and long delays in switching people with virological failure to second-line regimens.[70] This means there is probably an expanding pool of people with acquired HIVDR who can then transmit drug-resistant virus to susceptible individuals. Our findings therefore support calls to focus on improving the quality of HIV services.[71] This needs to be rooted within a broader multisectoral response, informed by high quality transdisciplinary research, that addresses the social and structural drivers of the epidemic.[72]

Interpretation of our findings should be subject to some limitations beyond those already discussed. Firstly, certain provinces were over-represented in our analysis, particularly KwaZulu-Natal and Gauteng, and estimates from the latter years were dominated by two large population-based surveillance studies from KwaZulu-Natal. Findings from the national PDR survey in 2013-14 suggested substantial heterogeneity between the provinces in levels of PDR, and therefore our estimates may not reflect the situation throughout the country.[8] Secondly, we pooled results from a number of individual studies, not all of which were designed to evaluate PDR. We did not account for individual study design in our analysis and derived only pooled crude estimates of prevalence. Our estimates should therefore not be taken to represent population prevalence. Lastly, we analysed only sequence data and were unable to explore differences by sex, age, CD4+ cell count, and duration of infection, as this information was not available for the majority of sequences.

In conclusion, we present evidence that the prevalence of PDR has risen substantially in South Africa in the past few years. Whilst this is predominantly NNRTI resistance, there is also evidence of rising levels of resistance to tenofovir and lamivudine/emtricitabine, although the absolute prevalence of PDR to these drugs remains low. Our findings support the decision to transition to a new, DTG-based first-line ART regimen. If the association between neural tube defects and DTG is confirmed, and NNRTIs continue to be recommended for women of childbearing age,[73] this evidence would suggest the need for additional interventions, such as pre-treatment genotypic resistance testing or early VL testing. These findings also highlight the need for broader strengthening of HIV services within the public health system if we are to eliminate HIV/AIDS as a public health threat by 2030.

Contributors

BC, RJL, S-YR and TDO were responsible for the study conception and design; BC, RJL, S-YR, AK, GH, PK, JM, and TDO were responsible for acquisition of data; BC, RJL, S-YR, JG, KN, LL, RS, AV, and TDO were responsible for data analysis; BC, RJL, S-YR, JG, AK, KN, LL, CC, DK, KAA, KD, RS, GH, AV, BS-P, MG, TM, PK, GR, JL, MK, LM, UMP, JWM, RWS, DK, PM, RKG, DP, SSAK and TDO were responsible for data interpretation, and critically revising the manuscript for important intellectual content. All authors approved the final version of the manuscript.

Declaration of interests and role of the funding source

We declare no competing interests. The funder had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding authors had full access to all the data and had final responsibility for the decision to submit the manuscript for publication.

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The manuscript in chapter 4 describes increasing levels of PDR in South Africa, most marked from 2010 when ART rollout was scaled-up, highlighting the decision to adopt a new first-line regimen that does not contain NNRTIs. However, changing regimens alone is only one key factor in addressing the glaring gaps challenging the ARV programme, viz, drug stock-outs, inadequate viral load monitoring and access to baseline resistance testing limited to research settings, amongst others. Currently, there is limited knowledge on the impact of DRMVs which are not reliably detected by Sanger sequencing, but could be selected for following ART initiation due to selective drug pressure. NGS has the ability to detect these DRMVs, but their clinical relevance is not well understood. The following manuscript is based on a study that aimed at assessing if standard genotypic resistance testing underestimates pretreatment resistance, and the impact DRMVs on clinical outcomes. This included testing the sensitivity and specificity of HIVDR thresholds in predicting virologic failure. The manuscript supplementary material is provided in Appendix 4.

CHAPTER 5: IMPACT OF PRE-TREATMENT DRUG RESISTANT MINORITY VARIANTS ON ANTIRETROVIRAL THERAPY IN HIV/TB CO-INFECTED PATIENTS

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Impact of HIV pretreatment drug resistant minority variants on antiretroviral therapy outcomes in HIV/TB co-infected patients

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Running title: Impact of HIV drug resistant minority variants on ART

Abstract

Objectives: To determine the impact of pretreatment drug resistant minority variants (DRMVs) on virological response to antiretroviral therapy (ART), and assess how thresholds of pre-ART DRMVs predict treatment failure.

Methods: A case-control study using plasma samples from adult HIV/TB co-infected patients. Cases were patients with confirmed viral loads ≥1,000 copies/mL after ≥6-months on ART, and controls were patients that achieved virological suppression throughout 24-months of ART follow-up. Samples were sequenced by Sanger sequencing and Illumina MiSeq next generation sequencing (NGS). Mutations were assessed using the Stanford HIV drug resistance database, and were analyzed at 2%, 5%, 10% and 20% thresholds. Associations between drug-class resistance and treatment response were assessed, and predictive accuracy of pre-treatment resistance for prediction of subsequent ART failure was estimated.

Results: Samples from 177 patients were analyzed (52 cases and 125 controls). Drug resistance prevalence was 6.2% (11/177) at pre-ART (i.e. 5 cases and 6 controls) by Sanger sequencing and NGS at 20%. The prevalence increased to 23.2% (41/179) when DRMVs at 2% were included (i.e. 14 cases and 27 controls). NNRTI-DRMVs at 5% were associated with ART failure (P=0.02). Lowering the detection threshold reduced the specificity from 97% (CI: 92-99) at 20%, to 93% (CI: 87-97) at 5% threshold.

Conclusions: NNRTI-DRMVs affect virological response to ART. NGS improved detection of drug resistance, but reduced the ability to identify patients at risk of virologic failure at lower thresholds. More studies assessing mutation thresholds predictive of virologic failure are required to inform use of NGS in treatment decisions.

Introduction

South Africa is one of the countries most affected by HIV and has the largest antiretroviral therapy (ART) program globally.[1] Tuberculosis remains the leading cause of death in people living with HIV in low- and middle-income countries (LMICs) such as South Africa.[2,3] HIV/TB co-infected patients have a higher risk of failing ART due to increased pill burden, overlapping toxicities, and programmatic challenges in integrating HIV/TB-care services.[3–6] This also increases their chances of developing HIV drug resistance (HIVDR).

Sanger sequencing has been the conventional method used for detecting HIVDR mutations, but it does not reliably detect mutations that occur at <20% of the viral population,[7] i.e. variants that are not well represented in the viral quasispecies. Various NGS platforms have been developed over the years, using different chemistries,[8] but all have the ability to produce high throughput data at relatively lower costs compared to Sanger sequencing, and have the ability to detect low frequency viral variants, also known as drug resistant minority variants (DRMVs).[8] However, the clinical impact of the DRMVs on clinical outcomes remains unclear and understudied in HIV-1 subtype C, and in HIV/TB co-infected patients.

A few studies have shown that pretreatment non-nucleoside reverse transcriptase inhibitor (NNRTI)-DRMVs have an impact on NNRTI-based ART outcomes,[9–13] whilst the CASTLE study (a prospective study in patients on first-line ART) showed no significant effect of transmitted DRMVs on PI-based regimens,[13] suggesting lack of adherence as a major cause of ART failure rather than pretreatment DRMVs.[14] Such inconsistencies warrant further research on DRMVs, if NGS technologies are to be used in routine clinical practice to inform treatment decisions. In this study we sought to assess the impact of pre-ART DRMVs at different thresholds in a cohort of HIV/TB co-infected patients (using NGS and Sanger sequencing), by comparing pretreatment drug resistance (PDR) profiles in patients that achieved viral suppression on ART to those that experienced virologic failure.

Patients and Methods

Ethics

This research study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference number: BF340/17). Ethical approval for the SAPiT (reference number: E107/05) and TRuTH (reference number: BF051/09) studies was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, and participants gave informed consent for sample storage and sample use for future studies.

Study design and study population

This was a nested case-control study aimed at assessing the impact of pretreatment DRMVs on ART. De-identified remnant plasma samples were obtained from a representative sample of HIV/TB co-infected adults (>18 years) from the Starting Antiretroviral therapy at three Points in Tuberculosis (SAPiT) trial. The study was an open-label, randomized, controlled trial conducted by the Centre for AIDS Programme Research in Africa (CAPRISA) between June 2005 and July 2008, at the eThekwini Clinical Research Site (ECRS) in Durban, South Africa. The study investigated the effect on mortality of antiretroviral therapy started during TB treatment (in two integrated-therapy groups) or after the completion of TB treatment (in one sequential-therapy group). Some SAPiT participants who went on to develop virological failure were identified through a subsequent study known as the TB Recurrence upon Treatment with HAART (TRuTH). Details of the SAPiT and TRuTH studies have been published previously.[15–18]

Samples from adult HIV/TB co-infected participants were selected in a 1:2 case control ratio. The cases included all participants enrolled in the SAPiT trial who had at least one viral load (VL) ≥1,000 copies/mL after ≥6 months on ART. The controls were unmatched randomly selected participants enrolled in the SAPiT trial who had 6-monthly VL's <1,000 copies/mL throughout follow-up for 24 months. The cases had two samples each, one at ART initiation (pre-ART) and another at virologic failure (ART failure), whilst the controls had only one sample from the pre-ART time-point. In cases where samples were not available at first high VL, a subsequent sample was accessed based on availability of remnant plasma.

Laboratory methods

Samples with VL ≥1,000 copies/mL were obtained for drug resistance testing. In summary, stored plasma samples were retrieved from storage at -80oC and thawed to room temperature prior to viral RNA extraction. For each sample, 500ul of plasma was centrifuged at 23,000g for 1 hour at 4oC to pellet the virus. Viral RNA was extracted from 200ul of pelleted plasma using a NucliSens EasyMAG HIV-1 (bioMerieux, France) extraction system. Protease (PR) and reverse transcriptase (RT) gene amplification was done using Southern African Treatment Resistance Network custom primers, as described previously.19 Successfully amplified polymerase chain reaction (PCR) products were purified using a Qiagen PCR purification kit (Qiagen, Germany), according to manufacturer's instructions. To limit sample variability in final sequencing product, purified PCR products of each sample were aliquoted for sequencing using Sanger sequencing and NGS.

Sanger sequencing

In preparation for capillary electrophoresis, sequencing reactions were done using a BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA), and sequence reaction purifications

using a BigDye XTerminator v3.1 purification kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's instructions. Capillary electrophoresis was done on a ABI 3730 genetic analyzer and the quality of sequences was assessed using Geneious software v8.1.9 (Biomatters Ltd, New Zealand).[20] Sequences with incomplete PR (codons: 1-99) and RT (codons: 1-254) genes were excluded as having poor sequence quality. Drug resistance mutations were detected using the Stanford University HIV drug resistance database.[21]

Next generation sequencing

For NGS, PCR product concentrations were determined using a Qubit 3.0 fluorometer (Life Technologies, Malaysia). The amplicons were diluted to 0.2ng/ul and library preparation was done using the Nextera-XT DNA Library Preparation kit and Nextera Index kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. In summary, library preparation involved kit-based enzymatic fragmentation of DNA, dual indexing of fragmented DNA, and bead-based purification of amplicons using AMPure beads (Beckman Coulter, Brea, CA, USA). Quality control steps were carried out using the LabChip GX Touch (PerkinElmer, Hopkinton, MA, USA) to determine the amplicon size, and library concentrations were determined by Qubit 3.0 fluorometer (Life Technologies, Malaysia). Each sample library was normalized to 4nM concentration and the normalized libraries were pooled and diluted to a final concentration of 10pM. The library at 10pM concentration was spiked with 5% PhiX control and run on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using the MiSeq Nano Reagent Kit v2 for 500 cycles.

Paired end sequencing analysis was done using the Polymorphism Analysis Sequencing (PASeq) software, which is a web-based accessible cloud based system (https://paseq.org/). In summary, the software used Trimmomatic for quality control in filtering sequences and for adapter trimming, and checking for external contamination. Gene coverage plots were generated, calling of deep variants was done, and querying of the Stanford HIVdb program with consensus sequence was done to assess resistance mutations. A report of the variants was provided with interpretations at different thresholds. The quality of NGS sequences was assessed in PASeq software and the depths of coverage were assessed in Genome Detective,[22] a web-based tool for analysis of molecular sequence data (https://www.genomedetective.com). Sequences with <1,000X depth of coverage or having incomplete PR (codons: 1-99) and RT (codons: 1-254) genes, were excluded as having poor sequence quality.

Drug resistance was defined as having a major PI resistance mutation, NRTI resistance mutation or a NNRTI resistance mutation. Patients with failed HIV genotyping at either time-point were excluded, in order to analyze complete sequence pairs from pre-ART to ART failure. Resistance mutations were analyzed at 2%, 5%, 10% and at 20% thresholds, to assess their effect on treatment outcome.

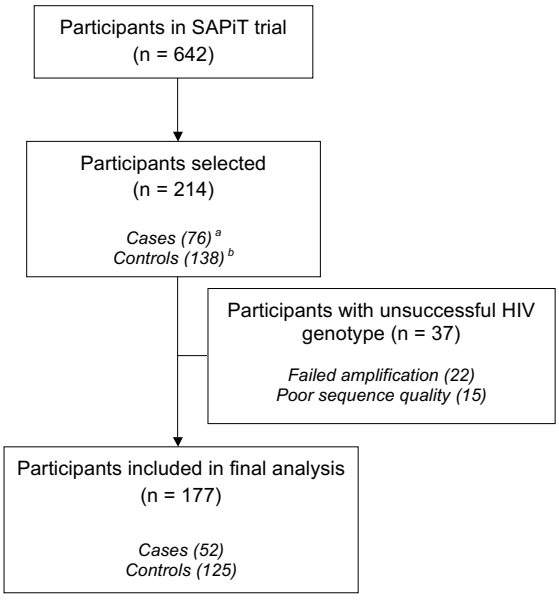
Data analysis

Statistical analysis was done using STATA v13 (StataCorp, College Station, TX, USA). We used the Fisher Exact test and Wilcoxon rank sum test (for categorical and continuous covariates respective) to compare baseline demographics (i.e. sex and age) and clinical characteristics (CD4 count, viral load, months on ART and SAPiT randomization arm) between cases and controls. Fisher's exact test and exact logistic regression were used to assess associations between participant demographic and clinical characteristics, and ART failure. The predictive accuracy of the different pre-ART thresholds in determining subsequent ART failure were assessed and presented as measures of sensitivity and specificity. Sensitivity represents the accuracy of the threshold in detecting patients that have pre-treatment drug resistance who experience virologic failure. Specificity represents the accuracy of the threshold in detecting patients that do not have pretreatment drug resistance who maintain virologic suppression.

Results

Participants characteristics

Two hundred and ninety samples were obtained from 214 participants, i.e. 152 case samples (from 76 participants) and 138 control samples. Two hundred and fifty-five of 290 samples were successfully amplified and 229 had complete NGS and Sanger sequence pairs; 104 case samples (from 52 participants), and 125 control samples (Figure 1).



[a] Cases included all participants enrolled in the SAPiT trial that had viral loads ≥1,000 c/ml after ≥6 months on ART

[b] Controls were randomly selected from SAPiT trial participants to match cases at a 1:2 ratio

Figure 1 Summary flow chart of participants from selection to analysis

All sequences were HIV-1 subtype C. All except 2 participants (175/177) received efavirenz (EFV), with lamivudine (3TC) and didanosine (ddI) at ART initiation. Table 1 summarizes the demographic and clinical characteristics of the participants included in the final analysis. No significant differences in these demographic and clinical characteristics were observed when comparing cases and controls.

Table 1 Baseline characteristics of participants included in final analysis

Characteristic	**Total (n=177)**	**Cases (n=52)**	**Controls (n=125)**	**p-value**
Female, *n (%)*	103 (58.2)	33 (63.5)	70 (56.0)	0.41
Age in years at baseline, median (IQR)	34 (29-40)	35 (27-39)	34 (29-42)	0.47
Viral load (log_{10} copies/mL) at baseline, median (IQR) [a]	5.3 (4.8-5.7)	5.2 (4.8-5.7)	5.3 (4.8-5.7)	0.58
CD4 count (cells/mm^3) at baseline, median (IQR)	140 (60-223)	107 (42-218)	150 (78 - 228)	0.22
Months on ART before virologic failure, median (IQR)	-	16 (9-37)	-	-
Treatment arms				
Early, *n (%)*	52 (29.4)	14 (26.9)	38 (30.4)	0.80
Post-intensive, *n (%)*	67 (37.9)	19 (36.5)	48 (38.4)	
Post continuation, *n (%)*	58 (32.8)	19 (36.5)	39 (31.2)	

ART, antiretroviral therapy; CD4, cluster of differentiation 4; IQR, interquartile range;

[a]1 case and 2 controls with missing viral loads at pre-ART

Pre-ART resistance data

Overall, of 177 pre-ART sequences, 11 (6.2%) had at least one drug resistance mutation detected by Sanger sequencing and NGS at 20% (5 cases and 6 controls). All except one sequence (case) had single-class resistance at pre-ART, and the most common major mutation was K103N, which was detected in 4 of 11 sequences with NGS resistance at 20%. When DRMVs were included in the analysis, the levels of pre-ART drug resistance increased from 1.1% (at 20%) to 6,2% (at 2%) for PIs, from 1.1% (at 20%) to 11.9% (at 2%) for NRTIs**,** and from 4.5% (at 20%) to 9.0% (at 2%) for NNRTIs. Table 2 summarizes the pre-ART drug-class mutations observed by NGS at different mutation thresholds.

Table 2. Proportion of pre-ART drug-class resistance by NGS mutation thresholds

	Detection threshold			
	2%	**5%**	**10%**	**20%**
Overall resistance (n=177)				
Any resistance, *n (%)*	41 (23.2)	19 (10.7)	14 (7.9)	11 (6.2)
Any PI major resistance, *n (%)*	11 (6.2)	5 (2.8)	2 (1.1)	2 (1.1)
Any NRTI resistance, *n (%)*	21 (11.9)	7 (4.0)	5 (2.8)	2 (1.1)
Any NNRTI resistance, *n (%)*	16 (9.0)	9 (5.1)	8 (4.5)	8 (4.5)
Controls (n=125)				
PI major resistance, *n (%)*	9 (7.2)	4 (3.2)	2 (1.6)	2 (1.6)
NRTI resistance, *n (%)*	12 (9.6)	4 (3.2)	4 (3.2)	1 (0.8)
NNRTI resistance, *n (%)*	9 (7.2)	3 (2.4)	3 (2.4)	3 (2.4)
Cases (n=52)				
PI major resistance, *n (%)*	2 (3.9)	1 (1.9)	0 (0)	0 (0)
NRTI resistance, *n (%)*	9 (17.3)	3 (5.8)	1 (1.9)	1 (1.9)
NNRTI resistance, *n (%)*	7 (13.5)	6 (11.5)	5 (9.6)	5 (9.6)

PI, protease inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; ART, antiretroviral therapy

The proportion of DRMVs were determined at variant frequency thresholds of <20%. Thirty of 177 (17.0%) pre-ART sequences had DRMVs only, with no mutations at 20%. DRMVs by drug class proportion at pre-ART were as follows: 4.5% (8/177) for PIs (1 case and 7 controls), 10.2% (18/177) for NRTIs (7 cases and 11 controls), and 4.0% (7/177) for NNRTIs (2 cases and 3 controls). The most common DRMV at pre-ART was the K65R mutation, occurring in 6 of 177 (3.4%) sequences. The median frequency of K65R at pre-ART was 2.8% (interquartile range (IQR): 2.2 – 3.6), occurring as the only mutation in 5 of the 6 sequences. Pre-ART NNRTI resistance at 5% was significantly associated with ART failure (P=0.02; OR: 5.3). Figure 2 shows the prevalence of pre-ART DRMVs only, by cases and controls.

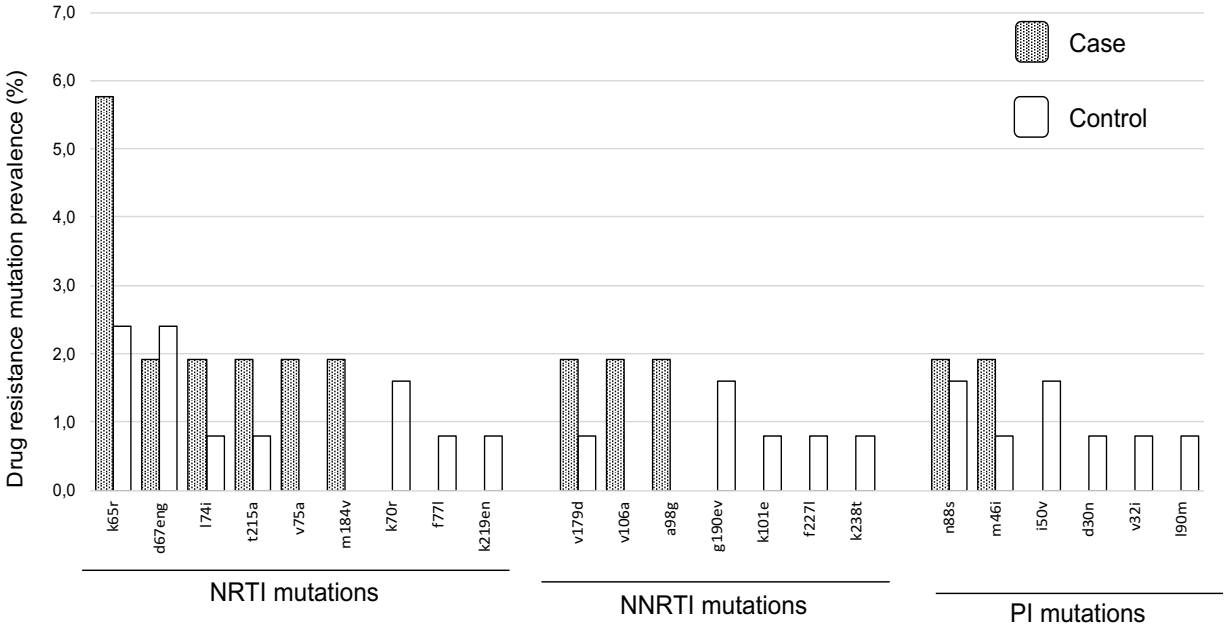


Figure 2 DRMVs observed in sequences with pre-ART resistance

ART failure resistance data

At ART failure, 78.9% (41/52) of sequences had drug resistance mutations detected at 2%, with 73.1% (38/52) having drug resistance at 20%. Seven of the 52 cases had switched from a NNRTI- to a PI-based regimen at time of virologic failure, and only 2 of the 7 had drug resistance mutations at 20%, with no PI resistance mutations observed (Supplementary Table S1). The median time on ART was 16 months (IQR: 9-37), and there was no significant association between duration on ART and presence of drug resistance mutations at 20% (P=0.58). Sex, age, CD4 counts and VLs were not significantly associated with ART failure (Supplementary Table S2).

Twenty-seven of 52 (51.9%) had dual-class resistance and 11 (21.2%) had single class resistance at 20%, at ART failure. The most common major NNRTI mutation at ART failure was V106M, occurring in 44.2% (23/52) of sequences, whilst M184VI was the most common NRTI mutation, occurring in 40.4% (21/54) of sequences, with no PI mutations detected at ART failure. There was no clear trend in selection of DRMVs between pre-ART and ART failure time-points, with only 4 of the 52 cases having pre-ART DRMVs occurring at 20%, at ART failure. The mutations selected for were NRTI mutations, K65R, D67N and L74I, and an NNRTI mutation V106AI, which occurred as a V106M mutation at ART failure (Supplementary Table S3).

Predictive accuracy of pre-ART resistance

We tested accuracy measures of the different thresholds in determining treatment failure outcome. Lowering the detection threshold from 20% to 2% resulted in an increased sensitivity from 9% (95%

confidence interval (CI): 3-20) to 33% (95% CI: 21-48), with a reduction in specificity from 97% (95% CI: 92-99) to 79% (95% CI: 71-86). Among participants classified as having resistance at 2% threshold, 40.9% (18/44) went on to experience virologic failure, whilst among those below this threshold 73.3% (99/135) maintained viral suppression on treatment. We observed a large reduction in specificity when shifting from the 5% to 2% threshold (93% to 79%) with a corresponding 3-fold increase in sensitivity (11% to 33%). However the 20% threshold showed the highest discriminative power (maximum diagnostic odds ratio) as shown in Table 3.

Table 3 Measures of sensitivity and specificity of pretreatment drug resistance thresholds

DRM threshold	Sensitivity	Specificity	Diagnostic OR
20%	9% (3-20)	97% (92-99)	3.1 (0.9-11.1)
10%	9% (3-20)	94% (89-98)	1.7 (0.6-5.4)
5%	11% (4-23)	93% (87-97)	1.6 (0.6-4.6)
2%	33% (21-48)	79% (71-86)	1.9 (0.9-3.9)

DRM, drug resistance mutation; OR, odds ratio

Discussion

NGS increased detection of resistance at pre-ART, and showed high concordance for detecting mutations at 20% when compared to Sanger sequencing. Pre-ART NNRTI resistance at 5% showed a significant association with developing virologic failure on ART. This is similar to previous studies that suggest NNRTI-DRMVs impact ART outcomes,[9,23,24] but is also contradictory to studies such as the OCTANE 2 trial,[25] which showed no impact on ART, with a recent study from the ANRS 12249 trial showing dual-class resistance (NRTI and NNRTI) prolonging time to viral suppression, rather than NNRTI resistance alone.[26] However, measures of predictive accuracy of each drug resistance threshold showed reduced specificity from 97% (CI: 92-99) at 20%, to 93% (CI: 87-97) at 5%, with a further reduction to 79% (CI: 71-86) at 2%. These results are relatively consistent with a multi-country nested case control study that showed the specificity reduce from 98% (CI: 95-99) at 20% threshold, to 96% (CI: 92-98) at 5% threshold.[27] Such reductions in specificity will have huge cost implications especially in LMICs which have the highest numbers of patients requiring ART, and could pose challenges to ART programs, in maintaining optimal treatment monitoring and retention of patients in care.

Notably, K65R was the most common DRMV, occurring at only 2% threshold in all 6 sequences with the K65R mutation at pre-ART. Considering the mutation occurred at very low frequencies at pre-ART and was not readily selected by ART drug pressure, there is a chance the K65R DRMVs (i.e. at 2%) detected resulted from PCR and sequencing errors.[28,29] Previous studies have shown that HIV-1 subtype C is more likely to develop the K65R mutation due to a homopolymeric region between RT

codon positions 63 and 65. This causes preferential pausing of the RT enzyme at the "AAG-to-AGG" position, resulting in dislocation mutagenesis that causes the K65R mutation.[30] However, the stringent quality control measures in trimming sequences, and including only sequences with >100X coverage, increased the confidence in calling mutations at low frequencies, up to 2%. K65R was also the third most common NRTI mutation at ART failure, which is concerning as it causes intermediate to high-levels of resistance to almost all NRTIs except AZT, and importantly, high-levels of resistance to tenofovir (TDF),[21] a drug that has become the main NRTI agent in current and future first-line regimens.[31] High rates of K65R DRMVs have also been reported recently in a Malawian cohort,[32] and previously as major mutations in South African patients failing first-line ART,[33,34] warranting further research on pre-ART K65R mutations.

Interestingly, NNRTI mutations had the least pre-ART DRMVs, but the most prevalent major mutations at pre-ART and at ART failure (Figures 2). The most common NNRTI mutations at ART failure occurred at positions where mutations are known to be highly selected for by EFV (i.e. positions 103, 106, 188 and 190).[21] High-levels of NNRTI resistance in first-line ART failures have been reported previously in a South African national survey,[34] with common mutations at positions 103 and 106. Among participants failing ART on PIs with no major mutations and good ART adherence, investigating linked-mutations outside the pol gene could determine the cause of failure, as previous studies have shown mutations in Gag cleavage sites [35,36] that are linked with PI-resistance. This suggests utility of whole genome sequencing in people failing ART.

This study had limitations which should be taken into consideration when interpreting these findings. The proportion of participants with pre-ART NNRTI-DRMVs at 5% was quite similar to those with mutations at 20% (Figure 1), reducing our certainty in associating NNRTI-DRMVs at 5% to ART failure. The similarity could suggest major mutations as the main contributor to ART failure, rather than the mutations at 5% threshold. Another limitation was the lack of participant drug concentration levels to definitively show that they were taking their treatment at time of ART failure. However, intensive counseling and adherence support were provided to the study participants, with an approximately 97% adherence rate according to monthly pill counts, as reported previously.[15] This would suggest adequate drug pressure for the selection of mutations, making the study population ideal to assess impact of pretreatment DRMVs on ART. However, factors such as drug-drug interactions, and GI toxicity leading to malabsorption could have affected the ability to determine the effect of the mutations on ART outcomes, warranting further research in HIV/TB co-infected patients.

Most participants in this study initiated ART on ddI, a drug that is not commonly used in current regimens. This is because remnant plasma samples were used in order to identify a significant number of HIV/TB co-infected patients that initiate and fail treatment at a later time-point. However, EFV

remains a common first-line regimen for the foreseeable future,[37] despite recommendations to switch to dolutegravir (DTG), a cheaper and better tolerated antiretroviral drug.[38,39] This is because of the risk of neural tube birth defects when DTG is used in pregnancy,[31,40] as well as the lack of evidence supporting use of DTG in patients on HIV/TB treatment.[41] The INSPIRING study has shown good efficacy and safety of twice daily DTG in HIV/TB co-infected adults treated with rifampicin.[42] However, more studies on use of DTG in HIV/TB co-infected patients and in women of child bearing potential are required.[43]

The participants had a relatively short follow-up period, median 16 months (Table1) on ART, as determined by the parent study.[15] Long-term follow-up is suggested in future studies, and more studies in individuals on recent regimens are required, to assess the importance of these DRMVs, regardless of the introduction of INSTIs. Lastly, we excluded 37 participants (Figure 1) due to failed amplification and poor sequence quality, most of which were case samples (24/37). This could have affected the sensitivity, given the smaller number of ART failure events when stratified by thresholds. Larger studies testing these diagnostic measures are required.

In conclusion, NNRTI-DRMVs have the potential to cause ART failure. These results suggest that a detection threshold of 5% in NNRTI pre-ART resistance could be considered to inform treatment response. However, more research is required to determine an optimal threshold that could be used for predicting virologic failure. Our findings add to the paucity in knowledge around the impact of DRMVs on ART, and suggest the need for studies addressing this research question.

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Transparency declarations

None to declare

Conflicts of Interest

We declare no conflicts of interest.

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6.1 Key Themes

Surveillance for HIVDR remains an integral part of successful ART programs. Attempts to treat HIV, without monitoring viral response to ART regimens, may be undermined by development of drug resistant virus, an inevitable phenomenon in any setting where ART is used. More people with HIV infection have been initiated on ART following the adoption of the UTT strategy. However, the supporting structures of ART programs seem to be failing due to the increasing demands of an extensive ART roll-out, especially in LMICs like South Africa. This is due to challenges such as reaching HIV positive individuals, testing and initiating them on ART, having continual linkage and retention in care, maintaining consistent access to ARV drugs and viral load testing, adherence support, and a timely response to switching patients to effective ART regimens once they develop treatment failure (Figure 11). Achieving all these steps in the cascade of care is challenging, especially in an HIV epidemic, such as in South Africa, and strengthening of the healthcare systems is imperative to the control of HIVDR.

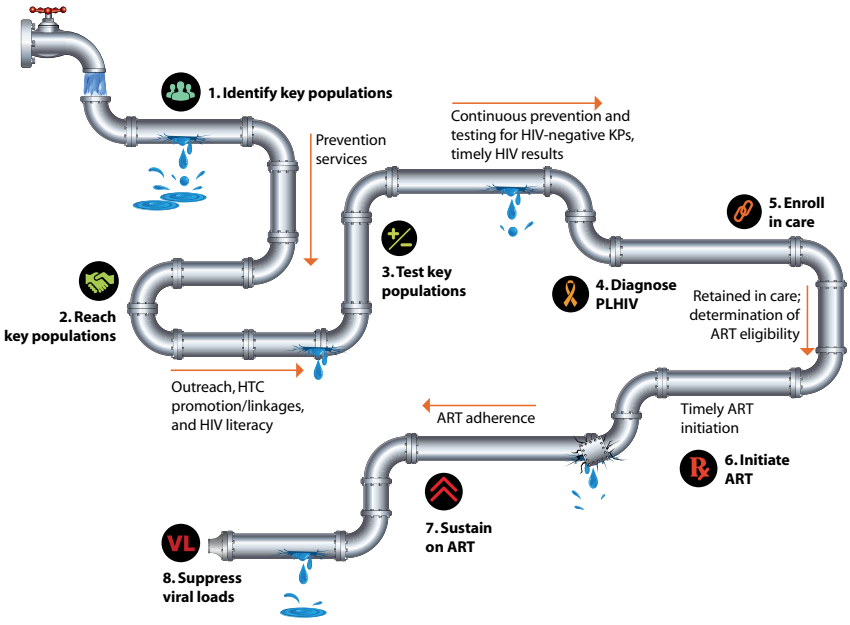

Figure 11 Healthcare system challenges in the HIV prevention, treatment and care
(Reproduced as is from the HIV cascade framework for key populations; USAID and PEPFAR, 2015: https://www.fhi360.org/sites/default/files/media/documents/linkages-hiv-cascade-framework-oct15.pdf)

The weaknesses of the health care system are evident by the increasing levels of drug resistance presented in chapters 3 and 4. Results presented in these chapters show that PDR levels exceeded 10% by 2014 in South Africa, the threshold considered by the WHO as indicating the need for urgent public health action. The public health action entails replacing NNRTI drugs in first-line ART once NNRTI-PDR has reached ≥10%, or testing for drug resistance mutations in all HIV positive people, before initiating them on ART. Drug resistance testing is costly and is available as a specialized test in most LMICs. One of the major limitations to the analysis of PDR was the lack of information on drug concentration levels from the different studies. Such information is important to determine prior ART exposure. However, considering that drug concentration level tests are not done in routine practice when people are initiated on ART, these results are still representative of the general population of people that are initiated on treatment as ART naïve. Furthermore, there is a high burden of HIV in South Africa, where approximately 270,000 new HIV infections occurred in 2017 [8]. Thus, changing the standard first-line ART regimen may be a more feasible option to pretreatment drug resistance testing and drug concentration level tests. Additionally, the levels of PDR exceeded the WHO critical threshold approximately 4 years before the findings were actually reported, which suggests that implementation of routine HIVDR surveillance in South Africa is needed to proactively identify such outcomes more rapidly, such as with the roll-out of DTG in the public sector.

In addition to surveillance monitoring, rapid switching of regimens in patients with virologic failure on TDF/FTC/EFV (TEE) is essential to reduce the risk of TDF-resistance associated mutations (TRAMs) developing and being transmitted. This is supported by phylogenetic results presented in chapter 3, suggesting that, most drug resistance transmission events are occurring from treatment experienced to treatment naïve individuals. Despite the noticeable increase in PDR shown in chapter 4, including that of NRTIs, the levels of TDF resistance remained relatively low over the years. However, when separated into three time periods (i.e. 2000 to 2008, 2009 to 2012 and 2013 to 2016), the levels of TRAMs increased significantly over time, prompting further research to understand whether or not this increase was due to transmission of TRAMs, or due to more people cycling in and out of care. This is of concern for DTG-based ART, as TDF is the main NRTI-backbone in current first-line regimens and in the impending DTG-based ART regimen, which will constitute TDF/3TC/DTG (TLD) [95]. This further emphasizes the need for surveillance of TDF resistance, especially among people already on TEE, as high-levels of the K65R mutation that confers resistance to TDF have been reported previously [96,97]. We included the M46I/L mutation in the analysis of sDRMs in chapter 3 despite the revision in the WHO global report in 2012 excluding the mutation. This could have slightly over estimated the levels of PI resistance, but still includes all possible mutations that could confer resistance to LPV/r and ATV/r, commonly used PI drugs in South Africa.

The results in chapter 5 show K65R as the most common DRMV at pre-ART, occurring at only 2% threshold. It has been suggested previously that subtype C virus is more prone to develop this mutation due to a homopolymeric region ending in the K65R position. This causes preferential pausing of the reverse transcriptase enzyme at the "AAG-to-AGG" position, resulting in dislocation mutagenesis that causes the K65R mutation [98]. Moreover, as the detection threshold is reduced, the chances of sequencing errors which could result in inaccurate mutation calls increases [99], which could explain the lack of selection of the mutation under drug pressure, as shown in chapter 5. However, the stringent quality control measures taken in trimming sequences, as well as including only sequences with a sequencing depth >100X, increased the confidence in calling mutations at low frequencies, up to 2% threshold. It is possible that these K65R DRMVs are the reason why patients failing on TDF-based ART in South Africa have high rates of K65R mutations [96,97], suggesting potential selection of the K65R DRMVs to become major resistance mutations at ART failure, due to drug pressure. Unfortunately, the study in chapter 5 could not support this hypothesis as only one patient had a pre-ART K65R DRMV selected for to become a major mutation at ART failure.

However, the study did show that as the detection threshold is reduced to <20%, more mutations are detected, at the expense of correctly predicting which patients will fail treatment. The paper shows a reduction in specificity from 97% (92-99) at 20% threshold, to 94% (89-98) at 10% threshold, to 93% (87-97) at 5% threshold, and to 79% (71-86) at 2% threshold. Therefore, reducing the detection threshold increases the chances of detecting more drug resistance mutations, but the mutations do not seem to affect virologic response to ART. Therefore, the accuracy of resistance as a predictor of virologic failure, decreases with a reduction in the detection threshold. Similar findings were described by a hypothetical case [100], which suggested that reducing the detection threshold from 20% to 1% could result in a 300% increase in patients misclassified as likely to fail treatment [100]. Therefore, in a population of 100,000 patients starting treatment, 5400 patients with mutations at 1% threshold will be incorrectly predicted as patients likely to fail ART, which may have huge cost implications as more people might not benefit from cheaper first-line drug options, if low frequency mutations are considered. Thus in South Africa which has a high HIV prevalence and where standard ART regimens are used for treatment initiation, careful consideration of the benefits and shortcomings of detecting DRMVs is vital for public health decision making.

The paper in chapter 5 also showed that having any pre-ART NNRTI resistance (at ≥2%) was significantly associated with ART failure, although cautious interpretation of the effect of the DRMVs is required, considering that only a few patients had pre-ART NNRTI-DRMVs occurring without mutations at 20%. These results are consistent with previous studies (in non-TB patients) showing that NNRTI-DRMVs are associated with ART failure [78–80,101]. However, there are conflicting findings regarding the impact of these mutations in different settings. In contrast to the OCTANE

Trial 1, which demonstrated an increased risk of virologic failure due to underlying K103N and Y181C DRMVs in patients initiated on NVP-based first-line ART following single-dose nevirapine exposure for prevention of mother-to-child transmission of HIV [102], the OCTANE Trial 2 showed no such effect of DRMVs on ART [103]. Thus understanding the impact of DRMVs on ART is complex and may not be properly explored in a single study but through incremental studies in different contexts, involving patients with different clinical characteristics. For example, some mutations can be selected for more than others, due to their low fitness cost, whilst the effect could also be due to the number of copies of a particular DRMV (i.e. mutational load), prior exposure to treatment and patient adherence to treatment. Therefore, understanding factors which contribute to poor treatment outcomes in patients with DRMVs is a process, which may require several studies.

In addition to understanding the complex effect of DRMVs on ART, NGS should become more feasible and accessible to clinicians caring for patients, especially in LMICs. Data analysis of NGS results is challenging and time consuming, partly due to the numerous sequence reads that are generated, requiring expert analysis [104]. NGS pipelines for data analysis such as Genome Detective [105], PASeq and HyDRA [104,106], which require minimal bioinformatics support are becoming increasingly relevant, in reducing the time and expertise required to analyse vast NGS data, as well as reducing variability in data analysis through automation. Moreover, considering that modern production scale NGS sequencers such as the Illumina NovaSeq 6000 produce approximately 6000 GB of output data per run (illumina.com), storage of the large amounts of data is costly and might require high performance computing systems for data processing. These challenges may be addressed through combined efforts of scientists, data analysts and policy makers.

6.2 Recommendations for policy

Considering the increasing levels of NNRTI-associated drug resistance shown in chapters 3 and 4, and the significant association between NNRTI-DRMVs and ART failure in chapter 5, changing first-line regimens from NNRTI- to DTG-based ART (i.e. TEE to TLD)) may be the most logical public health response in South Africa, especially with neighbouring Botswana already using DTG in the public health sector [107]. However, considering the HIV burden in South Africa, existing ART program structures need to be able to support the policies which will be implemented in transitioning to TLD, i.e. the system in which the ART program will prioritize patients that initiate TLD is critical. Venter et al., in 2017 posed three scenarios for transitioning to DTG-based ART; i) a conservative approach, which includes starting new patients on DTG-based ART and transitioning all treatment experienced patients over a 3-year period, ii) a moderate approach, which includes starting new patients on DTG-based ART and transitioning treatment experienced patients over a 2-year period, iii) and an aggressive approach, which includes everyone receiving DTG-based ART within a year [108].

A conservative approach in South Africa may be a better option, especially with such a high HIV burden. However, such an approach may cause undesirable delays in realizing the benefits of DTG.

The WHO currently recommends use of DTG in first-line ART for treatment initiators, in second-line ART following exposure to first-line NNRTI, and in third-line ART with ritonavir-boosted darunavir and NRTIs [109]. DTG is therefore an integral part of ART programs for the future. Since DTG will continue being used in combination with other ARV drugs, maintaining virus susceptibility to NRTIs and PIs is important to the success of DTG. If mutations to other drug classes are not closely monitored, there is a risk of DTG being used as the only fully functional ARV drug in a regimen. Such a situation may result in DTG monotherapy, which must be avoided especially because of high rates of resistance selection which occur due to DTG-monotherapy [110]. Therefore, strategies which strengthen the HIV treatment cascade must be enforced to alleviate the extent of drug resistance to less potent drugs and to ensure the success of DTG-containing regimens. Furthermore, optimizing VL monitoring is crucial as more people are started on ART and are likely to transmit mutations by remaining on failing regimens for prolonged periods.

A study from South Africa reported patients remaining on in-effective first-line ART regimens for of up to 27 months (interquartile range: 17 – 40) [55] before the ART regimen was changed. Strategic policy which enforces improvements in the coverage and quality of VL monitoring, management of virologic failure, and early switching of all patients showing virologic failure on NNRTI-based ART is recommended. It is important to continually attend to the objectives of the national strategic plan, which suggest the need for both routine and non-routine population and sentinel surveys, i.e. HIV prevalence surveys, antenatal surveys, and drug resistance surveys, to name a few [111]. These surveys are important to generate periodic estimates of HIV, but require coordination and routine implementation if they are to be effective. Facility based sentinel surveys (such as in antenatal women) may be easier to implement, but do not clearly reflect accurate measures of HIV in the general population. Despite the surveillance strategy used, implementation of surveys should be simplified, and should focus on timely monitoring that gives accurate measures within the general population, without adding further strain to the HIV treatment program.

Future targeted interventions should be intensified for areas known to have high HIV prevalences, such as in KZN province. Moreover, as NGS slowly replaces Sanger sequencing as the preferred genotyping method, there should be considerations of the cost of setting up and maintaining NGS structures that are sustainable. Therefore, as studies address questions around the importance of DRMVs in policy decisions, strategies in making NGS available and accessible are also required. Centralizing NGS testing may help to reduce infrastructural costs and improve accessibility of NGS in most LMICs. Furthermore, as multiplexing of samples using NGS reduces the cost of genotyping

[68], centralizing NGS will help achieve the required numbers of samples in a relatively shorter time, reducing both the cost of the assay as well as the turn-around time of results. Therefore, in the context of HIVDR, it is important for policies to focus on enhancing HIVDR surveillance monitoring, implementing and enforcing viral load monitoring, as well as considering how to integrate NGS testing in informing treatment decisions, in a timely manner.

6.3 Recommendations for future research

Future research focusing on three main areas is required. Firstly, developing workable solutions for real-time monitoring and surveillance, secondly, assessment of treatment outcomes on DTG-containing ART, and thirdly, continual assessment of the effect of pretreatment DRMVs on ART. If surveillance of drug resistance was being done regularly in South Africa, then the levels of resistance reported in chapters 3 and 4 could have contributed to the WHO's recommendation for the use of DTG, as was suggested for Uganda, Zimbabwe, Namibia, Nicaragua, Guatemala and Argentina, all of which had reported pre-ART NNRTI resistance at ≥10%, by 2017 [53]. Prior to this study, only two South African studies reported levels of NNRTI-PDR close to 10%. These are the studies done on antenatal samples in 2016 [72] and in the Western Cape on adult ART naïve samples in 2017 [73], both of which reported NNRTI-PDR levels at 8.3%. Most sequences contributing to the high levels of NNRTI resistance (chapters 3 and 4) were from patients living in HIV hyper-endemic areas of the KZN province. This highlights the need for more research in understudied provinces in South Africa, with continual research in areas where HIV incidence rates are known to be high.

The levels of resistance before and after the implementation of DTG based ART in the South African ART programme should be assessed to better inform future public health policy. Therefore, studies specifically investigating and reporting mutation profiles in patients who fail DTG-based ART will be important in making decisions on future drug regimens. Further research is needed to investigate drug resistance in patients receiving DTG with multiple resistance to NRTIs, NNRTIs and PIs, to assess whether they still manage to maintain viral suppression on DTG. In addition, studies assessing how DRMVs contribute to treatment response, such as the individual and cumulative effect of DRMVs, drug-class mutational loads, clinically relevant detection thresholds, as well as DRMV patterns in patients with HIV co-infections, such as TB, are crucial. Among patients that fail treatment with no mutations (and have good ART adherence), further research on linked-mutations outside the pol gene; such as in the Gag cleavage sites [112,113], is required to determine the cause of failure. The study in chapter 5 had very few patients on PIs at ART failure, and could not investigate this linkage as sequencing was only done for the *protease* and *reverse transcriptase* genes. This also suggests the importance of HIV whole genome sequencing at ART failure, to investigate all possible linked mutations across the viral genome.

In conclusion, the work presented in this thesis provides the first evidence of high level NNRTI-PDR (>10%) in KZN and South Africa, and further evidence of virologic failure due to pre-ART NNRTI resistance in HIV/TB coinfected patients in KZN. South Africa needs to enroll another 3 million people on ART in addition to all those who become newly infected over the next few years, to effectively control the HIV epidemic. Furthermore, to minimize chances of these new infections, PrEP will need to be implemented and scaled up, preferably with the same ARVs which are used in first-line ART. However, for ART to be an effective treatment as prevention strategy, more potent ARVs need to be made readily available and accessible. In addition, it will be crucial to implement surveillance of HIVDR, further innovative research, and improve laboratory capacity to perform large scale HIVDR testing. Whilst the response to these research findings may include modification of the standard first-line ART regimen, these findings also present a broader public health implication for improving the quality of HIV prevention and treatment care services, thus ending HIV by 2030, in South Africa.

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APPENDICES

Appendix 1 Biomedical research ethics approval

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06 July 2018

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Dear Mr Chimukaranga

Title: Impact of HIV-1 Drug Resistant Minority Variants on Antiretroviral Therapy (ART).
Degree: PhD
BREC Ref No: BE340/17

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 11 July 2018
Expiration of Ethical Approval: 10 July 2019

I wish to advise you that your application for Recertification received on 24 May 2018 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

This approval will be **ratified** by a full Committee at its meeting taking place on **14 August 2018.**

Yours sincerely

Prof V Rambiritch
Chair: Biomedical Research Ethics Committee

cc Supervisor: tullodna@gmail.com
cc Postgraduate Administrator: dudhrajhp@ukzn.ac.za

Appendix 2 Supplementary material to manuscript entitled "Moderate to high levels of pre-treatment HIV drug resistance in KwaZulu-Natal Province, South Africa"

Table S1. Estimated prevalence of pre-treatment HIV drug resistance by sex and age

	Pre-treatment HIVDR Prevalence					
	Female		Male		Total	
	n/N	% (95% CI)	n/N	% (95% CI)	n/N	% (95% CI)
Study A						
15-24 years	13/121	10.7 (5.8-17.7)	2/24	8.3 (1.0-27.0)	15/145	10.3 (5.9-16.5)
25-34 years	17/162	10.5 (6.2-16.3)	1/55	1.8 (0.0-9.7)	18/217	8.3 (5.0-12.8)
35-44 years	7/84	8.3 (3.4-16.4)	3/41	7.3 (1.5-19.9)	10/125	8.0 (3.9-14.2)
45+ years	8/88	9.1 (4.0-17.1)	5/36	13.9 (4.7-29.5)	13/124	10.5 (5.7-17.3)
Study B[a]						
15-24 years	34/248	14.1 (8.6-19.5)	6/66	7.9 (0.9-14.9)	40/314	12.2 (7.9-16.5)
25-34 years	52/299	17.0 (11.9-22.1)	20/208	8.1 (4.2-12.0)	72/507	11.9 (8.6-15.1)
35-44 years	22/203	8.5 (4.4-12.6)	8/116	7.5 (1.8-13.2)	30/319	8.0 (4.5-11.5)
45+ years	9/64	11.2 (1.1-21.3)	5/30	15.0 (1.2-28.7)	14/94	13.0 (4.9-21.1)

[a] Data for study B have been weighted to adjust for the survey design and for non-response across age and gender categories

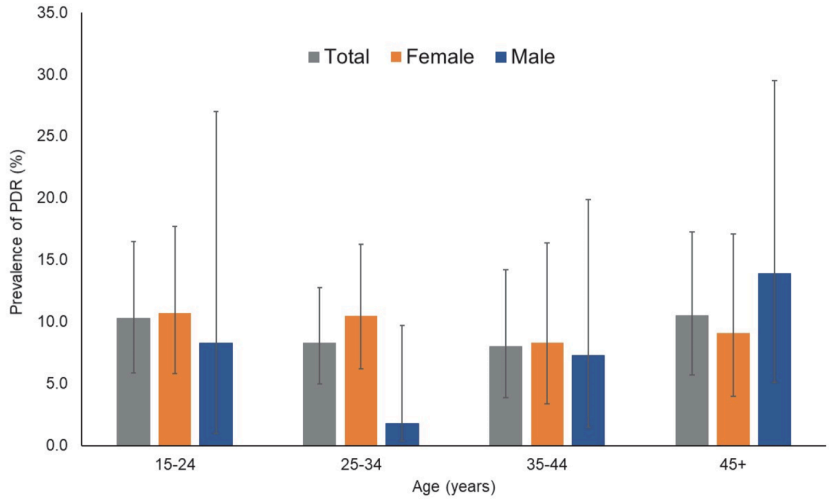
Table S2. Most frequently observed patterns of surveillance drug resistance mutations

Mutations	Study A	Study B	Overall
K103NS	24 (42.9)	76 (48.7)	100 (47.2)
M46IL	1 (1.8)	11 (7.1)	12 (5.7)
V106AM	1 (1.8)	8 (5.1)	9 (4.2)
M184V, K103NS, P225H	4 (7.1)	3 (1.9)	7 (3.3)
M184V, K103NS	3 (5.4)	2 (1.3)	5 (2.4)
G190AS	1 (1.8)	3 (1.9)	4 (1.9)
K101EP	0	3 (1.9)	3 (1.4)
Y181C	0	3 (1.9)	3 (1.4)
M230L	0	3 (1.9)	3 (1.4)
K103NS, P225H	1 (1.8)	2 (1.3)	3 (1.4)
M41L	1 (1.8)	2 (1.3)	3 (1.4)
Other	20 (35.7)	40 (25.6)	60 (28.3)
Total	56	156	212

Specific SDRM patterns are listed if observed in three or more participants overall

Figure S1

A


35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Prevalence of PDR (%)
Total
Female
Male
15-24
25-34
35-44
45+
Age (years)

B

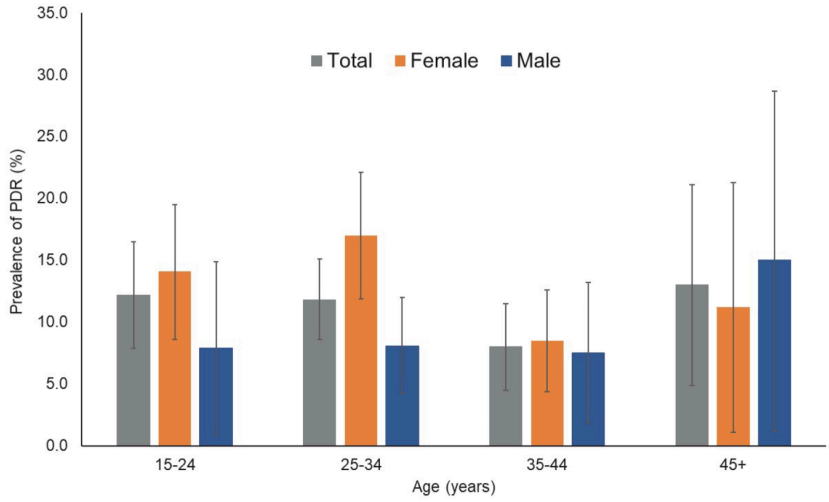
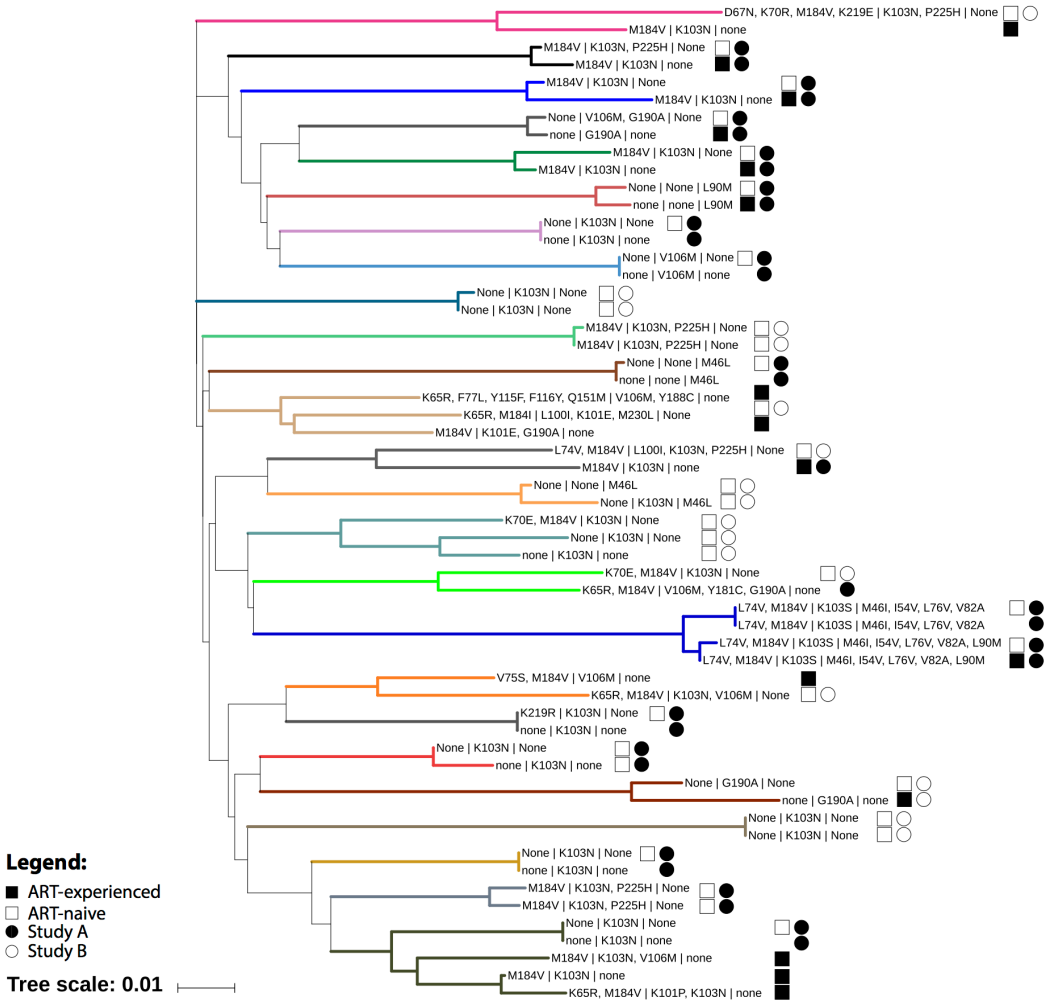

35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Prevalence of PDR (%)
Total
Female
Male
15-24
25-34
35-44
45+
Age (years)

Figure S2


D67N, K70R, M184V, K219E | K103N, P225H | None
M184V | K103N | none
M184V | K103N, P225H | None
M184V | K103N | none
M184V | K103N | None
M184V | K103N | none
None | V106M, G190A | None
none | G190A | none
M184V | K103N | None
M184V | K103N | none
None | None | L90M
none | none | L90M
None | K103N | None
none | K103N | none
None | V106M | None
none | V106M | none
None | K103N | None
None | K103N | None
M184V | K103N, P225H | None
M184V | K103N, P225H | None
None | None | M46L
none | none | M46L
K65R, F77L, Y115F, F116Y, Q151M | V106M, Y188C | none
K65R, M184I | L100I, K101E, M230L | None
M184V | K101E, G190A | none
L74V, M184V | L100I, K103N, P225H | None
M184V | K103N | none
None | None | M46L
None | K103N | M46L
K70E, M184V | K103N | None
None | K103N | None
none | K103N | none
K70E, M184V | K103N | None
K65R, M184V | V106M, Y181C, G190A | none
L74V, M184V | K103S | M46I, I54V, L76V, V82A
L74V, M184V | K103S | M46I, I54V, L76V, V82A
L74V, M184V | K103S | M46I, I54V, L76V, V82A, L90M
L74V, M184V | K103S | M46I, I54V, L76V, V82A, L90M
V75S, M184V | V106M | none
K65R, M184V | K103N, V106M | None
K219R | K103N | None
none | K103N | none
None | K103N | None
none | K103N | none
None | G190A | None
none | G190A | none
None | K103N | None
None | K103N | None
None | K103N | None
none | K103N | none
M184V | K103N, P225H | None
M184V | K103N, P225H | None
None | K103N | None
none | K103N | none
M184V | K103N, V106M | none
M184V | K103N | none
K65R, M184V | K101P, K103N | none
Legend:
ART-experienced
ART-naive
Study A
Study B
Tree scale: 0.01

Description on timing of recruitment and ascertainment of antiretroviral therapy status for the two population-based studies

Study A

In 2013, samples were collected between 22 January and 27 November, and the median date of sample collection was 5 June 2013, whilst in 2014, samples were collected between 21 January and 30 November, and the median date of sample collection was 18 July 2014.

To complement the population-based surveillance research, the Africa Health Research Institute (formerly Africa Centre for Population Health) has maintained a clinical database for all people treated with antiretroviral therapy (ART) at 17 primary health care clinics and one district hospital in the Hlabisa sub-district. This database holds records for people who have received ART since 2004, the start of the public sector ART roll-out. Data from the clinical database are linked with the population-based surveillance data by deterministic record linkage (using the unique South African ID number, if recorded) or probabilistic record linkage (using first name, surname, date of birth, and sex). The database has a variable for date of ART initiation, so for the purposes of this analysis, we could determine whether there had been any use of ART (for treatment) prior to the date of surveillance sample collection used for genotypic resistance testing. The clinical database does not hold information on antiretroviral regimens for prevention of mother-to-child transmission (pMTCT) prior to 2013, i.e. single-dose nevirapine regimens with or without zidovudine and/or single-dose tenofovir/emtricitabine. It also does not hold information on use of antiretrovirals for pre-exposure or post-exposure prophylaxis, but use in these circumstances was very low over the study period. The database does not hold information about people who accessed ART in the public sector outside Hlabisa sub-district. The database also does not hold information about people who accessed ART in the private sector. Private sector ART use is low in the study area, due to the low levels of private health insurance and good access to ART in the public sector.

Study B

In 2014, samples were collected between 7 January and 12 December, and the median date of sample collection was 26 August 2014, whilst in 2015, samples were collected between between 4 January and 6 December, and the median date of sample collection was 28 April 2015.

In the HIV Incidence Provincial Surveillance System (HIPSS), the survey included questions about antiretroviral use, which were asked to any participant who reported being HIV positive. The first question asked was ‘Has a doctor or nurse told you that

you need to take ARVs?' If the answer to this question was yes, then the participant was asked 'Are you still on ARVs?' In addition, female participants were asked the question, 'Have you ever been pregnant while you were HIV positive?' If the answer to this was yes, they were asked 'Which of the following services did you access while HIV positive and pregnant?' One option for this question was 'Medication to prevent mother-to-child transmission'. The survey did not ask questions about use of antiretrovirals for pre-exposure or post-exposure prophylaxis.

From these questions, we determined whether there had been any use of antiretrovirals for treatment or pMTCT prior to the date of sample collection.

Appendix 3 Supplementary material to manuscript entitled "Trends in pretreatment HIV-1 drug resistance in antiretroviral therapy-naive adults in South Africa, 2000 – 2016: a pooled sequence analysis"

Supplementary Table 1 PRISMA-IPD Checklist

PRISMA-IPD Checklist of items to include when reporting a systematic review and meta-analysis of individual participant data (IPD)

PRISMA-IPD Section/topic	Item No	Checklist item	Reported on page
Title			
Title	1	Identify the report as a systematic review and meta-analysis of individual participant data.	Yes
Abstract			
Structured summary	2	Provide a structured summary including as applicable:	Yes
		Background: state research question and main objectives, with information on participants, interventions, comparators and outcomes.	
		Methods: report eligibility criteria; data sources including dates of last bibliographic search or elicitation, noting that IPD were sought; methods of assessing risk of bias.	
		Results: provide number and type of studies and participants identified and number (%) obtained; summary effect estimates for main outcomes (benefits and harms) with confidence intervals and measures of statistical heterogeneity. Describe the direction and size of summary effects in terms meaningful to those who would put findings into practice.	
		Discussion: state main strengths and limitations of the evidence, general interpretation of the results and any important implications.	
		Other: report primary funding source, registration number and registry name for the systematic review and IPD meta-analysis.	
Introduction			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Yes
Objectives	4	Provide an explicit statement of the questions being addressed with reference, as applicable, to participants, interventions, comparisons, outcomes and study design (PICOS). Include any hypotheses that relate to particular types of participant-level subgroups.	Yes
Methods			
Protocol and registration	5	Indicate if a protocol exists and where it can be accessed. If available, provide registration information including registration number and registry name. Provide publication details, if applicable.	N/A
Eligibility criteria	6	Specify inclusion and exclusion criteria including those relating to participants, interventions, comparisons, outcomes, study design and characteristics (e.g. years when conducted, required minimum follow-up). Note whether these were applied at the study or individual level i.e. whether eligible participants were included (and ineligible participants excluded) from a study that included a wider population than specified by the review inclusion criteria. The rationale for criteria should be stated.	Yes
Identifying studies - information sources	7	Describe all methods of identifying published and unpublished studies including, as applicable: which bibliographic databases were searched with dates of coverage; details of any hand searching including of conference proceedings; use of study registers and agency or company databases; contact with the original research team and experts in the field; open adverts and surveys. Give the date of last search or elicitation.	Yes
Identifying studies - search	8	Present the full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Yes
Study selection processes	9	State the process for determining which studies were eligible for inclusion.	Yes
Data collection processes	10	Describe how IPD were requested, collected and managed, including any processes for querying and confirming data with investigators. If IPD were not sought from any eligible study, the reason for this should be stated (for each such study).	Yes
		If applicable, describe how any studies for which IPD were not available were dealt with. This should include whether, how and what aggregate data were sought or extracted from study reports and publications (such as extracting data independently in duplicate) and any processes for obtaining and confirming these data with investigators.	
Data items	11	Describe how the information and variables to be collected were chosen. List and define all study level and participant level data that were sought, including baseline and follow-up information. If applicable, describe methods of standardising or translating variables within the IPD datasets to ensure common scales or measurements across studies.	Yes
IPD integrity	A1	Describe what aspects of IPD were subject to data checking (such as sequence generation, data consistency and completeness, baseline imbalance) and how this was done.	Yes
Risk of bias assessment in individual studies.	12	Describe methods used to assess risk of bias in the individual studies and whether this was applied separately for each outcome. If applicable, describe how findings of IPD checking were used to inform the assessment. Report if and how risk of bias assessment was used in any data synthesis.	Yes
Specification of outcomes and effect measures	13	State all treatment comparisons of interests. State all outcomes addressed and define them in detail. State whether they were pre-specified for the review and, if applicable, whether they were primary/main or secondary/additional outcomes. Give the principal measures of effect (such as risk ratio, hazard ratio, difference in means) used for each outcome.	Yes
Synthesis methods	14	Describe the meta-analysis methods used to synthesise IPD. Specify any statistical methods and models used. Issues should include (but are not restricted to): • Use of a one-stage or two-stage approach. • How effect estimates were generated separately within each study and combined across studies (where applicable). • Specification of one-stage models (where applicable) including how clustering of patients within studies was accounted for. • Use of fixed or random effects models and any other model assumptions, such as proportional hazards. • How (summary) survival curves were generated (where applicable). • Methods for quantifying statistical heterogeneity (such as I^2 and τ^2).	Yes

		• How studies providing IPD and not providing IPD were analysed together (where applicable). • How missing data within the IPD were dealt with (where applicable).	
Exploration of variation in effects	A2	If applicable, describe any methods used to explore variation in effects by study or participant level characteristics (such as estimation of interactions between effect and covariates). State all participant-level characteristics that were analysed as potential effect modifiers, and whether these were pre-specified.	Yes
Risk of bias across studies	15	Specify any assessment of risk of bias relating to the accumulated body of evidence, including any pertaining to not obtaining IPD for particular studies, outcomes or other variables.	Yes
Additional analyses	16	Describe methods of any additional analyses, including sensitivity analyses. State which of these were pre-specified.	Yes
Results			
Study selection and IPD obtained	17	Give numbers of studies screened, assessed for eligibility, and included in the systematic review with reasons for exclusions at each stage. Indicate the number of studies and participants for which IPD were sought and for which IPD were obtained. For those studies where IPD were not available, give the numbers of studies and participants for which aggregate data were available. Report reasons for non-availability of IPD. Include a flow diagram.	Yes
Study characteristics	18	For each study, present information on key study and participant characteristics (such as description of interventions, numbers of participants, demographic data, unavailability of outcomes, funding source, and if applicable duration of follow-up). Provide (main) citations for each study. Where applicable, also report similar study characteristics for any studies not providing IPD.	Yes
IPD integrity	A3	Report any important issues identified in checking IPD or state that there were none.	Yes
Risk of bias within studies	19	Present data on risk of bias assessments. If applicable, describe whether data checking led to the up-weighting or down-weighting of these assessments. Consider how any potential bias impacts on the robustness of meta-analysis conclusions.	Yes
Results of individual studies	20	For each comparison and for each main outcome (benefit or harm), for each individual study report the number of eligible participants for which data were obtained and show simple summary data for each intervention group (including, where applicable, the number of events), effect estimates and confidence intervals. These may be tabulated or included on a forest plot.	Yes
Results of syntheses	21	Present summary effects for each meta-analysis undertaken, including confidence intervals and measures of statistical heterogeneity. State whether the analysis was pre-specified, and report the numbers of studies and participants and, where applicable, the number of events on which it is based.	Yes
		When exploring variation in effects due to patient or study characteristics, present summary interaction estimates for each characteristic examined, including confidence intervals and measures of statistical heterogeneity. State whether the analysis was pre-specified. State whether any interaction is consistent across trials.	
		Provide a description of the direction and size of effect in terms meaningful to those who would put findings into practice.	
Risk of bias across studies	22	Present results of any assessment of risk of bias relating to the accumulated body of evidence, including any pertaining to the availability and representativeness of available studies, outcomes or other variables.	Yes
Additional analyses	23	Give results of any additional analyses (e.g. sensitivity analyses). If applicable, this should also include any analyses that incorporate aggregate data for studies that do not have IPD. If applicable, summarise the main meta-analysis results following the inclusion or exclusion of studies for which IPD were not available.	Yes
Discussion			
Summary of evidence	24	Summarise the main findings, including the strength of evidence for each main outcome.	Yes
Strengths and limitations	25	Discuss any important strengths and limitations of the evidence including the benefits of access to IPD and any limitations arising from IPD that were not available.	Yes
Conclusions	26	Provide a general interpretation of the findings in the context of other evidence.	Yes
Implications	A4	Consider relevance to key groups (such as policy makers, service providers and service users). Consider implications for future research.	Yes
Funding			
Funding	27	Describe sources of funding and other support (such as supply of IPD), and the role in the systematic review of those providing such support.	Yes

A1 – A3 denote new items that are additional to standard PRISMA items. A4 has been created as a result of re-arranging content of the standard PRISMA statement to suit the way that systematic review IPD meta-analyses are reported.

Supplementary Table 2 Search strategy in OVID Medline

#	Searches	Results
1	exp HIV/	93755
2	HIV·mp [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	304579
3	1 or 2	304579
4	South Africa/	37442
5	South Africa·mp [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	42835
6	4 or 5	42835
7	exp Drug Resistance, Viral/	45943
8	exp Sequence Analysis/	340523
9	Genes, pol/	1114
10	Genotyping Techniques/	4991
11	exp Molecular Epidemiology/	33833
12	exp Genetic Variation/	998697
13	phylogenetic*·mp [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	105550
14	resistan*·mp [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier, synonyms]	882013
15	7 or 8 or 9 or 10 or 11 or 12 or 13 or 14	2062123
16	3 and 6 and 15	856

Supplementary Table 3 List of WHO 2009 surveillance drug-resistance mutations (SDRMs), with eight additional tenofovir resistance-associated mutations (TRAMs)

NRTI mutations	NNRTI mutations	PI mutations
M41L	L100I	L23I
A62V	K101E	L24I
K65R	K101P	D30N
K65N	K103N	V32I
D67N	K103S	M46I
D67G	V106M	M46L
D67E	V106A	I47V
S68G	V179F	I47A
S68D	Y181C	G48V
S68N	Y181I	G48M
T69D	Y181V	I50V
T69ins	Y188L	I50L
K70R	Y188H	F53L
K70E	Y188C	F53Y
K70Q	G190A	I54V
K70T	G190S	I54L
L74V	G190E	I54M
L74I	P225H	I54A
V75M	M230L	I54T
V75T		I54S
V75A		G73S
V75S		G73T
V75L		G73C
F77L		G73A
Y115F		L76V
F116Y		V82A
Q151M		V82T
M184V		V82F
M184I		V82S
L210W		V82C
L215Y		V82M
L215F		V82L
L215I		N83D
L215S		I84V
L215C		I84A
L215D		I84C
L215V		I85V
L215E		N88D
K219Q		N88S
K219E		L90M
K219N		
K219R		

NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor

Mutations in italics represent the eight additional tenofovir resistance-associated mutations

Supplementary Table 4 Details of excluded studies

Source	Sampling years	Province(s)	Study type	Study population	Number of HIV-1 *pol* sequences	Reason for exclusion
Abrahams[1]	2004-2005	KZN	Acute infection cohort	Adults with acute HIV infection	5	<10 *pol* sequences
Eshleman[2]	1993-2001	Not stated	Laboratory validation study	Asymptomatic blood donors	15	Samples collected prior to 2000
Hora[a]	2007-2008	Not stated	Unpublished	Not known	6	<10 *pol* sequences
Iweriebor[3]	2008	LP	Drug resistance study	ART-naïve adult	1	<10 *pol* sequences
Li[4]	2001-2005	GT	Prevention of mother-to-child transmission study	ART-naïve women prior to single dose nevirapine	99	Targeted sequencing for K65R mutation only
Liu[5]	Not stated	KZN	Acute infection cohort	Adults with acute HIV infection	9	<10 *pol* sequences
Musyoki[6]	2009	LP	Case report (recombinant strain)	Single ART-naïve female	1	<10 *pol* sequences
Orrell[7]	2003-2006	WC	PDR study	ART-naïve adults	120	Sequences not obtained
Rademeyer[8]	2007	KZN	Acute infection cohort	Adults with acute HIV infection	4	<10 *pol* sequences
Rousseau[9]	2000-2006	KZN	Chronic infection cohort	ART-naïve adults receiving HIV care (and specifically women in antenatal care)	248	Sequences duplicated from Matthews
Van Harmelen[10]	Not stated	KZN, WC	Acute and chronic infection cohorts	Recently and chronically infected adults	4	<10 *pol* sequences
Wilkinson[11]	1998-2004	WC	Laboratory samples from multiple sources	HIV-positive adults (multiple cohorts)	7	<10 *pol* sequences
Wright[12]	2003-2006	KZN	Chronic infection cohort	ART-naïve adults	405	Protease sequence only (no *RT* sequences)
Wright[13]	2008-2009	KZN	Acute infection cohort	Adults with acute HIV infection	32	Protease sequence only (no *RT* sequences)

ART, antiretroviral therapy; EC, Eastern Cape; FS, Free State; GT, Gauteng; KZN, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NS, not stated; NW, North West; PDR, pretreatment drug resistance; WC, Western Cape

[a] Unpublished

Supplementary Table 5 Additional details of included datasets

Dataset ID	Author	Urban/rural	Ages[a]	CD4+ cell count (cells/μL)	Recent vs chronic infection	Specimen type	Sequencing method[b]	VL criterion for sequencing (copies/mL)
1	Bessong	Rural	20-53 yrs	Median 334	-	Plasma	In house	NS
2	Bessong	Rural	20-53 yrs	NS	-	Plasma	In house	NS
3	Chimukangara	Rural	15-88 yrs	NS	Previous negative HIV ELISA (surveillance)	DBS	In house	10 000
4	Chimukangara	Rural	16-78 yrs	NS	Previous negative HIV ELISA (surveillance)	DBS	In house	10 000
5	Chimukangara	Rural	15-49 yrs	NS	-	Plasma	In house	1000
6	Gordon	Urban & rural	Mean 38 yrs	Mean 366	-	Plasma/DBS	Viroseq	NS
7	Hamers	Urban & rural	-	Median 94-140[c]	-	Plasma	In house	NS
8	Huang	Urban & rural	Mean 36 yrs	Mean 271	-	Plasma	Viroseq/In house	NS
9	Hunt	Urban & rural	18-24 yrs	NS	BED assay	Serum	In house	NS
10	Hunt	Urban & rural	18-21 yrs	NS	BED assay	Serum	In house	NS
11	Hunt	Urban & rural	18-22 yrs	NS	BED assay	Serum	In house	NS
12	Hunt	Urban & rural	18-24 yrs	NS	BED assay	Serum	In house	NS
13	Hunt	Urban & rural	18-21 yrs	NS	BED assay	Serum	In house	NS
14	Hunt	Urban & rural	Median 19 yrs	NS	-	NS	-	NS
15	Hunt	Urban & rural	<25 yrs	NS	-	NS	-	NS
16	Hunt	Urban & rural	<21 yrs	NS	-	NS	-	NS
17	Iweriebor	Urban	6-52 yrs	NS	-	Plasma	In house	NS
18	Jacobs	Urban	NS	NS	-	Plasma	In house	NS
19	Jacobs	Urban	21-50 yrs	Mean 375	-	Plasma	In house	NS
20	Manasa	Rural	18-57 yrs	Median 413	Previous negative HIV ELISA (surveillance)	Plasma	In house	NS
21	Manasa	Rural	Mean 34 yrs	NS	Previous negative HIV ELISA (surveillance)	DBS	In house	10 000
22	Manasa	Rural	Mean 34 yrs	NS	Previous negative HIV ELISA (surveillance)	DBS	In house	10 000
23	Matthews	Urban	NS	Median 387	-	Plasma	In house	NS
24	Msimanga	Rural	16-41 yrs	Mean 450	-	Plasma	In house	NS
25	Musyoki	Urban	NS	NS	-	Plasma	In house	100 000
26	Nwobegahay	Urban & rural	18-69 yrs	Mean 138	-	Plasma	In house	NS

27	Papathanasopoulos	Urban	21-55 yrs	Median 403	-	Plasma	In house	NS
28	Parboosing	Urban	15-20 yrs	NS	-	Plasma	Viroseq	NS
29	Parikh	Urban	18-40 yrs	NS	-	Plasma	Viroseq/In house	200
30	Pillay	Urban	NS	Median 479	-	Plasma	In house	NS
31	Pillay	Urban & rural	18-21 yrs	NS	-	Serum	In house	NS
32	Pillay	Urban & rural	18-21 yrs	NS	-	Serum	In house	NS
33	Seoighe	Urban	NS	NS	-	NS	In house	NS
34	Steegen	Urban & rural	Median 34 yrs	Median 149	-	Plasma	In house	NS
35	Treurnicht	Urban	NS	Median 558	Acute infection[d]	Plasma	In house	NS
36	van Zyl	Urban & rural	Mean 34 yrs	Median 337	-	Plasma	In house	NS
37	Wilkinson	Urban & rural	21-61 yrs	NS	-	Plasma	In house	NS
38	Wilkinson	Urban & rural	21-61 yrs	NS	-	Plasma	In house	NS

BED, BED IgG-Capture Enzyme Immunoassay; DBS, dried blood spots; ELISA, enzyme-linked immunosorbent assay; NS, not stated; VL, viral load

[a] Ages are stated as range, unless otherwise stated; [b] All in-house sequencing systems used Sanger sequencing methods; [c] Range of median CD4+ cell count across three South African study sites; [d] Acute infection defined as the detection of HIV-1 antibodies within five months of a previously negative HIV-1 test, or evidence of HIV-1 viral replication in the absence of HIV-1 antibodies

Supplementary Table 6 Details of publications and HIV sequence accession numbers

Dataset ID	Source	Journal	PMID	GenBank accession numbers	PopSet
1	Bessong	AIDS Res Hum Retro	15665650	AY510031-AY510056	40846255, 40846281
2	Bessong	AIDS Res Hum Retro	17209775	DQ222243-DQ222317	77812531, 77812451
3	Chimukangara	AIDS Res Hum Retro	30430843	NA	NA
4	Chimukangara	AIDS Res Hum Retro	30430843	NA	NA
5	Chimukangara	AIDS Res Hum Retro	30430843	NA	NA
6	Gordon	J Virol	12551997	AY136957-AY137008, AY196498-AY196517	28557514
7	Hamers	Lancet Infect Dis	21802367	HQ994353-HQ994917	NA
8	Huang	Antivir Ther	19918101	KT736966-KT737213	1004353616
9	Hunt	Clin Infect Dis	22544199	NA	NA
10	Hunt	Clin Infect Dis	22544199	NA	NA
11	Hunt	Clin Infect Dis	22544199	NA	NA
12	Hunt	Clin Infect Dis	22544199	NA	NA
13	Hunt	Clin Infect Dis	22544199	NA	NA
14	Hunt	Comm Dis Surv Bull	NA	KY060489-KY060546, KY060662-KY060711	NA
15	Hunt	Comm Dis Surv Bull	NA	KY060053-KY060129, KY060309-KY060389, KY060547-KY060596, KY060712-KY060773, KY061063-KY061127,	NA
16	Hunt	Comm Dis Surv Bull	NA	KY060016-KY060052, KY060130-KY060157 KY060390-KY060488, KY060597-KY060661, KY060774-KY061062, KY061128-KY061145,	NA
17	Iweriebor	Arch Virol	22189822	GU201754-GU201826	284434133, 282895359
18	Jacobs	AIDS Res Hum Retro	18593350	EF602162-EF602301	148612215, 148612233, 149394577
19	Jacobs	PLoS ONE	24609015	KF793121-KF793185	NA
20	Manasa	AIDS Res Hum Retro	22251009	JN664970-JN665041	374094941
21	Manasa	AIDS Res Hum Retro	27002368	NA	NA
22	Manasa	AIDS Res Hum Retro	27002368	NA	NA
23	Matthews	J Virol	18596105	FJ199532-FJ199992	212382549

28	Parboosing	56	56	56	56
29	Parikh	353	352	352	352
30	Pillay	37	37	0	0
31	Pillay	58	58	0	0
32	Pillay	43	43	0	0
33	Seoighe	300	300	0	0
34	Steegen	277	277	277	277
35	Treurnicht	15	15	15	15
36	van Zyl	59	59	59	59
37	Wilkinson	29	29	29	29
38	Wilkinson	32	32	32	32
	Total	**7025**	**6880**	**6501**	**6294**

Supplementary Table 8 Patterns of single class, dual class and triple class resistance in HIV-1 sequences with any drug resistance mutation

Pattern of resistance	Number of sequences	Proportion (95% CI)
Single class resistance	**389**	**81·4 (77·6-84·8)**
NRTI	*50*	*10·5 (7·9-13·6)*
NNRTI	*289*	*60·5 (55·9-64·9)*
PI	*50*	*10·5 (7·9-13·6)*
Dual class resistance	**87**	**18·2 (14·8-22·0)**
NRTI/NNRTI	*79*	*16·5 (13·3-20·2)*
NRTI/PI	*4*	*0·8 (0·2-2·1)*
NNRTI/PI	*4*	*0·8 (0·2-2·1)*
Triple class resistance	**2**	**0·4 (0·1-1·5)**
NRTI/NNRTI/PI	*2*	*0·4 (0·1-1·5)*

CI, confidence interval; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor

Supplementary Table 9 Pooled prevalence of specific non-nucleoside reverse transcriptase inhibitor resistance mutations

Mutation	n	% of sequences with any DRM (n=478)	% of all sequences (n=6880)
L100I	5	1·05	0·07
L101E	27	5·65	0·39
L101P	2	0·42	0·03
K103N	271	56·69	3·94
K103S	7	1·46	0·10
V106M	46	9·62	0·67
V106A	1	0·21	0·01
V179F	0	0·00	0·00
Y181C	34	7·11	0·49
Y181I	0	0·00	0·00
Y181V	0	0·00	0·00
Y188L	11	2·30	0·16
Y188H	0	0·00	0·00
Y188C	5	1·05	0·07
G190A	25	5·23	0·36
G190S	2	0·42	0·03
G190E	0	0·00	0·00
P225H	23	4·81	0·33
M230L	6	1·26	0·09

DRM, drug resistance mutation

Supplementary Table 10 Pooled prevalence of specific drug resistance mutations by time period

Drug resistance mutations	2000-2008 (N = 2480)		2009-2012 (N = 2219)		2013-2016 (N = 2181)		p value
	n	% (95% CI)	n	% (95% CI)	n	% (95% CI)	
NRTI resistance mutations							
M184VI	4	0·16 (0·04-0·41)	20	0·90 (0·55-1·39)	47	2·15 (1·59-2·86)	<0·001
TRAMs	3	0·12 (0·02-0·35)	11	0·50 (0·25-0·89)	23	1·05 (0·67-1·58)	<0·001
TAMs	10	0·40 (0·19-0·74)	10	0·45 (0·22-0·83)	19	0·87 (0·53-1·36)	0·071
L74V or Y115F	0	-	2	0·09 (0·01-0·33)	7	0·32 (0·13-0·66)	0·008
NNRTI resistance mutations							
K101EP	5	0·20 (0·06-0·47)	9	0·41 (0·19-0·77)	15	0·69 (0·39-1·13)	0.038
K103NS	37	1·49 (1·05-2·05)	81	3·65 (2·91-4·52)	160	7·34 (6·28-8·51)	<0.001
V106AM	2	0·08 (0·01-0·29)	17	0·77 (0·45-1·22)	28	1·28 (0·85-1·85)	<0.001
Y181C	13	0·52 (0·28-0·89)	10	0·45 (0·22-0·83)	11	0·50 (0·25-0·90)	0.934
G190ASE	7	0·28 (0·11-0·58)	9	0·41 (0·19-0·77)	11	0·50 (0·25-0·90)	0.477

CI, confidence interval; TAMs, thymidine analogue mutations; TRAMs, tenofovir resistance-associated mutations

Supplementary Table 11 Pooled prevalence of specific nucleoside reverse transcriptase inhibitor resistance mutations

Mutation	n	% of sequences with any DRM (n=478)	% of all sequences (n=6880)
M41L	14	2·93	0·20
A62V	10	2·09	0·15
K65R	21	4·39	0·31
K65N	0	0·00	0·00
D67N	10	2·09	0·15
D67G	4	0·84	0·06
D67E	2	0·42	0·03
S68G	0	0·00	0·00
S68D	0	0·00	0·00
S68N	0	0·00	0·00
T69D	4	0·84	0·06
T69ins	0	0·00	0·00
K70R	8	1·67	0·12
K70E	4	0·84	0·06
K70Q	0	0·00	0·00
K70T	2	0·42	0·03
L74V	6	1·26	0·09
L74I	1	0·21	0·01
V75M	0	0·00	0·00
V75T	0	0·00	0·00
V75A	2	0·42	0·03
V75S	0	0·00	0·00
V75L	0	0·00	0·00
F77Le	0	0·00	0·00
Y115F	4	0·84	0·06
F116Y	1	0·21	0·01
Q151M	1	0·21	0·01
M184V	65	13·60	0·94
M184I	6	1·26	0·09
L210W	3	0·63	0·04
L215Y	2	0·42	0·03
L215F	3	0·63	0·04
L215I	0	0·00	0·00
L215S	0	0·00	0·00
L215C	0	0·00	0·00
L215D	2	0·42	0·03
L215V	1	0·21	0·01
L215E	1	0·21	0·01
K219Q	3	0·63	0·04
K219E	6	1·26	0·09
K219N	2	0·42	0·03
K219R	6	1·26	0·09

DRM, drug resistance mutation

Supplementary Table 12 Pooled prevalence of specific protease inhibitor resistance mutations

Mutation	n	% of sequences with any DRM (n=478)	% of all sequences (n=6294)[a]
L23I	1	0·21	0·02
L24I	1	0·21	0·02
D30N	0	0·00	0·00
V32I	1	0·21	0·02
M46I	19	3·97	0·34
M46L	16	3·35	0·28
I47V	2	0·42	0·04
I47A	0	0·00	0·00
G48V	0	0·00	0·00
G48M	0	0·00	0·00
I50V	1	0·21	0·02
I50L	0	0·00	0·00
F53L	0	0·00	0·00
F53Y	1	0·21	0·02
I54V	0	0·00	0·00
I54L	0	0·00	0·00
I54M	0	0·00	0·00
I54A	0	0·00	0·00
I54T	1	0·21	0·02
I54S	0	0·00	0·00
G73S	1	0·21	0·02
G73T	0	0·00	0·00
G73C	0	0·00	0·00
G73A	0	0·00	0·00
L76V	0	0·00	0·00
V82A	1	0·21	0·02
V82T	0	0·00	0·00
V82F	2	0·42	0·04
V82S	0	0·00	0·00
V82C	0	0·00	0·00
V82M	0	0·00	0·00
V82L	0	0·00	0·00
N83D	1	0·21	0·02
I84V	0	0·00	0·00
I84A	0	0·00	0·00
I84C	0	0·00	0·00
I85V	6	1·26	0·11
N88D	0	0·00	0·00
N88S	1	0·21	0·02
L90M	5	1·05	0·09

DRM, surveillance drug resistance mutation

[a] Denominator is number with complete PR sequences

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Appendix 4 Supplementary material to manuscript entitled "Impact of HIV pretreatment drug resistant minority variants on antiretroviral therapy in HIV/TB co-infected patients

Table S1 Subset of patients receiving PI-based ART at time of virologic failure

Sample ID	2nd line regimen	Date 2nd line ART initiation	Date sample collected	Months on 2nd line ART	Mutations at 20%
SAP026	ABC+AZT+LPV/r	04/02/2010	11/11/2010	9.3	None
SAP098	ddI+3TC+LPV/r	19/08/2008	03/02/2009	5.6	K65R, D67N, V106M, Y188C
SAP100	TDF+AZT+LPV/r	05/08/2010	07/06/2012	22.4	K103N
SAP194	ABC+TDF+LPV/r	14/10/2008	21/10/2008	0.2	None
SAP200	TDF+AZT+LPV/r	08/12/2008	05/01/2009	0.9	None
SAP206	ABC+AZT+LPV/r	01/06/2009	06/07/2009	1.2	None
SAP221	TDF+AZT+LPV/r	16/04/2009	27/02/2012	35.9	None

ABC, abacavir; ART, antiretroviral therapy; AZT, zidovudine; ddI, didanosine; ID, identification; LPV/r, ritonavir-boosted lopinavir; TDF, tenofovir

Note: Dates are formatted as dd/mm/yyyy

Table S2 Associations between demographic and clinical characteristics with ART failure

	p-value	Odds ratio	95% confidence interval
Continuous variables			
Age	0.47	0.986	0.948 – 1.024
CD4 count (cells/mm^3)	0.22	0.998	0.995 – 1.001
Viral load (log_{10} copies/mL)	0.58	0.880	0.566 – 1.374
Categorical variables			
Sex	0.41	1.362	0.669 – 2.828

ART, antiretroviral therapy mm^3, CD4, cluster of differentiation 4; cubic millimeter; mL, milliliter

Note: Exact logistic regression was used for continuous variables and Fisher's exact test for categorical variables

Table S3 Subset of case samples with DRMVs selected for between pre-ART and ART failure time points

Sample ID	Pre-ART VL (copies/ml)	ART failure VL (copies/ml)	DRMs at pre-ART (frequency in %)	DRMs at ART failure (frequency in %)
SAP017	355 000	6 970	**K65R (3.1)**	**K65R (21.2)**
			V106A (2.2)	D67G (54.5)
				V106M (97.8)
				V179D (20.9)
				M184V (83.8)
				Y188C (69.8)
				F227L (22.4)
SAP098	124 000	80 300	**D67N (2.3)**	K65R (99.9)
				D67N (27.2)
				V106M (99.8)
				Y188C (99.8)
SAP185	107 000	33 600	M46I (4.7)	70Q (99.6)
			A98G (4.5)	L74I (95.7)
			K103N (74.2)	**V106M (99.6)**
			V106I (2.7)	V179D (26)
			V179D (99.1)	M184V (96.8)
SAP208	171 000	152 000	**L74I (2.4)**	**L74V (99.5)**
				K103N (99.7)
				V106M (99.3)
				M184V (98.1)

ART, antiretroviral therapy; DRMs, drug resistance mutations; ID, identification; VL, viral load

Note: Text in bold represents mutations that were selected for between pre-ART and ART failure time-points