Chapter 5

The application of Seq2Res to evaluate ultra deep pyrosequencing as a large-scale, cost-effective alternative to conventional HIV resistance genotyping

# Introduction

HIV exists in an infected individual as a complex heterogeneous population called quasispecies (Yin et al., 2012) primarily arising as a result of HIV’s high replication rate and the high error rate of the viral reverse transcriptase (Bebenek et al., 1993; Ji and Loeb, 1992; Preston et al., 1988). During the replication process, HIV develops random mutations (Bebenek et al., 1989; Bebenek et al., 1993; Berkhout et al., 2001; Roberts et al., 1988) in its genes that can result in viral resistance against one or more antiretroviral drugs (Clavel and Hance, 2004; D’Aquila et al., 2003; Kantor and Katzenstein, 2004; Sebastian and Faruki, 2004). Viral variants containing DRMs can be present at varying levels in the viral quasispecies (Devereux et al., 1999; Johnson et al., 2008; Metzner et al., 2009) with these variants emerging to dominate the viral population in response to treatment (Adje et al., 2001; Adje-Toure et al., 2003; Johnson et al., 2008; Marconi et al., 2008).

Approximately 8 million HIV infected individuals in resource-limited countries are receiving antiretroviral therapy by the end of 2012 (UNAIDS, 2012) following the scale-up of treatment programmes in 2002 (Beck et al., 2006; Ferradini et al., 2006; Gilks et al., 2006; Stringer et al., 2006). The number of HIV infected individuals with transmitted drug resistant mutations (DRMs) is increasing (Aghokeng et al., 2011; Phillips et al., 2013; Zaidi et al., 2013) with studies showing that while antiretroviral (ART) drugs increase life expectancy of infected individuals, this increase life expectancy increases the risk of transmission of drug resistant HIV variants to uninfected individuals (Zaidi et al., 2013).

The history of HIV treatment in 1980s has shown that the therapy with a single dose antiretroviral drug or a combination of drugs from a single drug class usually results in treatment failure (Kellam et al., 1994; Larder et al., 1989; Larder et al., 1991; Larder and Kemp, 1989; Larder et al., 1987). This has shown that administration of a single antiretroviral drug selects the drug resistant variants and increases the chance of rapid drug failure (Hamers et al., 2012; Jackson et al., 2000; Partaledis et al., 1995; Tisdale et al., 1993). A perfect example of this is the emergence of resistance in single dose nevirapine (NVP) programmes. NVP, an NNRTI, is prescribed to HIOV positive pregnant woman in order to prevent HIV transmission from mother to child in resource poor settings (Audureau et al., 2013; Chi et al., 2013; Shapiro et al., 2010; Stringer et al., 2010a; Zolfo et al., 2010). The drug is effective in reducing the viral transmission as has been reported in several research reports (Connor et al., 1994a; Connor et al., 1994b; Guay et al., 1999; Jackson et al., 2003; Lallemant et al., 2004). However, studies have shown that the use of single dose NVP to prevent mother to child transmission of the virus could show nevirapine associated resistant mutations (Coovadia et al., 2009; Eshleman et al., 2004b; Eshleman et al., 2005; Eshleman et al., 2001; Flys et al., 2005; Havlir et al., 1996; Jackson et al., 2000; Loubser et al., 2006; Martinson et al., 2007; Richman et al., 1994; Tisdale et al., 1993). The persistence of NVP resistant virus in the mothers and children treated with single dose NVP (Hauser et al., 2011) compromises the treatment with subsequent NVP containing highly active antiretroviral therapy (HAART) (Arrive et al., 2007; Chi et al., 2007; Lehman et al., 2012; Martinson et al., 2007). Thus, studies have shown that the first line therapy, which is a combination of at least three fully active ART drugs from different drug classes – Non-Nucleotide Reverse Transciptase Inhibitors (NNRTIs) and Nucleotide Reverse Transcriptase Inhibitors (NRTIs) are necessary for optimum suppression of HIV from replication and resistance development (Gupta et al., 2009; Hamers et al., 2012; Robbins et al., 2003; Shafer et al., 2003; van Leeuwen et al., 2003; van Leth et al., 2004). For this, the World Health Organization (WHO) recommends drug resistance testing before prescribing ART drugs.

Resistance testing reveals the drug resistance mutations in the HIV quasispecies. HIV with DRMs is present at varying prevalence levels in the quasispecies. The conventional HIV resistance genotyping is limited to detecting the mutations in HIV with prevalence of 20% or greater (Booth and Geretti, 2007; Liang et al., 2011; Wang et al., 2007). Ultra deep pyrosequencing (UDPS) technology has ability to detect HIV with prevalence to as low as 1% or below (Archer et al., 2009; Balduin M, 2011; Bansode et al., 2013; Dudley et al., 2012; Gilles et al., 2011; Hedskog et al., 2010; Hoffmann et al., 2007; Huse et al., 2007; Ji et al., 2012; Ji et al., 2010; Lataillade et al., 2010; Le et al., 2009; Liang et al., 2011; Wang et al., 2007).

Here, we describe the application of the computational tool Seq2Res to HIV resistance testing to a significant dataset generated using UDPS.

# Methods and Materials

The datasets used in this study had been generated as part of the CIPRA-SA study (Comprehensive International Program for Research in AIDS in South Africa) which was a prospective, unblinded, randomized controlled trial of comparing “doctor-initiative-doctor monitored” and “doctor-initiative-nurse-monitored” strategies for antiretroviral drug monitoring in resource poor setting (Sanne et al., 2010). The study population consisted of 831 HIV infected individuals with a CD4+ count less than 350-cells/mm3 or AIDS-defining illness were enrolled on the study. HIV positive mothers with previous exposure of single dose nevirapine (NVP) drug for prevention of viral transmission from mother to child (PMTCT) during their pregnancy were also included in the study.

562 patients were followed up with the remainder not included in the study for reasons such as drug toxicity, death, and withdrawal of consent or loss to follow-up. Baseline blood samples were retrieved from all 562 patients (sampled from 2005 – 2006). In this instance, baseline describes samples obtained from individuals immediately before initiation of first line antiretroviral therapy (ART). 71% of these patients received the drug combination D4T-3TC-EFV, 20% received D4T-3TC-NVP, 8% received D4T-3TC-LPV/r and 1% received D4T-3TC-NLF.

Virologic failure (VF) to the treatment was defined as decline of viral load less than 1.5 log10 from baseline to 12 weeks of treatment or two consecutive samples from a patient taken four weeks apart have viral load greater than 1000 RNA copies/ml (Sanne et al., 2010). VF to first line ART was identified in 79 patients, with 15 patients failing second-line therapy (Sanne et al., 2010). Blood samples had been retrieved for all of these individuals upon failure detection.

From all the obtained samples, the entire protease and reverse transcriptase genes of HIV had been amplified as three fragments - PR (HXB2 *pol* position 169 - 480) RT1 (HXB2 *pol* position 466 – 795) and RT2 (HXB2 pol position 796 – 1185) using HIV subtype C specific primers. Ten UDPS sequencing runs using the Roche/454 Junior platform (hereafter only Junior) platform had been attempted for 471 samples using MID tags to pool 48 samples per sequencing plate. Further, sequencing was attempted for 630 samples using the FLX platform (hereafter only FLX). 12 FLX runs were undertaken, dividing each plate into 8 distinct sections with 8 MID tagged samples per section for each sequencing run.

Conventional genotyping results were also available for 349 of the samples. All of the sequence data had been generated by our collaborators in the laboratory of Prof Maria Papathanasopoulos at the University of the Witwatersrand Medical School, South Africa.

Sequence data from all samples (baseline and first line VF samples) were analyzed using Seq2Res. To facilitate direct comparisons with the Sanger data the prevalence cutoff was set to 20%, consistent with the reported ability of Sanger-based sequencing to detect resistant variants to a level of 20% in the viral population (Hudelson et al., 2010; Larder et al., 1993; Leitner et al., 1993; Schuurman et al., 1999; Van Laethem et al., 1999). The presence of resistance in the UDPS genotypic data was further explored at prevalence levels of 15%, 10%, 5% and 1% of the amplified and sequenced viral population.

Every sequence read was tested for drug resistance using the Stanford HIV drug resistance database resistance interpretation algorithm (Liu and Shafer, 2006; Rhee et al., 2003; Shafer, 2006). The percentage of sequences that were predicted as resistant for a particular antiretroviral drug by the algorithm was calculated. Using a number of prevalence cutoffs (20%, 15%, 10%, 5% and 1%), if the percentage of sequences predicted as resistant to a drug from the total number of sequences analyzed for a sample, was greater or equal to the cutoff, the sample was predicted resistance for that drug.

As the baseline regimens of each of the individuals was known, resistance was defined as predicted resistance to one or more of the drugs in the individuals regimen. In the very rare event of an individual’s regimen not being recorded, resistance was defined as predicted resistance to one or more of the entire spectrum of drugs used as first line therapy in the study (D4T, 3TC, EFV, NVP, LPV/r or NLF).

In the case of VF, samples that failed the first line therapy, resistance was defined as predicted resistance to one or more of the drugs in the first line therapy. Similarly, individuals that failed second line therapy were predicted resistant if one or more of the drugs in the second line therapy were predicted to be resistant.

The number of samples with and without predicted resistance was obtained and statistical significance was calculated using Fisher’s exact test.

# Results

In the preliminary assessment of the sample’s sequence data from FLX and Junior, the samples in which protease (PR) or reverse transcriptase (RT) or both were not amplified were not considered for subsequent analysis. A total of 599 samples and 468 samples that were sequenced in FLX and Junior platform respectively from both baseline and first line VFs had both PR and RT sequences (**Table 5.1**) and were considered for analysis.

Out of the samples that were eligible for analysis, 464 samples were sequenced using both the FLX and Junior platforms, 327 samples using both the FLX and population based Sanger genotyping method and 257 samples with both the Junior platform and population based Sanger genotyping method (**Table 5.2**).

## Analysis of baseline samples

### Genotyping of baseline samples using the Roche/454 FLX sequencing platform

FLX sequencing was successful for baseline samples from a total of 526 patients of which 187 samples had previous ARV exposure as a result of PMTCT therapy while the remaining 339 had no previous exposure to ARVs. The eventual clinical outcome of all of these individuals was known and showed that out of the 339 no-PMTCT patients, 50 had exhibited VF while 289 exhibited virologic success (VS) during the course of follow-up. On the other hand, out of 187 PMTCT exposed patients, 25 exhibited VF and 162 exhibited VS.

The number of samples with and without predicted drug resistant HIV in the PMTCT and no-PMTCT groups at varying prevalence cutoffs (1%, 5%, 10%, 15% and 20%) that were predicted drug resistant to at least one baseline drug increased when the prevalence cutoff was decreased to 1% (**Figure 5.1**). In the no-PMTCT VF group of 50, there was only one sample exhibiting resistant virus to at least one baseline drug at prevalence level range 5% to 20% but this increased to five samples (10%) at prevalence level of 1%. Similar increments in the number of individuals predicted as resistant as the prevalence levels decreased were observed in other groups as well (**Figure 5.1**).

At the 1% prevalence cutoff in the no-PMTCT group there was a significant difference between the number of clinical viral failures predicted as resistant when compared with the number of clinical viral successes predicted as resistant (p < 0.05, Fisher’s exact test) while a similar observation was observed at the 15% prevalence cutoff in the PMTCT exposed group (**Figure 5.1**).

### Genotyping of baseline samples using the Roche/454 Junior platform sequencing platform

407 patients were sampled at baseline and sequenced using Junior, 250 patients had no previous ARV exposure through PMTCT and 147 patients had previous PMTCT therapy. The clinical outcome showed that out of 250 non-PMTCT patients, 40 had VF and 210 had VS. On the other hand, out of 147 PMTCT exposed patients, 21 had VF and 136 had VS (**Figure 5.2**).

The obtained baseline blood samples were sequenced using Junior platform and again analyzed using Seq2Res. The observation on the number of samples with and without predicted resistant HIV showed that in the no-PMTCT VF group of 40, there was only one individual predicted resistant to at least one drug in baseline at prevalence cutoff 20% and that increased to four individuals at prevalence cutoff 1% (**Figure 5.2**). Similar increments in the number of samples with predicted resistant HIV was observed in the no-PMTCT VS group as well as in PMTCT VF and VS groups (**Figure 5.2**). The highest number of samples (16 samples) with predicted resistant HIV was observed in PMTCT VS group at a prevalence cutoff 1%. Further, in the No-PMTCT group there was a significant difference between the number of clinical viral failures predicted as resistant when compared with the number of clinical viral successes predicted as resistant (p < 0.05, Fisher’s exact test, **Figure 5.2**).

### Comparison of number of sequence reads per baseline sample generated by Roche/454 FLX and Roche/454 Junior

Sequencing had been successful on both FLX and Junior platforms for 464 samples (**Table 5.2**). The initial analysis focused on comparing the number of sequence reads generated by each platform for each sample and identifying if ‘deeper’ sequencing coverage resulted in more accurate prediction of resistance. We saw that the FLX platform generated on average 6412 sequence reads per sample (standard deviation 2297) while the Junior platform generated an average 1903 sequence reads per sample (Standard deviation 595, **Figure 5.3**). Thus, it is clear that the FLX platform produced significantly (P-value < 2.2-16) more reads per sample than the Junior platform.

### Comparison of genotyping results between Roche/454 FLX and Roche/454 Junior platforms on baseline samples

Baseline samples from 405 individuals had been sequenced using both the FLX and Junior platforms. 249 had no previous PMTCT therapy while 156 had previous exposure to ARVs as a result of PMTCT therapy. Of these 249 patients, 40 exhibited VF and 209 exhibited VS to the first line antiretroviral therapy regimen. Of the 146 PMTCT exposed patients, 21 exhibited VF and 135 exhibited VS in first line antiretroviral therapy (**Figure 5.4**).

Our results show that the number of samples with and without predicted resistance in both the PMTCT and no-PMTCT groups is not significantly different between the FLX and Junior sequencing platforms at all prevalence cutoffs (**Figure 5.4**).

Thus, despite the significantly higher numbers of sequence reads generated per individual for the FLX data, both the genotyping results from both platforms were completely comparable.

### Comparison of ultra deep pyrosequencing and population based Sanger method for resistance prediction using baseline samples

In section 3.1.4 we showed that there was no significant difference between the FLX and Junior platforms for the prediction of resistance. While genotyping results from the Junior and FLX sequencing platforms are comparable between each other, this is essentially meaningless unless these results are comparable to that of the current “gold-standard” of population based Sanger genotyping method, to be used as a replacement. Thus, we compared the Junior platform (now also referred to as UDPS) results with those from the Sanger-based genotyping.

239 of 302 baseline samples were sequenced using both Junior platform and conventional population based Sanger genotyping technology. 128 of them had no previous PMTCT therapy exposure and 111 had previous PMTCT therapy exposure. Out of 128 no-PMTCT patients, 15 exhibited VF and 113 exhibited VS in first-line antiretroviral therapy. Similarly, out of 111 previously PMTCT exposed patients, 10 exhibited VF and 101 exhibited VS (**Figure 5.5**).

The results from the Junior platform showed identical numbers of individuals predicted as resistant at the 20%, 15% and 10% prevalence levels with now significant difference from the resistance predictions from the Sanger-based genotyping (Figure 5.5). The numbers of individuals predicted as resistant by the Junior platform increased at the lower prevalence levels (Figure 5.5), however these observations were still not significantly different from the predictions of the Sanger-based genotyping.

Thus, it appears for baseline samples at least, the UDPS resistance genotyping approaches employed here are directly comparable to that of Sanger-based resistance genotyping.

## Analysis of virologic failure samples

Using the baseline samples, we showed that there was no significant difference between FLX and Junior platform and between UDPS and population based Sanger method. We repeated the platforms comparative analysis test using samples collected following clinical evidence of viral failure in individuals on 1st line therapy (Either a decline of < 1.5 log10 in viral load from baseline to 12 weeks of treatment or two consecutive viral loads 4 weeks apart of >1000 RNA copies/ml).

### Resistance genotyping of samples collected from individuals at first-line virologic failure (VF1) using Roche/454 FLX platform

51 of the first line ART VF1 samples had been sequenced using FLX technology. 15 of these had previous ARV exposure through PMTCT while 36 had no previous exposure through PMTCT.

Genotyping using the FLX platform predicted resistance to at least one of the first line drugs at all prevalence levels in 14 out of 15 the PMTCT samples (**Figure 5.6**). On the other hand, in the no-PMTCT sample, 23 out of 36 had predicted resistance to at least one of the first line drugs at all prevalence levels while 13 had no predicted resistance (**Figure 5.6**).

The observation of the number of samples with and without predicted resistance showed that there was a significant difference between the PMTCT and no-PMTCT groups at all prevalence cutoffs. The observation also showed that the viral resistance prediction in the samples from PMTCT group was more than in no-PMTCT group at prevalence cutoffs using FLX system.

### Resistance genotyping of samples collected from individuals at first line virologic failure (VF1) using Roche/454 Junior

Out of the 36 first-line therapy failure samples sequenced using the Junior platform, 23 had no previous PMTCT therapy exposure while 13 had previous PMTCT therapy exposure. The numbers of predicted resistant and non-resistant viral samples were calculated at all prevalence cutoffs (**Figure 5.7**).

We observed that the amplified and UDPS sequenced viral population in all 13 VF samples in PMTCT group were predicted resistant when the prevalence cutoff was below 20% (**Figure 5.7**). At the 20% cutoff, 12 out of 13 PMTCT samples (92.3%) were predicted resistant (**Figure 5.7**). Similarly in no-PMTCT group, 14 out of 23 samples (60.86%) were predicted resistant at prevalence cutoffs 20% down to 5%. The number of samples with predicted resistance increased to 15 out of 23 (65%) at a prevalence cutoff of 1%. The observation of the number of samples that were predicted drug resistant showed that there were significant differences between the PMTCT and no-PMTCT at all prevalence cutoffs. The result obtained was similar to the result in VF1 samples sequenced using FLX platform, which indicated that the likelihood of predicting resistance in PMTCT group is more than in no-PMTCT group.

Although we see significance at all prevalence levels in the number of samples predicted as resistant with the genotypic data from VF1 samples sequenced using Junior and FLX platforms, we further compared the platforms directly, only with the VF1 sample genotypic data sequenced in both the platforms.

### Comparison of genotyping results between Roche/454 FLX and Roche/454 Junior platforms on first line virologic failure samples

50 VF1 samples sequenced using both the FLX and Junior platforms were available. 36 of them had no previous exposure to the drug NVP and 14 had previous exposure to the drug NVP through PMTCT. The number of VF1 samples on both no-PMTCT and PMTCT groups that were predicted resistance were calculated at different prevalence cutoffs (**Figure 5.8**).

We observed the number of VF1 samples that were predicted resistant was consistent from 20% to 5% for both FLX and Junior platforms but increased by one at 1% cutoff (**Figure 5.8**). There was no significant difference at all prevalence cutoffs. 13 of 14 (93%) PMTCT samples and 69% or above no-PMTCT samples were predicted resistant by both FLX and Junior. The observation showed that both Junior and FLX are comparable for HIV drug resistance genotyping of viral failure samples.

### Comparison of the genotyping performance of the Roche/454 Junior platform and conventional population based Sanger genotyping method using first line virologic failure samples

Genotypic data from both Junior platform and conventional population based Sanger genotyping method were available from 13 VF1 samples. Out of the 13 patients, 6 had no previous PMTCT therapy and 7 had PMTCT therapy exposure. The observation of the number of VF1 samples that were predicted with or without resistant for both approaches at 20% prevalence cutoff showed that there was 100% concordance across all clinical outcome categories between the Sanger-based and UDPS-based resistance genotyping approaches (**Figure 5.9**).

## Resistance to nevirapine is more likely to be present at baseline in individuals previously exposed to nevirapine through PMTCT

We compared the resistance predictions for PMTCT versus no-PMTCT therapy exposed individuals and identified the percentage of individuals with predicted resistance to nevirapine at baseline for conventional Sanger genotyping method, FLX and Junior platform (**Figure 5.10**). In all comparisons we found that the percentage of individuals with predicted resistance to nevirapine was always significantly higher in the PMTCT therapy exposed group when compared with the no-PMTCT therapy exposed group. This discordance became more evident at the ‘deeper’ prevalence cutoff (**Figure 5.10**), suggesting a large number of PMTCT-exposed individuals were harboring low-abundance NVP resistant viruses.

To ascertain whether the prediction of NVP resistance in PMTCT exposed individuals correlates with the time since NVP exposure, we compared the time since NVP exposure in baseline PMTCT samples with predicted NVP resistance and those predicted as susceptible to NVP. At prevalence thresholds of 15% and below (for both FLX and Junior platforms) we found that the prediction of NVP resistance significantlycorrelates with time since NVP exposure (**Table 5.3**). The median number of days since PMTCT exposure was observed to be 674 days for those individuals predicted as susceptible to NVP and 172 days for those predicted as resistant.

# Discussion and Conclusions

We have analyzed 562 baseline samples and 79 first line ART viral failure (VF1) samples using UDPS (both FLX and Junior platforms) and conventional population based Sanger genotyping method. The baseline samples were collected in 2005 – 2006. The samples were grouped as – individuals with prior exposure to ARVs through PMTCT therapy and individuals without prior exposure to ARVs.

## Genotyping results from the Roche/454 Junior platform are comparable to the Roche/454 FLX platform

The junior platform is a desktop sequencing platform with a single sequencing plate that has the capacity of generating up to 100,000 reads per run (70,000 for amplicon data) and a throughput of ~35 megabyte data (www.454.com). The FLX platform has similar sequencing chemistry like Junior platform and is a larger sequencing platform that has the capacity of generating up to 1,000,000 reads per run and the throughput of ~450 megabytes ([www.454.com](http://www.454.com)). The number of reads per run and the depth of sequencing per sample limit the number of samples that can be sequenced in Junior and FLX platforms. As the Junior platform has lower throughput than the FLX platform, more samples can be pooled for sequencing on the FLX platform than in the Junior platform for the same sequencing coverage per sample.

In our analysis the FLX data was not generated to achieve equal coverage as the Junior data but was, in fact, generated to explore whether ‘deeper’ sequencing (i.e. higher coverage) is a more sensitive approach for HIV drug resistance genotyping. Despite the FLX platform producing significantly higher number of sequence reads per sample than Junior (almost three times as many reads per individual), there was no significant difference in the resistance prediction in 405 baseline samples at all prevalence levels (20%, 15%, 10%, 5% and 1%). This implies that, in our data at least, the FLX approach is not sequencing at a ‘deeper’ level and, thus, finding very lowly abundant variants but is more likely sequencing re-sampled data generated by the PCR step. This is further supported by the observation that the prevalence of many DRMs is equivalent between the FLX and Junior data. Thus, the higher coverage of the FLX approach doesn’t give any significant advantage over the lower number of reads generated by the Junior platform and, in a resource limited setting at least, the lower coverage from the Junior platform is sufficient for HIV resistance genotyping. Whether this holds true in populations with a longer history of ARV programmes and a wider selection of available ARVs is not known, and should be explored further in a similar manner and depth of sampling to our study.

Given its easier setup and usability and lower setup costs the Junior platform is undoubtedly preferred over the FLX platform for HIV resistance genotyping in resource limited settings. The price of a Junior platform (~$125,000) is much cheaper than FLX (~$500,000). Two publications have previously evaluated the Junior platform in terms of cost in relation to HIV resistance genotyping with Dudley et al obtaining high quality HIV genotypic data sequencing 48 samples in a single Roche/454 Junior platform at the cost of $20 per sample (Dudley et al., 2012) while Hezhao Ji et al sequenced only HIV PR gene from 96 samples using 1/16th of the full PicoTiterPlate at the cost of $32.46 per sample and HIV PR and RT genes at same sequencing capacity for $52.75 per sample (Ji et al., 2010). The cost per sample included both labor cost and material cost. While the Roche/454 platform has historically been used for HIV drug resistance genotyping because of its longer read lengths, the read lengths of a number of other platforms such as the Illumina and Ion Torrent platforms are increasing thus reducing Roche/454’s advantage. This is further compounded by the fact that Roche have recently announced that they are discontinuing the 454 sequencing platform by 2016 to focus on the development of a desktop PacBio system. Further, the significantly higher levels of coverage on other approaches means that there can potentially be even further cost savings using these sequencing approaches for HIV drug resistance genotyping in resource limited settings with a high burden of HIV.

## Evidence of minor drug resistant HIV variants in baseline samples

Numerous studies have shown that HIV infected patients that are drug naïve could be harboring viral variants containing primary DRMs (Balduin M, 2011; Bansode et al., 2011; Hamers et al., 2011; Kozal et al., 2011; Metzner et al., 2011; Simen et al., 2007; Simen et al., 2009; Varghese et al., 2009). Simen et al (Simen et al., 2009) studied the risk of VF in the subsequent ART treatment due to minor drug resistant variants at baseline using conventional Sanger genotyping and UDPS methods. The authors observed 113 mutations below 20% prevalence in the sample; 45 of them (39.8%) were found at 5% or lower prevalence in the viral population. 7 of 84 (8.33%) patients having low abundance NNRTI resistant mutations (<20% mutation prevalence) at baseline experienced VF at the first line ART. Consistent to this report, we also observed 5 of 50 (10%) of the baseline no-PMTCT samples that were predicted resistant at 1% prevalence cutoff indicating that they harbored the low abundant drug resistant HIV variants. Kozal et al (Kozal et al., 2011) also reported that 147 patients in the cohort of 411 had DRMs when sequenced to 0.4% prevalence level in the viral population. Similarly, Metzner et al (Metzner et al., 2011) reported 20 of 246 (13.7%) drug naïve patients had minor drug resistant viral variants.

Thus, the studies described above showed that primary DRMs in baseline samples from drug naïve patients could be expected. In the high HIV burden regions like sub-Saharan African, the drug resistant viral variants at baseline could be expected even higher. This is supported by Hamers et al (Hamers et al., 2011) whose study on PASER-M cohort findings showed that high prevalence of primary DRMs in treatment naïve individuals can be expected in ART rolled out regions. The authors had pretreatment genotypic data for 2436 baseline samples collected from different African countries. They observed primary DRMs in 5.6% of 2436 patients (range: 1.1% of 176 in South Africa to 12.3% of 179 in Uganda). According to the authors, the high prevalence of primary DRMs in Uganda could related to earlier roll out of ART in the country than other countries (South Africa, Nigeria, Kenya, Zambia and Zimbabwe). Transmission of drug resistant HIV variants to healthy individuals in the ART scaled up region could have lead to high prevalence of primary DRMs (Varghese et al., 2009). More supportive data came from the ART scaled up country – Thailand where a study showed 4.9% of 499 patients had primary HIV-1 drug resistance (Sungkanuparph et al., 2012). Gupta et al (Gupta et al., 2012) and Aghokeng et al (Aghokeng et al., 2011) observed high rate of increase in prevalence of DRMs in ART scaled up regions around the world. The drug resistant HIV variants at baseline has necessitated the surveillance of primary DRMs in the ART scaled up region and it is recommended (Hamers et al., 2011; Sungkanuparph et al., 2012).

The use of NVP in PMTCT therapy has been observed to result in viral variants with the drug-associated mutations in the HIV quasispecies (Arrive et al., 2007; Flys et al., 2005; Johnson et al., 2005; Martinson et al., 2007). Thus, as expected we observed a higher number of samples with predicted resistance to at least one drug in the PMTCT-exposed group than in the no-PMTCT group at 1% prevalence cutoff. We observe 2.6% of no-PMTCT baseline samples had predicted resistance to at least one drug while 12.2% of PMTCT had predicted resistance to at least one drug using genotypic data from FLX. Similarly, a higher number of baseline samples were predicted resistant from the PMTCT group than the no-PMTCT group using genotypic data from Junior platform. Various studies also showed that high number of individuals treated with sdNVP in PMTCT therapy had NVP resistance. For example Arrive et al (Arrive et al., 2007) studied NVP resistance 4-8 weeks after receiving sdNVP and they observed 35.7% of PMTCT mothers has the drug-associated resistance. Flys et al (Flys et al., 2005) studied NVP resistance in individuals that received sdNVP after 1 year or more. The authors observed high level NVP resistant mutation K103N in 8 of 9 women and 4 of 5 infants 6-8 weeks after sdNVP. The mutation persisted in 3 of 9 women and 1 of 5 infants after 12-24 months of sdNVP administration. Johnson et al (Johnson et al., 2005) studied genotypic data from 50 South African women before and after sdNVP administration using conventional population based Sanger genotyping method. They found that the NVP resistance emerged in at least 65% of them. They expected the prevalence of NVP resistance would be more using UDPS method. The prevalence of NVP resistance in these studies varied from our observation due to variation in the sample size. But these all studies including our finding showed that NVP resistance is high in sdNVP exposed individuals.

## The presence of NVP resistance correlates with the time since sdNVP exposure

The World Health Organization (WHO) recommends the use of a single dose nevirapine for HIV infected pregnant mother for HIV prevention from mother to child (WHO, 2008). However, a number of research publications have shown that the single dose NVP treatment rapidly develops HIV variants with NVP resistant mutations (Eshleman et al., 2001; Hudelson et al., 2010; Jackson et al., 2000; Palmer et al., 2006). The single dose NVP is provided one time to the pregnant women before labor. The effect of NVP decreases in those women in PMTCT therapy as the time goes on and thus NVP resistant viral variants decline in them (Eshleman et al., 2001; Kassaye et al., 2007). However, studies have shown the persistence of the minor NVP resistant viral variants (Flys et al., 2005; Flys et al., 2006; Loubser et al., 2006; Palmer et al., 2006; Rowley et al., 2010) and may compromise the subsequent first line therapy that contains the NNRTI drug (Boltz et al., 2011; Ciaranello et al., 2011; Jourdain et al., 2004; Lockman et al., 2007; Moorthy et al., 2011). A study by Chi et al (Chi et al., 2007) showed the time correlation (less than six months) before initiation of ART treatment in NVP exposed individuals as a risk factor that might produce poor treatment outcome. We then assessed the time correlation of NVP exposure with resistance prediction at different prevalence cutoffs. We knew the ART treatment initiation date and the date of PMTCT for the individuals in the study. We then calculated the time of sdNVP exposure before ART initiation for the individuals. We observed that at 15% and lower prevalence cutoff, the prediction of NVP resistance significantly correlates (p < 0.05) with time since NVP exposure (**Table 5.3**). According to our finding, for median time of 671 days NVP exposure, the samples were predicted NVP sensitive and for median time of 174 days NVP exposure, the samples were predicted NVP resistant. Our finding is supported by various earlier studies. Coovadia et al (Coovadia et al., 2009) studied the effect of sdNVP exposure to the virologic response to NVP based first line ART and observed that women who received sdNVP 18-36 months prior to NVP based first line ART initiation had likelihood of sustained virologic suppression. A study by Stringer et al (Stringer et al., 2010b) provides more support for our observation on correlation between time and resistance prediction. The authors studied the prevalence of VF after NVP containing first line therapy in the patients experienced with single dose NVP. They also observed that the time elapse between NVP exposure and initiation of NVP containing ART therapy were correlated. The authors observed VF in 47 of 116 (40%) of women with six or less months of time elapse, 25 of 67 (37%) of women with seven to 12 months of time elapse and 42 of 172 (24%) of women with more than 12 months of time elapse between NVP exposure and initiation of NVP containing ART therapy. Their observation showed that as the time elapsed increased, the rate of VF in NVP containing ART was declined. The authors concluded that risk of VF in recent drug exposed patients was high and suggested that NVP should not be included in the subsequent first line therapy for the drug exposed patients before 12 months of the therapy. The authors’ conclusion was highly consistent with our observation of time elapse and resistant prediction.

Thus, over the time of sdNVP exposure in PMTCT therapy, the prevalence of the drug resistant variants decreases in the viral population (Eshleman et al., 2004b) until eventually they are no longer present. When the selection pressure of the drug NVP is removed, the resistant variants get less fit in the viral quasispecies (Mammano et al., 2000; Quinones-Mateu and Arts, 2002) and the sensitive wild type viruses reemerge to dominate in the viral population. Therefore, we hypothesize that long term virologic suppress can be achieved with the time exposure to the sdNVP drug over six months before initiating first line ART.

## First line therapy failure correlates to historical antiretroviral drug use

Pregnant mothers who were administered sdNVP as a HIV prophylaxis to prevent the viral transmission from mother to child (Guay et al., 1999; Jackson et al., 2003) harbored NVP resistant mutations after the therapy (Eshleman et al., 2004a; Eshleman et al., 2004b; Eshleman et al., 2001; Johnson et al., 2005). Studies have showed that the presence of the drug resistant viral variants could relate to the poor clinical outcomes in the first line ART (Casado et al., 2000; Chi et al., 2007; Johnson et al., 2008; Lecossier et al., 2005; Metzner et al., 2009). We studied the resistance in first line antiretroviral treatment failure samples using UDPS genotypic data.

Our finding showed that the frequency of samples with predicted resistance in VF1 PMTCT samples was significantly higher than VF1 no-PMTCT samples (93.33% vs. 63.88%) using FLX platform. The finding was consistent from prevalence cutoff 20% to 1%. Lockman et al (Lockman et al., 2007) had also studied the response of NVP based first line ART on the samples exposed to the drug through PMTCT therapy. They observed that 5% women that received placebo and 18.4% women that received single dose nevirapine experienced first line VF in the first six month of the initiation of the ART treatment. They defined NVP resistance as the presence of any high level NVP resistant mutations: 100I, 103N, 106A/M, 108I, 181C/I, 188L/C/H, or 190A. Genotypic data from 16 of 20 (80%) mothers who received sdNVP and experienced VF within six months of first line ART showed that 12 had NVP resistance at the time of failure, one had baseline NVP resistance but no NVP resistance was detected at the time of VF and three had no detectable NVP resistance before ART and at the time of VF. Genotyping using conventional Sanger method could the reason of undetectable NVP resistance in the some VF mother.

In more support to our finding, Geretti et al (Geretti et al., 2009) also showed detection of resistance before pretreatment was highly associated with virologic failure of drug regiment . The authors studied 93 HIV infected patients diagnosed in the median year 2000, received drug cocktail after 1 median year and had median CD4 count of 218. Before the highly active antiretroviral treatment, they were under nevirapine or efaverinz treatment. In the pretreatment samples, the common mutation found was K103N that provide high-level resistance to NVP and EFV. Using population based sequencing, four of 18 virologic failure samples harbored K103N mutation while none of 75 virological success samples had the mutation. Using sensitive genotyping method, the association of resistance and VF was increased. Seven of 18 VF sample had the mutation while none of 75 virologic success samples had the mutation.

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