Introduction

The attainment and maintenance of sustained viral suppression is the ultimate goal of antiretroviral therapy (ART). The optimal response to therapy is the substantial decrease in viral load (VL) to below the technical specificities of the VL assays which results in the avoidance of emergence of drug resistance mutations [1]. The treatment guidelines in resource rich settings recommend performing VL monitoring every 3–6 months [2]. However, in resource constrained settings like sub-Saharan Africa and India, treatment efficacy is typically monitored through immunological response to therapy is the substantial decrease in viral load to below the technical specificities of the VL assays which results in the avoidance of emergence of drug resistance mutations [1]. The treatment guidelines in resource rich settings recommend performing VL monitoring every 3–6 months [2]. However, in resource constrained settings like sub-Saharan Africa and India, treatment efficacy is typically monitored through immunological markers such as measurement of CD4+ T cell count, [3] whereas VL monitoring is usually restricted to research settings. Despite this caveat, the short term outcomes of cART in resource-constrained settings are comparable to those in resource-rich settings [4,5]. The data on long term outcome, however, are limited as most of the studies have been conducted within the first two years after initiation of ART [5].

The National AIDS Control Organisation (NACO) sponsored by the government of India, initiated the National AIDS Control Program (NACP) to provide free ART through established ART-centres in April 2004 [6]. Currently, nearly 384,000 HIV-1 infected individuals are receiving free ART with one non-nucleoside reverse transcriptase inhibitor (NNRTI), either nevirapine (NVP) or efavirenz (EFV), in combination with two nucleoside reverse transcriptase inhibitors (NRTI); zidovudine (AZT) or stavudine (d4T), and lamivudine (3TC) [7].

India has the third largest HIV-1 epidemic in the world with a burden of nearly 2.5 million people, where HIV-1 subtype C is the dominant subtype.
predominating subtype [8]. A previous study with a small number of patients (n = 40) using generic ART identified a rapid suppression of HIV-1 viral load [9]. However data on long term efficacy of the national first line regimen is lacking.

Therefore, to understand the clinical impact of the Indian national ART guideline, the primary purpose of our study was to investigate long term virological outcome among Indian HIV-1 infected patients on first-line therapy, following initial viral suppression. In addition, we evaluated the factors associated with virological rebound, its consequences, and emergence of drug resistance mutations.

Materials and Methods

Study sample

The sample described in this paper was derived from a large observational clinical cohort of 533 patients from two tertiary care hospitals in the southern part of India, which adhere to the national therapeutic guidelines [10,11]. The criteria used for inclusion in these analyses were: (i) Adult HIV-1 infected patients initiated on first-line ART for a minimum period of 6 months, and (ii) a viral load <100 copies/ml at the time of entry into the study. First line ART included two NRTI and one NNRTI drug. A total 323 patients met these inclusion criteria. The patients were followed up for every 3 months and evaluated for self-reported adherence, adherence barriers and other health behaviours, and lab samples were collected every 6 months for a total follow-up period of 2 years.

CD4+ T-cell count, viral load and drug resistance genotyping

Blood samples were collected in EDTA tubes. CD4 count was measured using a single platform flow cytometric assay (PCA system; Guava Technologies Inc., Hayward, CA, USA). Viral load was measured by an in-house real time polymerase chain reaction with TAQMAN assay at Molecular Diagnostics and Genetics, Reliance Life Sciences, Mumbai, India. Given the lower detection limit of the VL assay (<100 copies/mL), intermittent viral rebound was defined as a single episode of detectable viral load >100 copies/mL. Virological failure was defined as two successive viral load values >400 copies/mL. Patients with VL >2000 copies/mL were genotyped for drug resistance using in-house techniques [12,13]. The drug resistance genotyping was successfully performed on 16 patients samples during their first viremia and a further six in the next visit who had viral load >2000 copies/mL. The mutations were interpreted using International AIDS Society updated drug resistance mutations in 2011 (IAS_2011) [14]. The resistance data was not used for treatment decisions.

HIV-1 subtyping

HIV-1 subtyping was determined using the maximum likelihood (ML) phylogenetic tree using the reference sequences downloaded from Los Alamos Database (www.hiv.lanl.gov).

Adherence measurements

Self-reported adherence was measured using a Visual Analogue Scale (VAS) which has been found to predict virological failure better than other self-report measures in this setting [15]. In the present study, two types of adherence measurements were combined into one dichotomous “perfect adherence” variable: Percent of prescribed pills taken, assessed with the Visual Analogue Scale (VAS) and Treatment interruptions, defined as having missed medications for more than 48 hours in the past three months. To be classified as “perfectly adherent”, patients had to report 1) taking 100% of prescribed doses in the past three months (past one month at baseline) at each study visit in which they were present and 2) zero treatment interruptions during the 2-year study period.

Statistical analysis

Differences between groups on continuous variables were examined by independent sample t-test or Mann–Whitney U-test, while categorical variables were analyzed via frequencies and cross-tabulations, with χ2 tests to assess the significance of the associations. All analyses were conducted in PASW Statistics version 18.

Ethical Considerations

The study was approved by the Committee for Human Research at University of California, San Francisco, USA and the Institutional Ethical Review Board of St John’s Medical College and Hospital, Bangalore, India. Written informed consent was obtained from all the study participants before enrolling into the study.

Results

Patient demographics and clinical characteristics

The demographic and clinical characteristics of the 323 patients at baseline and during the two year follow-up period are presented in Table 1. Almost three quarters of participants were male, and mean (SD) age at baseline was 38 (8.5) years. Most participants (87.9%) were on a NVP-based regimen. The mean (SD) duration of treatment at baseline was 23 (11) months. Median CD4+T cell count at baseline was 370 cells/mm³ (IQR: 243–525). During the study period 35.3% (114/323) patients maintained a good immunological profile (CD4 >350 cells/mm³) at all waves in which they were present. The median duration of viral suppression was 44 months (IQR 36–54) and 15.8% (51/323) of patients showed intermittent viral rebound without failure during the study period. Viral failure was observed in only 2.8% (9/323) of patients. Among the patients, 75.2% (243/323) was able to maintain ≥95% adherence throughout the study and 47.4% (153/323) were able to maintain perfect adherence (100% adherence by VAS and no treatment interruptions) during the same time (Table 1). Patients with a single episode of viral rebound had a significantly lower mean viral load compared to the failure group during their first viremia (3.2 vs 4.0 Log copies/mL; p = 0.047) (Table 2). Mean CD4+T cell counts during first viral rebound were also low in the viral failure group compared to the viral rebound group (284 cells/mm³ vs. 404 cells/mm³), but the difference was not statistically significant (p = 0.147) due to lack of statistical power, given the small number of patients (n = 9) with viral failure.

HIV-1 subtyping

HIV-1 subtyping using the Maximum likelihood (ML) phylogenetic tree with the best fitting general time reversible substitution model with inverse gamma distribution (GTR+G+I) with 500 bootstrapped data sets identified all the study sequences as HIV-1 subtype C (Figure 1), which is predominant in India [8].

Correlates of viral rebound/failure

Comparisons between participants with and without viral rebound/failure (Table 3) showed that about half of those without rebound/failure reported maintaining perfect adherence (100% adherence without any treatment interruption) during the study period, compared to less than a third of those who did experience
Drug resistance genotyping

Drug resistance mutations were identified in 9 of the 16 patients. Among the six patients with virological failure who had high viremia during their first viral rebound, four had accumulated more NRTI drug resistance mutations (DRMs) in their follow up visit (data not shown).

Discussion

Our results indicate a good overall long-term response to first line therapy reflecting an overall success of the standardized Indian national program for individuals with HIV infection. By the end of the two-year study period, 81.4% of the patients remained virologically suppressed for a total median duration of nearly four years (based on time on ART at baseline + two-year study period) and this efficacy is likely to continue be longer as most of the patients reached the study endpoint with good immuno-virological conditions. As the study was conducted in two different settings, one public tertiary care hospital and one non-profit private tertiary care hospital, which follow the same therapeutic guidelines, recommended by the NACO, it is likely that the results are generalizable to other Indian clinics that follow the same protocols.

Previous studies on short term efficacy of antiretroviral therapy from resource limited settings, within two years after initiation, have obtained comparable results to studies conducted in resource rich settings in Europe and America [4,5]. A recent systemic review showed that in sub-Saharan Africa, 67% of the patients achieved virological success after 2 years of therapy [5]. However, long term follow up data after 2 years of therapy, are limited. A study from Botswana, estimated virological failure in 22.1% and 30.1% of HIV-1 subtype C infected patients at 3 and 5 years respectively [16]. A study from South Africa reported that 61% of the patients achieved virological suppression at 3 years [17]. A study from Burkina Faso reported that 81.8% of the patients remained virologically suppressed at 3 years [18]. Our study showed that 81.4% of the patients who initially achieved viral load <100 copies in our setting, remained virologically suppressed for a median of almost four years, 15.8% had an intermittent viral rebound, and only 2.8% of patients experienced a true virological failure. This study is the first to document the favourable treatment response of a median of nearly four years duration with the national first line treatment regimen in India.

Non-adherence to therapy has been shown to be associated with intermittent viremia [19,20] in therapy experienced patients. A

| Table 1. Patients’ demographic, clinical and laboratory parameters at baseline and whole study period of 2 years. |
| --- |
| **Variable** | **Parameter** |
| **Baseline** | |
| Total number of patients, N (%) | 323 (100%) |
| Gender, N (%) | |
| Male | 235 (72.8%) |
| Female | 87 (26.9%) |
| Transgender | 1 (0.3%) |
| Age, Mean (SD), years | 38 (8.5) |
| Duration of treatment, Mean (SD), months | 23 (11) |
| CD4 cell count, Median (IQR), cells/mm³ | 370 (243–525) |
| Duration of viral suppression | 44 (36–54) |
| **Drug regimen at baseline, N (%)** | |
| d4T+3TC+LPV | 123 (38.1%) |
| AZT+3TC+LPV | 160 (49.5%) |
| d4T+3TC+EFV | 21 (6.5%) |
| AZT+3TC+EFV | 17 (5.3%) |
| FTC+TDF+EFV | 1 (0.3%) |
| DDI+3TC+LPV | 1 (0.3%) |
| **Follow-up** | |
| Intermittent viral rebound, N (%) | 51 (15.8%) |
| Virological failure, N (%) | 9 (2.8%) |
| Treatment Interruptions, N (%) | 29 (9.0%) |
| ≥ 95% Adherence, N (%) | 243 (75.2%) |
| Perfect Adherence, N (%) | 153 (47.4%) |

Abbreviations: d4T, Stavudine; 3TC, lamivudine; AZT, zidovudine; FTC, emtricitabine; TDF, tenofovir; NVP, Nevirapine; EFV, Efavirenz; SD, Standard deviation; IQR, interquartile range; N, Number; PVL, plasma viral load; VAS, Visual analogue scale.

*Over the study period of 2 years, doi:10.1371/journal.pone.0055421.t001

viral rebound/failure (p = 0.007). None of the other variables were significantly associated with viral rebound/failure.

Drug resistant genotyping

Drug resistance mutations were identified in 9 of the 16 patients with VL > 2000 at their first instance of viremia during the study period (M184V: 8; K70R: 1; K65R: 1; G190A: 4; K103N: 3; Y181C: 3; V108I: 3; H221Y: 2) (Table 4). Among these 16 patients, seven had successfully controlled the viremia in their subsequent 6-month follow up study visit without any change of therapy. Of the seven controlled vireemics, three had multiple major NRTI/NRTI mutations [M184V+G190A; M104V+K103N; M104V+Y101C] during their viral rebound, but later supressed as they maintained >95% adherence both before and after the viral rebound. Further, all three patients maintained good immunological conditions (mean CD4+ T cell count 610 cells/mm³) in their remaining study visits for 12 months, 18 months and 6 months, respectively.

Among the six patients with virological failure who had high viremia during their first viral rebound, four had accumulated more NRTI drug resistance mutations (DRMs) in their follow up visit (data not shown).

| Table 2. Clinical and demographic parameters during first viral rebound for viral failure and intermittent viral rebound groups. |
| --- |
| **Parameter** | **Viral Failure (n = 9)** | **Viral Rebound (n = 51)** | **p value** |
| Age in years at baseline: mean (SD) | 35.4 (6.7) | 38.5 (10.1) | 0.390 |
| Male: n (%) | 8 (88.9) | 35 (68.6) | 0.423 |
| CD4-T cell count; cells/mm³: mean (SD) | 284 (241) | 404 (224) | 0.147 |
| Viral load (log transformed): mean (SD) | 4.0 (1.2) | 3.2 (1.0) | 0.047 |
| 100% adherence past month: n (%) | 5 (55.6) | 40 (80.0) | 0.195 |

*p-value based on t-test (d.f. = 58) for age and CD4 count and viral load, Fisher’s exact test (d.f. = 1) for male gender and adherence. doi:10.1371/journal.pone.0055421.t002
significant relationship between adherence (including treatment interruptions) and viremia was observed in our study, which is consistent with previous findings in this region [10]. However our study contradicts a recent study from British Columbia, Canada, which showed the probability of viral rebound was lower in patients who had longer duration of viral suppression and was independent of adherence level [21]. The discrepancies in the two settings might be due to the drug regimen used. Thus, our study showed that the risk of viral rebound in perfectly adherent patients is lower than in imperfectly adherent patients. Previous work in our setting has shown that individual-level adherence barriers (away from home, busy with other things, and simply forgetting) and medication side effects are the major obstacles for adherence [11,15,22].

Previous studies have shown that sub-optimal adherence, treatment interruptions and improper dosing can lead to drug resistance [10,23,24]. A recent meta-analysis on studies from resource limited settings showed that early identification of virological failure limits the emergence of drug resistance [25]. In our study, three patients with virological rebound showed major mutations, including M184V, K103N and Y181C. However, at the next visit, their viral load was suppressed to below the detectable limit. It is important to note that these three patients had good immunological status during that time and good adherence throughout the study periods. Studies from Sweden, Belgium and South Africa showed that despite the presence of NNRTI mutations such as M184V, viral re-suppression occurred in a fraction of patients with virological rebound [26–29]. Studies on subtype C virus from South Africa showed that despite the presence of NNTRI mutations such as K103N, V106M and G190A, a fraction of patients were successfully re-suppressed on the same regimen [28,29]. It was also proposed that supporting the adherence with special targeted counselling without any treatment change among such patients could be of cost benefit for the public health system in resource constrained settings. Our finding corroborates this idea. The patients in our settings are under good adherence counselling as part of a standardised national program and maintained an overall good adherence profile throughout the study period. Though 19% of the patients presented viral rebound, only 2.8% of the patients finally failed virologically. This combination of findings by us and others provides support for the conceptual premise that viral load measurement can act as an indicator for adherence. Early identification of viremia and targeted special adherence counsel-

Table 3. Viral rebound/failure and its associations with patients’ demographic, clinical, and behavioural parameters.

| Variables                                      | Viral rebound/failure (n = 60) | No viral rebound/failure (n = 263) | p valuea |
|------------------------------------------------|-------------------------------|-----------------------------------|----------|
| Age in years: mean (SD)                        | 38.0 (9.7)                    | 37.6 (8.3)                        | 0.703    |
| Male: n (%)                                    | 43 (71.7)                     | 192 (73.0)                       | 0.834    |
| Mean CD4 – T cell count in cells/mm³ over study period: mean (SD) | 410 (217)                     | 420 (221)                        | 0.758    |
| Regimen switch: n (%)                          | 29 (48.3)                     | 116 (44.1)                       | 0.553    |
| Perfect Adherence: n (%)                       | 19 (31.7)                     | 134 (51.0)                       | 0.007    |

*p-value based on t-test (d.f. = 321) for age and mean CD4 count, chi-square test (d.f. = 1) for male gender, regimen switch and perfect adherence.
**Table 4.** Characteristics of patients (n = 16) with VL > 2000 at first virological rebound/failure.

| Patient ID | CD4 count (cells/mm³) | VL at rebound (copies/mL) | VL at follow-up (copies/mL)* | Months on ART at viral rebound | Therapy | NRTI Resistance | NNRTI Resistance |
|------------|------------------------|---------------------------|-----------------------------|--------------------------------|---------|----------------|------------------|
| 1077       | 33                     | 70500                     | 38850                       | 34                             | AZT+3TC+NVP | M184V          | G190A            |
| 1104       | 711                    | 1144100                   | <100                        | 53                             | AZT+3TC+NVP | None           | None             |
| 1107       | 895                    | 3750                      | <100                        | 40                             | AZT/d4T+3TC+NVP | M184V          | G190A            |
| 1123       | 398                    | 2300                      | 1000                        | 36                             | d4T+3TC+NVP | None           | None             |
| 1127       | 74                     | 1699300                   | NA                          | 38                             | d4T+3TC+NVP | None           | None             |
| 1134       | 214                    | 4900                      | 2950                        | 36                             | d4T+3TC+NVP | K70R, M184V | V108I, Y181C, H221Y |
| 1153       | 160                    | 342300                    | <100                        | 48                             | d4T/AZT+3TC+NVP | None           | None             |
| 1162       | 176                    | 4726550                   | <100                        | 22                             | d4T+3TC+NVP | None           | None             |
| 1197       | 753                    | 5550                      | <100                        | 35                             | AZT+3TC+NVP | M184V          | K103N            |
| 2025       | 427                    | 5350                      | NA                          | 40                             | d4T+3TC+NVP | None           | None             |
| 2057       | 19                     | 3050                      | NA                          | 47                             | AZT/d4T/DDI+3TC+NVP | K65R, M184V | K103N, V108I |
| 2078       | 147                    | 14350                     | 15300                       | 41                             | d4T+3TC+NVP/EFV | M184V          | K103N            |
| 2082       | 21                     | 764100                    | 435100                      | 30                             | d4T+3TC+NVP/EFV | None           | A98G, K101E, V108I, Y181C, G190A, H221Y |
| 2140       | 560                    | 43000                     | <100                        | 13                             | d4T+3TC+NVP | None           | None             |
| 2230       | 324                    | 30700                     | <100                        | 30                             | AZT+3TC+EFV/NVP | M184V          | Y181C            |
| 2238       | 98                     | 333300                    | 62388                       | 29                             | AZT+3TC+EFV/NVP | M184V          | K101E, V106M, G190A |

*Abbreviations: 3TC, lamivudine; AZT, zidovudine; d4T, Stavudine; DDI, didanosine; EFV, Efavirenz; HAART, highly active antiretroviral therapy; NNRTI, non-nucleoside reverse-transcriptase inhibitors; NRTI, nucleoside reverse-transcriptase inhibitors; NVP, Nevirapine; VL, viral load. *VL follow-up after 6 months.*

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Author Contributions

Was the PI in the project and responsible for overall cohort maintenance, data accuation: SC MLE. Responsible virology and lab related tests: RS. Conceived and designed the experiments: UN A. Shet A. Sonnerborg MLE. Performed the experiments: UN EH SC RS. Analyzed the data: UN EH A. Shet A. Sonnerborg MLE. Contributed reagents/materials/analysis tools: SC RS. Wrote the paper: UN EH A. Shet A. Sonnerborg MLE.