Original article

Effect of early antiretroviral therapy during primary HIV-1 infection on cell-associated HIV-1 DNA and plasma HIV-1 RNA

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Background: Early initiation of combination antiretroviral therapy (ART) during primary HIV-1 infection may prevent the establishment of large viral reservoirs, possibly resulting in improved control of plasma viraemia rebound after ART cessation.

Methods: Levels of cell-associated HIV-1 DNA and plasma HIV-1 RNA were measured longitudinally in 32 acutely and recently infected patients, who started ART ≤120 days after the estimated date of infection, and interrupted ART after 18 months (median) of continuous therapy. Averages of HIV-1 DNA and RNA concentrations present in blood 30–365 days after therapy interruption (median duration 300 days, range 195–358) were compared between patients who started ART ≤60 days after the estimated date of infection (early starters), those who started between 61 and 120 days (later starters), and, for HIV-1 RNA only, with 89 untreated participants of the Swiss HIV Cohort Study with documented seroconversion and longitudinal measurements collected 90–455 days after the first positive HIV test.

Results: In early ART starters, average levels of plasma HIV-1 RNA and cell-associated HIV-1 DNA after treatment interruption were 1 $\log_{10}$ (P=0.008) and 0.4 $\log_{10}$ (P=0.03) lower compared with later starters. Average post-treatment plasma HIV-1 RNA levels in early starters were significantly lower, respectively, compared with untreated controls (-1.2 $\log_{10}$; P<0.0004).

Conclusions: Early treatment initiation within 2 months after HIV infection compared with later therapy initiation resulted in reduced levels of plasma viraemia and proviral HIV-1 DNA for ≥1 year after subsequent ART cessation. Plasma HIV-1 RNA levels in early starters were also significantly lower than in untreated controls.

Introduction

Successful antiretroviral therapy (ART) suppresses viral replication in patients infected with HIV-1 so that HIV-1 RNA in plasma can frequently be reduced to levels below the detection limit of standard assays, resulting in reduction of mortality and morbidity [1,2]. By contrast, HIV-1 DNA was found to remain detectable in peripheral blood mononuclear cells (PBMC) and in lymphoid tissues despite treatment and may be reactivated to produce infectious HIV particles after many years of successful ART [3–10]. This latent viral reservoir is typically established during primary HIV infection (PHI) [11,12].

It has been proposed that ART initiated during PHI may reduce the initial spread of the virus within

the body, prevent the establishment of large viral reservoirs, preserve HIV-specific immune responses and possibly increase the chance of durable replication control after ART interruption [13–16]. Indeed, one study found a more pronounced reduction in the size of the latent reservoir in early treated patients compared with those initiating ART during the chronic phase of HIV infection when comparing viral outgrowth assays and cell-associated HIV-1 DNA, respectively [6]. In chronically infected patients, the size of the latent reservoir as measured by cell-associated HIV-1 DNA has been shown to correlate with the magnitude of viral rebound after ART interruption [17]. A continued suppression of plasma HIV-1 RNA below detectable levels has been observed in small proportions of patients after cessation of ART initiated during PHI [18–22]. Follow-up periods of these studies were highly variable, mostly ranging from 3 to 12 months with some anecdotal reports of post-treatment controllers after a median follow-up of 75 months [22]. By contrast, other studies with follow-up ranging from 6 up to 24 months showed that virus control was not maintained in most patients after early ART interruption [23–27]. Nonetheless, since the viral setpoint following PHI has been shown to be predictive for the rate of disease progression and death [28,29], control of the virus in plasma as well as in cellular compartments during PHI may have the potential to improve disease outcomes of HIV-1 infection.

To investigate the possible benefit of early ART initiation at time of PHI, we assessed the longitudinal changes of cell-associated HIV-1 DNA and plasma HIV-1 RNA in 32 patients before ART, during early ART, and within 1 year after treatment interruption. Furthermore, we studied differential kinetics of cell-associated HIV-1 DNA and plasma HIV-1 RNA in patients who started ART ≤60 days after the estimated date of infection (EDI) and a group who started 61–120 days after the EDI. Finally, we compared viral RNA setpoints in the subset of patients who started ART within 60 days from the EDI with those in a control group of 89 untreated participants of the Swiss HIV Cohort Study (SHCS) with known dates of seroconversion.

Methods

Patients

Patients presenting with acute or recent HIV infection were enrolled in the Zurich Primary HIV Study (ZPHI) [30] between November 2002 and July 2007. The ZPHI study is an observational, open label, non-randomized, single-centre study (clinicaltrials.gov identifier NCT00537966). Written informed consent was obtained from each patient prior to inclusion.

Definitions

The diagnosis of PHI was performed based on established algorithms [31]. Acute HIV-1 infection was defined as: acute retroviral syndrome (ARS) and negative or indeterminate western blot (WB) in the presence of a positive p24 antigen and/or detectable plasma HIV-1 RNA (Fiebig stage I–IV); or a documented seroconversion with or without symptoms during the past 90 days. Recent HIV-1 infection was defined as: possible ARS, positive WB and detectable plasma HIV-1 RNA, in addition to a negative HIV-gp120 avidity or detuned assay (Fiebig V–VI); or documented acute HIV-1 infection but referral to our centre >90 days after the EDI. Of note, the avidity assay and the detuned assay both have a sensitivity and a specificity >95% in distinguishing between recent and chronic infection [32,33].

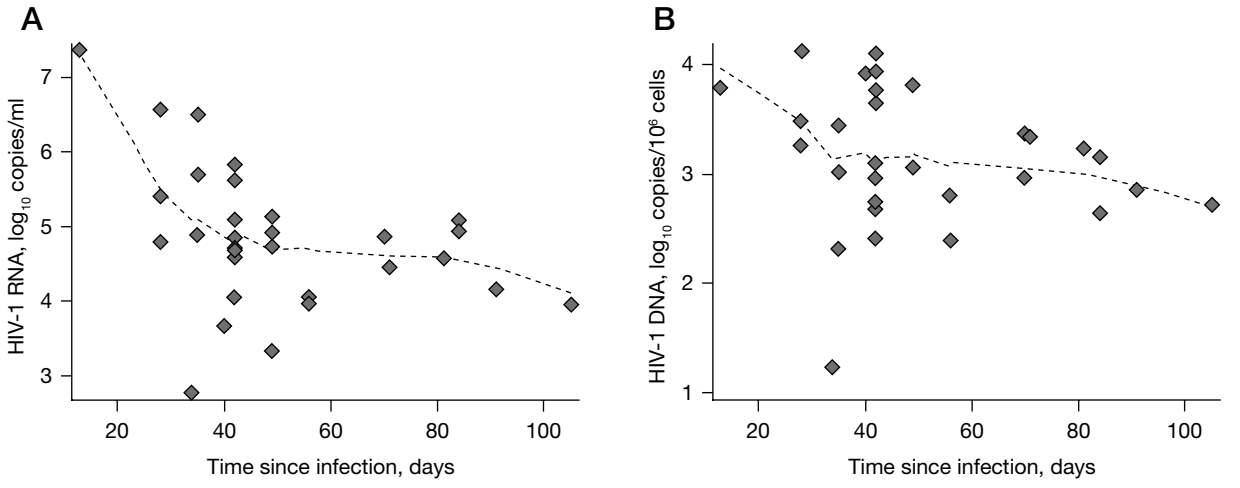
Estimated date of infection

For each patient a date of infection was estimated as previously described [34] by taking into account the pattern of different assay reactivities (first positive and last negative HIV test, negative, indeterminate and positive WB, positive p24 antigen, and avidity assay), patient's reports of unambiguous risk contacts, and timing of onset of ARS symptoms. With respect to WB results, three rules were applied to determine the EDI. First, with regard to fully negative WB (Fiebig stage I–III), if a single risk contact was reported within the last 3 weeks before the date of WB, this date was taken as the EDI; if no history of risk contacts was reported, infection was assumed to have occurred 14 days before the WB date. Second, with regard to indeterminate WB (Fiebig stage IV), if a single risk contact was reported between 2 and 6 weeks before the date of WB, this date was taken as the EDI; in case of several risk contacts, a higher and lower range was estimated and the mean of this range was taken as the EDI. Third, with regard to positive WB (Fiebig stage V–VI), if a single risk contact occurred 6 weeks or earlier before the date of the WB, this date was taken as the EDI if seroconversion was documented; if a seroconversion within 6 months was clearly documented without history of risk contact, the mean between the two tests (last negative and first positive HIV test) was taken as the EDI; if a patient had a former history of an ARS, a fully converted WB but no documented seroconversion and a negative detuned or avidity assay, the EDI was defined as the date 20 days before the onset of the ARS.

Antiretroviral therapy

Patients were offered standard first-line combination ART [35] independently of clinical indication and laboratory values (plasma viral load and $CD4^+$ T-cells) and, after 1 year of viral suppression below detection limits, they could choose to stop therapy. Patients who

Figure 1. Time dependence of baseline cell-associated HIV-1 DNA and plasma HIV-1 RNA measurements



Running means (broken lines) were calculated with locally weighted regressions (LOWESS). **(A)** Plasma viraemia (log_{10} HIV-1 RNA copies/ml) and **(B)** cell-associated HIV-1 DNA (log_{10} HIV-1 DNA copies/10^6 cells) plotted against the time elapsed between the estimated date of infection and the day of antiretroviral therapy initiation when the represented baseline parameters were measured.

never experienced a rebound of plasma HIV-1 RNA above 50 copies/ml while receiving therapy and then stopped treatment for >180 days were analysed in this study. They were stratified into two different subgroups: early starters (*n*=24), including patients who started ART ≤60 days after the EDI; and later starters (*n*=8), who started ART 61–120 days after the EDI. The cutoff point of 60 days after the EDI was based on our observation that the initially very high levels of viral HIV-1 RNA and DNA, which are a hallmark of acute HIV-1 infection [36,37], appear to level off after approximately 2 months post-infection (Figure 1).

Specimens

Longitudinal blood samples were collected from all patients at enrolment before ART, during treatment (monthly sampling until plasma HIV-1 RNA was undetectable and every 3 months thereafter), and after stopping therapy (monthly after cessation for the first 3 months, then every 3 months). EDTA blood from all time points was separated into PBMC and plasma according to standard procedures. Cellular material was divided into 2 million cells per aliquot and stored at -80°C.

Control group

The control group was selected from the SHCS [38], an ongoing multicentre, prospective, observational study and consisted of 89 untreated seroconverters with a negative and positive HIV test not longer than 90 days apart and at least two plasma HIV-1 RNA measurements while not on ART. Of note, methods of data collection and laboratory values within the two studies are comparable because individuals from the ZPHI study usually also participate in the SHCS and information is anonymously exchanged between the studies.

PCR assays

HIV-1 RNA in plasma was measured using Amplicor HIV-Monitor version 1.5 (Roche Diagnostics, Rotkreuz, Switzerland). Genomic DNA was prepared by incubation of PBMC in 150 µl cell lysis buffer [39]. DNA amplification was performed by quantitative real-time PCR (qPCR) in an IQ5 thermocycler (Biorad, Basel, Switzerland) using quadruplicate or duplicate reactions in a volume of 60 µl with 10 µl DNA template, HotStarTaq master mix (QIAGEN, Hilden, Germany) supplemented with PCR primers (1 µM each), probe (0.3 µM), and additional $MgCl_2$ (1.5 µM) by incubation for 15 min at 95°C and 60 cycles of 10 s at 95°C, 5 s at 55°C and 40 s at 60°C. Cellular input was quantified by β-actin PCR using the primers mf140 (CTCCCCCATGCCATCCTGCGTCTG) and mf141 (CTCGGCCGTGGTGGTGAAGC) with mf143 (f-ACCTGGCTGGCCGGGA-q) as the detection probe. Primers mf302 and mf299 [40], which were individually matched to patient-specific HIV-1 *pol* sequences derived from the SHCS genotypic drug resistance database [41], were used (primer and probe sequences available from the authors upon request). Fluorescent probes identical to individual patient-derived HIV sequences were chosen

from a collection of highly conserved probes with optimal performance in qPCR [40]. Individualized HIV-1 DNA standard curves for qPCR assays were prepared by serial threefold dilutions in DNA lysis buffer using baseline samples from each patient.

Calculations and statistics

HIV-1 DNA copy numbers were interpolated from cycle threshold (*Ct*) values using slopes (*S*) and the *y*-axis intercept (*I*) by applying the following equation: $Ct=I+S\times\log_{10}$ (copy number). *S* was calculated by linear regression of *Ct* values versus $\log_{10}$ of the input volumes of the original RNA sample at each dilution above the 50% end point (<2 replicates PCR positive). *I* was determined by the mean of all *Ct* values from samples at or below the 50% end point of PCR-positive dilutions. Based on previous validations [40,42,43], *I* reflects the *Ct* of reactions containing one HIV-1 DNA copy. HIV-1 DNA copy numbers resulting in nominal values <1 were censored to one copy per PCR as previously described [42,43]. Finally, HIV-1 DNA values were averaged over replicate PCR measurements, normalized to cellular input, and expressed in copies per 10^6 cell equivalents as obtained with β-actin PCR.

Viral setpoints were determined as the average of all post-treatment measurements of plasma viraemia (HIV-1 RNA) taken over 1 year, starting from day 30 after ART cessation for acutely and recently infected patients (median time of last measurement 300 days, range 195–358). Viral setpoints in the control group were estimated as the average of all HIV-1 RNA values taken over 1 year starting 90 days after the positive HIV test. For the controls, baseline was set at day 90 after the first positive HIV test to ensure that these individuals were no longer in the phase of acute infection when HIV-1 RNA plasma values are subject to fluctuations. Because post-treatment viral loads were not always stable and averages can mask significant longitudinal trends, we further performed comparisons on the highest values of post-treatment HIV-1 RNA and cell-associated HIV-1 DNA for each patient, which are less affected by possible dynamics. Differences between measurements taken before ART initiation (baseline) and post-treatment plasma HIV-1 RNA and cell-associated HIV-1 DNA setpoints were compared with the *t*-test (with the Welch correction for unequal variances) and linear regression, which was adjusted for the time between the EDI and baseline measurements. These adjustments either consisted of the inclusion of a linear term only to model linear decays, or of a linear term and a quadratic term to allow for non-linear dynamics of viral markers as they are often observed for plasma HIV-1 RNA. The final model was selected on the basis of adjusted R^2 values. The normality assumption implied by these tests of significance was checked with the Shapiro–Wilks test.

Statistical analyses were performed using Graph-Pad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA), and Stata 11/SE (Stata Corp., College Station, TX, USA). The level of significance was set at $P<0.05$, and all *P*-values were two-sided.

Results

Patient characteristics

A total of 32 patients with ART initiation during PHI was studied (Table 1). Most of them were male (*n*=28, 87.5%), had acquired HIV through homosexual contacts (*n*=21, 65.6%), and were infected with HIV subtype B (*n*=21, 65.6%). Eight patients (25%) were infected with CRF01_AE viruses. All but one patient (who was treated with unboosted nelfinavir) received boosted lopinavir in combination with two nucleoside reverse transcriptase inhibitors. The median (IQR) $CD4^+$ T-cell count at time of treatment initiation was 498 (434–563) cells/μl and individuals received therapy for a median (IQR) time of 18 months (16–20). In addition, we selected 89 control patients from the SHCS with documented seroconversion who remained naive to ART (Table 1).

Description of longitudinal changes in plasma HIV-1 RNA and cell-associated HIV-1 DNA

Figure 1 shows the time dependence of baseline cell-associated HIV-1 DNA and plasma HIV-1 RNA measurements from the EDI and treatment initiation (coinciding with the first set of laboratory measurements). Both baseline plasma HIV-1 RNA and baseline cell-associated HIV-1 DNA values exhibited a time-dependent spontaneous decay even in the absence of ART.

Comparisons of cell-associated HIV-1 DNA at three different time points – before ART, during early ART, and after therapy cessation – showed a distinct pattern, as illustrated in Figure 2B. The levels (mean [95% CI]) were generally highest at baseline with 3.1 (2.9–3.4) $\log_{10}$ copies/million cells, reached a minimum of 1.9 (1.6–2.1) $\log_{10}$ copies/million cells during treatment, and then rebounded to a setpoint of 2.4 (2.2–2.7) $\log_{10}$ copies/million cells after therapy was stopped. Similarly, baseline levels of HIV-1 RNA superseded post-treatment setpoints, with 4.8 (4.5–5.2) $\log_{10}$ copies/ml compared with 3.3 (2.8–3.8) $\log_{10}$ copies/ml, as well as the maximum values observed within the 1-year period after therapy cessation (3.8 [3.3–4.4] $\log_{10}$ copies/ml; Figure 2A).

Effect of early ART on plasma HIV-1 RNA or cell-associated HIV-1 DNA setpoints after therapy interruption

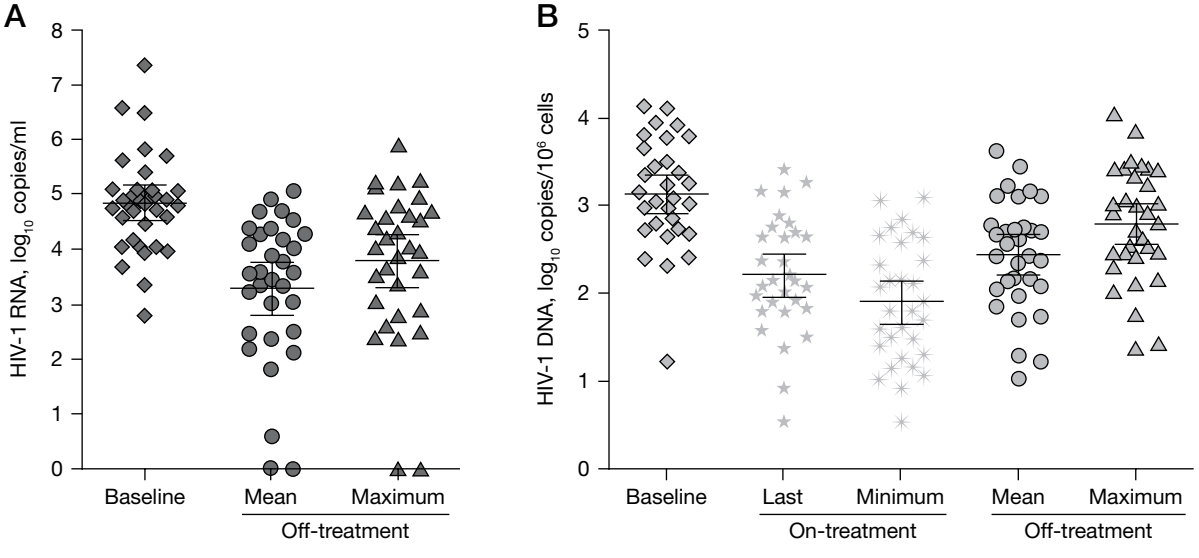
To investigate whether the initiation of ART during PHI lowered the setpoints of plasma HIV-1 RNA or cell-associated HIV-1 DNA after treatment interruption,

Table 1. Patient characteristics

Parameter	Treatment initiation during acute phase	Untreated controls
Patients	32 (100)	89 (100)
Male sex	28 (87.5)	73 (82.0)
Median age, years (IQR)	39 (36–42)	36 (34–38)
Mode of HIV acquisition		
Heterosexual risks	10 (31.3)	26 (29.2)
Homosexual risks	21 (65.6)	48 (53.9)
Other risks	1 (3.1)	15 (16.9)
HIV subtype		
Subtype CRF01	8 (25)	2 (2.2)
Subtype B	21 (65.6)	61 (68.5)
Other subtypes	3 (9.4)	26 (29.2)
First antiretroviral treatment		
Median year of ART initiation (IQR)	2004 (2003–2004)	NA
Median duration of first completed ART, months (IQR)	18.0 (15.7–20.4)	NA
First pre-ART laboratory values		
Median baseline CD4$^+$ T-cell count, cells/ml (IQR)	498 (434–563)	NA
Median baseline RNA, $\log_{10}$ copies/ml (IQR)	4.8 (5.0–5.2)	NA
Median baseline DNA, $\log_{10}$ copies/10^6 cells (IQR)	3.1 (2.9–3.4)	NA
Median time from infection to first laboratory measurements, months (IQR)	1.7 (1.4–1.9)	NA
Median time from infection to first ART, months (IQR)	1.7 (1.5–2.0)	NA
First measurement 30 days after stop of initial therapy		
Median CD4$^+$ T-cell count, cells/ml (IQR)	763.4 (678.5–848.3)	NA
Median RNA, $\log_{10}$ copies/ml (IQR)	2.6 (2.0–3.3)	NA
First measurement 90 days after first positive HIV test		
Median CD4$^+$ T-cell count, cells/ml (IQR)	NA	667.5 (612.6–722.3)
Median RNA, $\log_{10}$ copies/ml (IQR)	NA	4.7 (4.4–5.0)

Values are *n* (%) unless stated otherwise. ART, antiretroviral therapy; NA, not applicable.

Figure 2. Distribution of HIV-1 DNA and HIV-1 RNA before, during and after early ART



(A) Plasma viraemia ($\log_{10}$ HIV-1 RNA copies/ml). **(B)** Cell-associated HIV-1 DNA ($\log_{10}$ HIV-1 DNA copies/10^6 cells). Baseline, off-treatment mean, and off-treatment maximum values from the 32 patients who received early antiretroviral therapy (ART) for >180 days and then stopped therapy are shown for both parameters. For cell-associated HIV-1 DNA, measurements of last and minimum on-treatment levels are also displayed, but not so for plasma viraemia because these were all below the detection threshold of 50 copies/ml. Error bars show means and their 95% CIs.

Table 2. Regression analyses adjusted for the time span between infection and baseline measurement

		Student's *t*-test			Linear regression[a]		
Parameter 1	Parameter 2	Mean $\log_{10}$ difference	95% CI	*P*-value	Adjusted mean $\log_{10}$ difference	Adjusted 95% CI	Adjusted *P*-value
Baseline DNA	Mean off-treatment DNA	0.68	(0.49–0.87)	<0.001	0.22	(-0.15–0.59)	0.228
Baseline DNA	Maximum off-treatment DNA	0.33	(0.14–0.52)	0.002	0.04	(-0.40–0.41)	0.985
Baseline RNA	Mean off-treatment RNA	1.49	(0.94–2.04)	<0.001	0.59	(-0.58–1.75)	0.312
Baseline RNA	Maximum off-treatment RNA	1.00	(0.42–1.57)	0.001	0.29	(-0.97–1.56)	0.642

[a]Linear regression adjusted for time since HIV infection, which was centred at day 90. Thus the difference between parameters was estimated as what it would have been at day 90 after infection; that is, after the acute phase.

we performed paired analyses of the respective pre-treatment and post-treatment levels (Table 2), which yielded statistically significant differences. However, such comparisons are problematic because most baseline measurements were taken within a median time of 1.7 months (IQR 1.4–1.9) after EDI, when the immune response is still fully developing and viral replication is decelerating independently of ART (Figure 1) [36,37]. Therefore, we re-assessed the differences between levels of pre-treatment and post-treatment viral markers using regression analyses adjusted for the time span between the EDI and baseline (Table 2) to emulate a uniform sampling of baseline measurements at day 90 after the infection, when natural decay of nucleic acids was expected to have levelled off. These adjustments led to far smaller estimates for the difference between pre- and post-treatment of 0.22 (95% CI -0.15–0.59) $\log_{10}$ for cell-associated HIV-1 DNA and 0.59 (-0.58 –1.75) $\log_{10}$ for plasma HIV-1 RNA, which were no longer statistically significant.

Timing of early ART initiation and effect on plasma HIV-1 RNA and cell-associated HIV-1 DNA setpoints

Next, we investigated whether the timing of ART initiation during PHI affects post-treatment plasma HIV-1 RNA and cell-associated HIV-1 DNA levels. We hypothesized that initiation of treatment around peak viraemia within the first 60 days of infection (early starters; *n*=24) may be superior to starting treatment later (that is, within 61–120 days; later starters; *n*=8). When comparing post-treatment setpoints between these groups (Figure 3), plasma HIV-1 RNA levels (mean [95% CI]) were lower in the early starter group compared with the later starters (3.1 [2.4–3.7] versus 4.0 [3.6–4.4] $\log_{10}$ copies/ml; *P*=0.008). Similarly, patient-specific maximum HIV-1 RNA of all values collected over 1 year after ART interruption were also lower in the early starter group (3.6 [3.0–4.2] $\log_{10}$ copies/ml) when compared with later starters (4.3 [3.9–4.8] $\log_{10}$ copies/ml; *P*=0.0497). Of note, three patients (9%) in the group starting ART within 60 days from the EDI continuously maintained undetectable plasma HIV-1 RNA after cessation of ART (follow-up range 0.8–2.5 years). Post-cessation mean cell-associated HIV-1 DNA setpoints were also statistically different between early and later starters (2.3 [2.0–2.6] versus 2.7 [2.5–3.0] $\log_{10}$ copies/million cells; *P*=0.03), but the difference in maximum off-treatment DNA levels no longer reached statistical significance between early starters (2.7 [2.4–3.0] $\log_{10}$ copies/million cells) and later starters (3.0 [2.7–3.3] $\log_{10}$ copies/million cells; *P*=0.18).

Comparison between patients treated early during PHI and an untreated control group

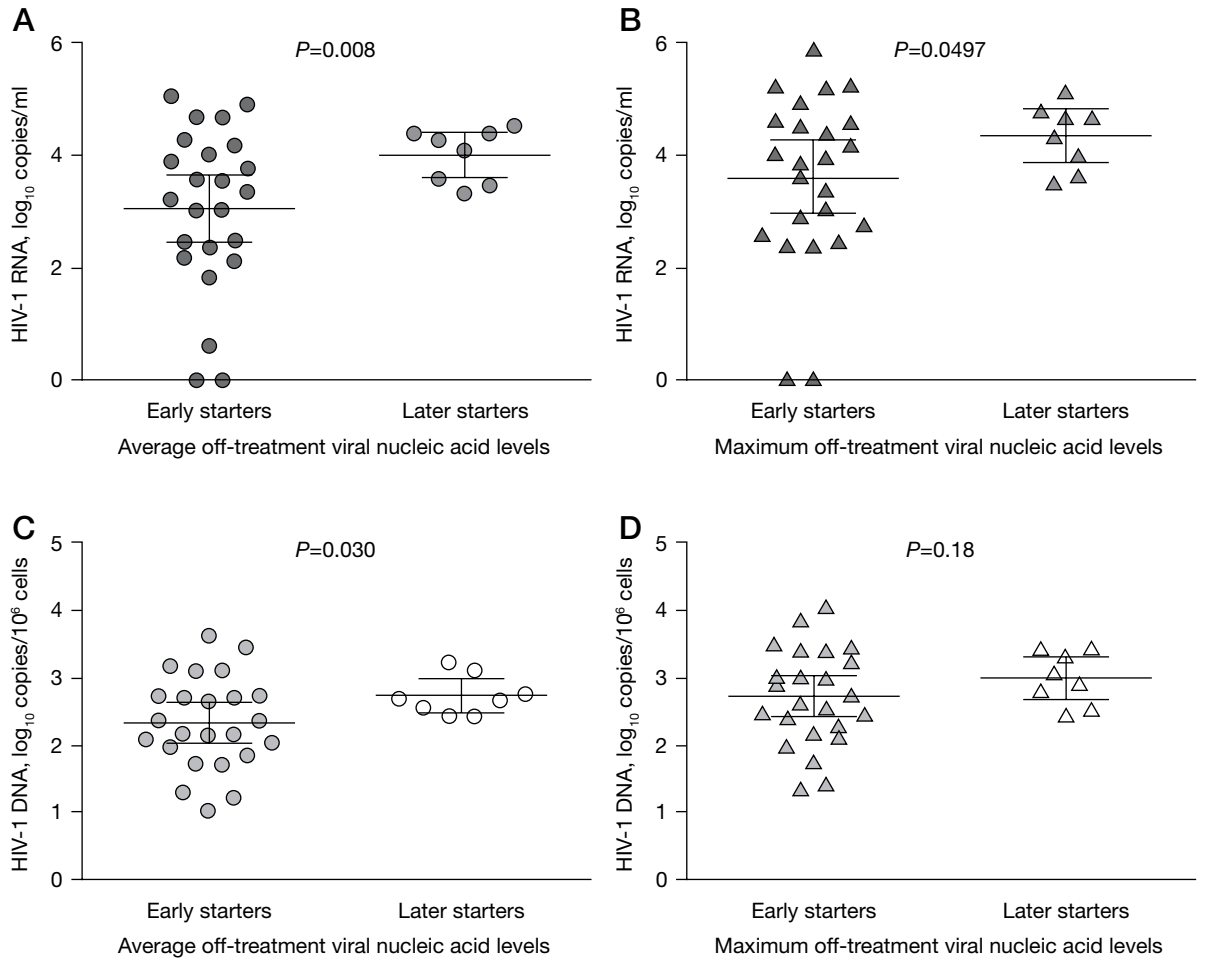
Baseline characteristics of early starters and the untreated control group (documented seroconverters), including sex, risk of HIV acquisition, and ethnicity, were not different. However, early starters tended to be older (median age 41 versus 36 years in controls), had a higher $CD4^+$ T-cell count (median 752 [IQR 586–990] versus 667.5 [612.6–722.3] cells/μl) and had lower plasma HIV-1 RNA levels (mean 2.6 [95% CI 1.7–4.1] versus 4.7 [4.4–5.0] $\log_{10}$ copies/ml) at day 30 after therapy cessation compared with the measurements taken around 90 days after diagnosis of HIV infection in untreated controls.

Plasma HIV-1 RNA setpoints were significantly different between the subgroup of patients starting ART within 60 days from the EDI and untreated controls (3.1 [2.4–3.7] versus 4.2 [4.1–4.4] $\log_{10}$ copies/ml; *P*<0.0004). Also, maximum off-treatment HIV-1 RNA was significantly different between these two groups of patients. In Figure 4, these findings are summarized by depicting differences in viral plasma RNA levels between early starters and later starters, as well as between early starters and the untreated control group. All comparisons reached statistical significance (*P*≤0.0497), and showed mean decrements of 0.8–1.3 $\log_{10}$ viral RNA levels in early starters versus the two other groups.

Discussion

Whether initiation of ART during PHI can attenuate the course of HIV disease remains unknown. Theoretically,

Figure 3. Post-treatment cessation HIV-1 nucleic acid levels from patients stratified according to the time of ART initiation



(A&B) Plasma viraemia (log_{10} HIV-1 RNA copies/ml). Early starters (antiretroviral therapy [ART] initiation ≤60 days after infection) are displayed by dark grey symbols and later starters (start >60 days after infection) by light grey symbols. **(C&D)** Cell-associated HIV-1 DNA (log_{10} HIV-1 DNA copies/10^6 cells). Early starters are displayed by filled grey symbols and later starters by open symbols. Circles show mean setpoints and triangles show maximum off-treatment measurements. *P*-values indicate the results of the Student's *t*-test using Welch's correction for unequal variance. Error bars show means and their 95% CIs.

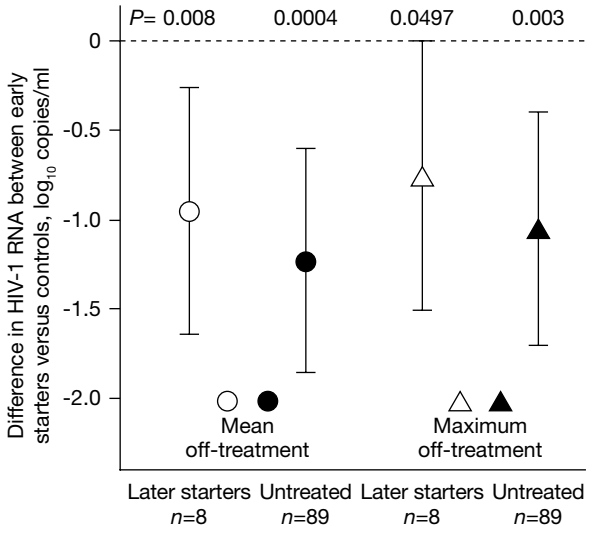
early intervention could decrease the severity of acute illness, alter the initial virus spread, could positively affect disease progression, and could reduce the rate of viral mutation, preserve immune function, and lower the risk for viral transmission [6,27,34,44–47].

We observed a time-dependent spontaneous decay of both plasma HIV-1 RNA and cell-associated HIV-1 DNA baseline values before early ART initiation. Consequently, the levels of plasma HIV-1 RNA and cell-associated HIV-1 DNA were consistently higher at baseline than post-treatment. However, our matched analyses of baseline with post-treatment markers may have been confounded by their highly time-dependent dynamics: in fact, early after HIV infection (at baseline in our study), viral replication is intrinsically decelerating independently of ART [36,37] as a consequence of $CD4^+$ T-cell depletion in gut-associated lymphoid tissue [48,49], and the effect of host cytotoxic and humoral immune response as well as complement-induced virolysis on systemic HIV replication [19,50–53]. After emulation of baseline viral nucleic acid levels to a time point when natural decay of nucleic acids was expected to have levelled off (90 days after the EDI), differences between baseline values and viral setpoints of plasma HIV-1 RNA or cell-associated HIV-1 DNA were no longer statistically significant. To circumvent this analytical caveat, the patients receiving early ART starting ≤60 days after the EDI were compared with a group of later starters (61–120 days after the EDI) and with an

untreated control group in order to estimate possible benefits of ART during PHI. Relative to later starters, average post-treatment levels of plasma HIV-1 RNA and cell-associated HIV-1 DNA after ART interruption were 1 and 0.4 $\log_{10}$ lower, respectively, in patients with early ART initiation. Moreover, viral setpoints in early starters showed significant differences of 1.2 $\log_{10}$ copies/ml for average HIV-1 RNA in comparison with untreated seroconverters. Reassuringly, differences of similar order of magnitude were observed even when a more conservative analytical approach was applied by only comparing the highest post-treatment levels per patient with corresponding measurements from the untreated control group. Thus, our finding provides robust evidence that ART initiation as early as possible during PHI (that is, within 60 days from the EDI) can impede the pervasion of viral reservoirs, and perhaps also the establishment of a pool of latently infected cells, thereby possibly enhancing control of HIV replication after treatment interruption. Whether these reductions in viral marker levels extend beyond the 1-year post-treatment phase studied here requires further investigation. However, the magnitude of reduction in plasma viraemia of around 1 $\log_{10}$ observed in our study (in both analyses, early starters versus later starters as well as early starters versus untreated control group) may probably be clinically significant, as suggested by previous studies in which HIV-1 plasma viral load has been identified and validated as a marker for HIV-1 disease progression [54,55]. In other studies, cell-associated HIV-1 DNA has been shown to be an independent prognostic marker of disease progression, correlated with body viral reservoir and dynamics of viral infection [6,17,56–59].

The body of evidence for an effect of early ART overall remains sparse and several studies reported contradictory results. A recent analysis [60] did not show any difference between acutely and recently infected patients with regard to plasma viraemia setpoints after cessation of ART. Nevertheless, in that study overall 40% of patients treated during acute or recent infection maintained a viral load <5,000 HIV-1 RNA copies/ml for 24 weeks, which suggests some sustained effect after treatment interruption. However, the two groups of acutely and recently infected patients described in this study were classified according to different criteria (acute infection until 4 weeks after the EDI, recent infection up to 6 months after the EDI) compared with the early starters and later starters in our study. Moreover, the follow-up was shorter (24 weeks versus up to 1 year in our study). It is therefore possible that immunological benefits may take longer than 24 weeks of treatment interruption to be appreciated.

Figure 4. Differences in post-treatment cessation levels of plasma viraemia in early starters versus later starters and untreated HIV-1 seroconverters



Differences in post-treatment levels of plasma viraemia in early starters (expressed as $\log_{10}$ copies/ml) from plasma HIV-1 RNA levels of later starters (open symbols) and from a control group (black symbols) of untreated individuals with known time of seroconversion and >2 viral load measurements within 90 and 356 days thereafter. Circles and triangles show differences in mean setpoints and maximum measurements, respectively. *P*-values show the results of the Student's *t*-test using Welch's correction for unequal variance. Error bars show means and their 95% CIs.

Hecht *et al.* [27] compared plasma HIV-1 RNA levels among acutely and recently treated HIV-1 seroconverters with an untreated control group. The authors concluded that both groups of early treated individuals had significantly lower viral loads and higher $CD4^+$ T-cell counts at 24 weeks, but the effects seemed to disappear in the recent group after longer follow-up (72 weeks). Also in this study, the two subgroups were defined in a different way from ours (acute treatment group started ART within 2 weeks and recent treatment group between 2 weeks and 6 months after antibody seroconversion). The duration of early ART was more variable (range 12–173 weeks for acute and up to 260 weeks for recent infection) than in our cohort.

Some limitations of our analysis should be noted. (i) The observational nature and the lack of a randomized, untreated control group may have led to systematic differences between compared groups, which are not reflected by the data. (ii) EDI determination could be biased by various factors, particularly by inaccurate reporting of the history of sexual risk contacts. (iii) Since WB assays have mostly been characterized for subtype B virus, the relatively high

proportion of non-B subtype present in our population could introduce further variability to the EDI. (iv) The early versus later treated analysis is limited by relatively small numbers, especially among the group who started ART between 61 and 120 days. (v) Moreover, evaluation of the early treated ZPHI group versus the untreated SHCS control group may have been confounded if ZPHI patients diagnosed during PHI were more symptomatic compared with controls from the SHCS, and thus presented earlier for medical attention. However, if such a bias was present, it would shift the results toward the null hypothesis, meaning that it would probably make the difference in viral setpoints between early starters and untreated controls even smaller (and as a consequence make our findings of reduced setpoints after ART interruption less statistically significant) because patients with severe ARS tend to have higher viral loads and are progressing more rapidly than patients with mild or absent ARS symptoms [61]. (vi) Since the time of receiving treatment was blanked out for patients in the treatment group, these patients were infected with HIV for a longer duration than the untreated group at each time point of the analysis. (vii) In addition, the dates of enrolment into the ZPHI and the SHCS may differ between studies; that is, enrolment into the ZPHI study started in 2002, whereas control patients were also selected from earlier time periods. However, since virulence of HIV-1 has been shown to be stable over a 20-year period in the SHCS [62], these differences most probably did not play a major role on the outcome of our analyses.

Major strengths of our study include the high resolution of plasma and PBMC sampling and the long follow-up period after ART interruption. Moreover, our patient-matched PCR approach allowed a more accurate quantification of cell-associated HIV-1 DNA than real-time PCR using standard probes and primers, by which copy numbers would probably be underestimated due to the relatively high prevalence of point mutations even in highly conserved regions of the HIV-1 genome [40].

In conclusion, our results support the concept of early ART initiation during PHI, particularly within 60 days after the EDI. Over an observational period of 1 year after cessation of therapy, patients with early ART initiation exhibited lower plasma HIV-1 RNA and cell-associated HIV-1 DNA setpoints relative to patients with later ART initiation or untreated controls. Whether these differences in viral markers will translate into clinical benefits for early treated patients requires further study. Nevertheless, a small HIV reservoir on ART may facilitate switching to simplified maintenance drug regimens and a low viral setpoint after cessation of therapy may increase the time window of planned treatment interruption, reducing toxicity, drug resistance development, and the cost of long-term ART. Moreover, suppressed HIV-1 RNA during early ART and a subsequent lower HIV-1 RNA setpoint after therapy interruption could partially prevent additional HIV transmission [34]. Future randomized controlled trials will show whether these promises of early treatment hold true.

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SG designed patient-matched primers and probes, carried out the HIV-1 DNA measurements, and participated in the design of the statistical analyses. SG and VvW wrote the manuscript. VvW participated in the study design, and performed statistical analyses. MF participated in design and coordination of this study, in primer and probe design, in the statistical analyses, and revised the manuscript. BN performed PCR assays. BJ participated in primer and probe design and revised the manuscript. HFG initiated and leads the ZPHI study, designed the present study, participated in data analysis and revised the manuscript. MB, EB, MC, AR, BH, PV and RW enrolled patients and revised the manuscript. All authors read and approved the final manuscript.

Disclosure statement

The authors declare no competing interests.

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