Establishment and Replenishment of the Viral Reservoir in Perinatally HIV-1-infected Children Initiating Very Early Antiretroviral Therapy

Marta Martínez-Bonet,[1,2,3,4] Maria Carmen Puertas,[5] Claudia Fortuny,[6] Dan Ouchi,[5] Maria José Mellado,[7] Pablo Rojo,[8] Antoni Noguera-Julian,[6] Mª Angeles Muñoz-Fernández,[1,2,3,4,a] and Javier Martinez-Picado[5,9,10,a]

[1]Hospital General Universitario Gregorio Marañón, [2]Instituto de Investigación Sanitaria Gregorio Marañón, [3]Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), [4]Spanish HIV HGM BioBank, [5]AIDS Research Institute IrsiCaixa, Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona, [6]Unidad de Enfermedades Infecciosas, Servicio de Pediatría, Hospital Sant Joan de Déu, Universitat de Barcelona, Esplugues del Llobregat, [7]Servicio de Pediatría Hospitalaria y E. Infecciosas y Tropicales Pediátricas. Hospital Universitario Infantil LA PAZ- H. Carlos III, Madrid, [8]Servicio de Pediatría. Hospital 12 de Octubre, Madrid, [9]Universitat de Vic – Universitat Central de Catalunya (UVic-UCC), and [10]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Background. Combination antiretroviral therapy (cART) generally suppresses the replication of the human immunodeficiency virus type 1 (HIV-1) but does not cure the infection, because proviruses persist in stable latent reservoirs. It has been proposed that low-level proviral reservoirs might predict longer virologic control after discontinuation of treatment. Our objective was to evaluate the impact of very early initiation of cART and temporary treatment interruption on the size of the latent HIV-1 reservoir in vertically infected children.

Methods. This retrospective study included 23 perinatally HIV-1-infected children who initiated very early treatment within 12 weeks after birth (n = 14), or early treatment between week 12 and 1 year (n = 9). We measured the proviral reservoir ($CD4^+$ T-cell–associated HIV-1 DNA) in blood samples collected beyond the first year of sustained virologic suppression.

Results. There is a strong positive correlation between the time to initiation of cART and the size of the proviral reservoir. Children who initiated cART within the first 12 weeks of life showed a proviral reservoir 6-fold smaller than children initiating cART beyond this time ($P < .01$). Rapid virologic control after initiation of cART also limits the size of the viral reservoir. However, patients who underwent transient treatment interruptions showed a dramatic increase in the size of the viral reservoir after discontinuation.

Conclusions. Initiation of cART during the first 12 weeks of life in perinatally HIV-1-infected children limits the size of the viral reservoir. Treatment interruptions should be undertaken with caution, as they might lead to fast and irreversible replenishment of the viral reservoir.

Keywords. HIV-1; vertical infection; early antiretroviral therapy; viral reservoir.

Received 14 April 2015; accepted 21 May 2015; electronically published 10 June 2015.

[a]M. A. M.-F. and J. M.-P. contributed equally to this work.

Correspondence: Javier Martinez-Picado, PhD, IrsiCaixa Foundation, Hospital Germans Trias i Pujol, Ctra. de Canyet s/n, Badalona, Barcelona 08916, Spain (jmpicado@irsicaixa.es).

Clinical Infectious Diseases® 2015;61(7):1169–78

DOI: 10.1093/cid/civ456

Perinatal infection by human immunodeficiency virus type 1 (HIV-1) can be diagnosed during the first days of life in newborns infected in utero or 2–6 weeks after delivery in infants infected during delivery. Compared with older children and adults [1], acute infection in babies is characterized by a sustained high-level plasma viral load (pVL) in the initial years of infection [2, 3] and a high risk of rapid progression to AIDS and death [4]. The rapid reduction of peak pVL that is characteristic of acute HIV-1 infection in adults occurs very slowly during the first months of an infant's life. These

age-associated disparities in the viral kinetics of HIV-1 and the clinical outcome of HIV infection are most likely attributable to developmental differences in the neonatal immune system [5, 6].

AIDS-related mortality in children has decreased significantly with the wide availability of combination antiretroviral therapy (cART). Initially, cART was strongly recommended only in infants with HIV-related symptoms [7]. However, during recent years, multiple studies have suggested the benefit of starting early cART in all HIV-1-infected infants [8–11]. Therefore, international guidelines are now recommending initiation of cART in all HIV-1-infected infants aged less than 1 year regardless of clinical and immunological conditions [12, 13].

Some HIV-1-infected infants who initiate cART soon after birth do not display HIV-1-specific antibodies or cellular responses, thus indicating early control of viral replication [14, 15]. Nevertheless, HIV-1 infection quickly establishes latent reservoirs, mainly in resting memory $CD4^+$ T cells. Although the memory T-cell population in peripheral blood is small in newborns [16], only to develop later in childhood [17], recent findings demonstrate the presence of HIV-1-susceptible memory $CD4^+$ T cells in the gut of newborns [18]. The limitations on establishment of reservoirs facilitated by early cART could play a critical role in achieving natural control of viral replication upon discontinuation of cART, which could be defined as "functional cure" [17, 19–22]. On the other hand, viral reservoirs could provide a persistent source of recrudescent viraemia despite temporary remission of HIV-1 infection after withdrawal of treatment [23], as observed in the so-called Mississippi baby [24]. Therefore, in order to design interventions aimed at achieving functional cure in this population, it is important to understand how very early initiation of cART can affect persistence of HIV-1 in older children with successfully suppressed viraemia.

We retrospectively studied a cohort of perinatally HIV-1-infected children who initiated cART within the first year of life. We compared initiation of cART within the first 12 weeks of life with initiation at a later date to assess the potential limitation of establishment of viral reservoirs in the long-term. Our data show that the extent of the latent infection can be limited by administration of optimal cART shortly after birth, which leads to rapid viral suppression. Some of the infants included in this cohort had undergone treatment interruptions. The analysis of the effects of interruptions on the dynamics of viral reservoir indicates that very low reservoir size is not a prognostic marker of long-lasting HIV remission and that reservoir replenishment driven by viral rebound may be fast and irreversible even in these cases.

MATERIALS AND METHODS

Study Participants

The study was based on 139 children with vertically transmitted infection from the Paediatric Spanish AIDS Research Network Cohort (coRISpe). Only samples from those children who initiated cART within the first year of life were included. The analysis was finally based on samples from 23 children who had maintained viral suppression (≤200 copies/mL) for at least 1 year before sampling. When possible, we selected samples that had been collected at multiple time points. Cryopreserved plasma and peripheral blood mononuclear cells (PBMC) and associated clinical data were provided by the Spanish HIV HGM BioBank [25] and by coRISpe [26]. Clinical classification of AIDS-defining events and immunologic categories were based on international guidelines [27]. The ethics committee of Hospital Gregorio Marañón in Madrid approved the study.

Quantification of Proviral HIV-1 DNA

To evaluate the size of the proviral reservoir, $CD4^+$ T cells were purified from PBMC by negative immunomagnetic separation ($CD4^+$ T Cell Isolation Kit; Miltenyi Biotech), and lysed extracts were used to measure cell-associated total HIV-1 DNA by droplet digital polymerase chain reaction (ddPCR) with 5′LTR or Gag primers and probes, depending on the efficiency of detection in each patient. The 2 primer-probe sets have been previously assessed to be comparable in terms of efficiency and sensitivity by ddPCR on a plasmid containing the IIIB reference HIV-1 sequence (standard 2LTR-CCR5 plasmid, kindly provided by M. Stevenson). However, mismatches or deletions in the viral sequences can prevent from efficient amplification in some patient samples. For that reason all samples were measured in parallel using the 2 primer-probe sets to ensure efficient and reliable proviral absolute quantification. The RPP30 cellular gene was quantified in parallel to normalize sample input. All primers and FAM/HEX-ZEN-Iowa BlackFQ dual-labeled double-quenched probes were purchased from Integrated DNA Technologies [28, 29].

Serological Assessment

The Inno-Lia HIV I/II Score assay (Innogenetics, Ghent, Belgium) was run as previously described [30] to evaluate humoral responses in plasma samples (taken simultaneously with stored PBMCs). The results of antibody testing for HIV-1 antigens gp120, gp41, p31, p24, and p17 were analyzed and classified as positive if at least 2 lines had a rating ≥1+, and both were envelope lines or at least 1 was an envelope antigen and the other p24. Other combinations were considered indeterminate.

Ultrasensitive Viral Load Test

Residual low-level viraemia was determined by ultracentrifugation of up to 7.5 mL of plasma at 170 000 *i* at 4°C for 30 minutes, followed by viral RNA extraction using the m2000sp Abbot device. HIV-1 RNA copies were quantified in the Abbot m2000rt instrument using the Abbott RealTime HIV-1 Assay and laboratory-defined applications software from the instrument. HIV-1 RNA copies in the low range were determined by

means of a calibration curve set between 1000 and 10 copies/mL (five points at 1/3 serial dilutions: 1000, 300, 100, 30, and 10 in triplicate). The quantification method was validated in triplicate with a positive control (prequantified standard HIV from the World Health Organization) in the range of 128–0.5 copies/mL (9 serial 1/2 dilutions). Similarly, the concentration protocol was validated by running direct vs diluted ultracentrifuged prequantified plasma samples, with viral load (VL) ranging from 400 to 10 HIV-1 RNA copies/mL.

Statistical Analysis

We compared numerical and categorical variables between groups using the Mann–Whitney test and Fisher exact test, respectively.

A multiple linear regression model was fitted to define the association between HIV-1 DNA and previously selected clinical variables. Selection was performed by means of a Spearman pairwise correlation test to identify those parameters that were significantly correlated with HIV-1 DNA and as independent as possible between them. The final model—$\log_{10}$ (copies HIV-1 DNA/10^6 $CD4^+$ T cells) = (Age at cART initiation) + (Time to virologic control)—was derived using a bootstrapped Akaike information criterion–based stepwise selection method (Supplementary Figure 1).

Longitudinal changes within groups of children who discontinued or did not discontinue cART and the comparison of the mean change between groups (slope coefficient) were evaluated using linear mixed models: $\log_{10}$ (copies HIV-1 DNA/10^6 $CD4^+$ T cells) = intercept + group + timepoint + group*timepoint. A statistically significant interaction term (group*timepoint) identified significantly different slopes between the groups. To perform these longitudinal analyses, timepoints were defined according to the time since the first determination: 0.5–2 years, 2–2.5 years, 2.5–3 years, and more than 3 years. All analysis were performed using the R package [31].

RESULTS

The study population comprised 23 perinatally HIV-1-infected children, who were grouped into 2 categories: those who initiated cART very early (VET, 0–12 weeks, 14 patients) or early (ET, 12–54 weeks, 9 patients). The first cART regimen consisted of a backbone of 2 nucleoside reverse transcriptase inhibitors (mostly lamivudine and zidovudine) plus either a nonnucleoside reverse transcriptase inhibitor (nevirapine) or a protease inhibitor (nelfinavir or lopinavir/ritonavir). Clinical and demographic characteristics are summarized in Table 1. At sampling, all patients responded effectively to cART and had been virologically suppressed (≤200 copies/mL) for more than 1 year (median time on suppression = 4.5 years [interquartile range (IQR): 3.3–6.9]). At sampling (median age = 8.0 years [IQR: 5.1–10.0]), 17 children were on a protease inhibitor–based regimen.

We first explored the potential association between proviral reservoir size and the main clinical parameters. We found a strong positive correlation between total HIV-1 DNA in $CD4^+$ T cells and the age of the children at initiation of cART (*P*-value <.0001; Figure 1*A*). Indeed, children treated within the first 12 weeks of life had a smaller reservoir than children treated later (*P*-value <.01; Figure 1*B*). A positive correlation was also found between total HIV-1 DNA in $CD4^+$ T cells and the time needed to achieve viral suppression after initiation of cART (*P*-value = .03; Figure 1*C*). However, we did not find statistically significant differences when children were classified as rapid controllers (≤1 year) or slow controllers (>1 year) (Figure 1*D*). A more accurate analysis, in which the main clinical parameters were fitted into a mixed linear model, confirmed that the most significant parameters contributing to proviral reservoir size were age at initiation of cART (*P*-value = .002) and time between initiation of cART and virologic control (*P*-value = .04) (Supplementary Figure 1). The resulting model predicts an increment of 0.1 log in the total HIV-1 DNA content of peripheral $CD4^+$ T cells driven by each 1-month delay in the initiation of cART (Figure 2*A*). Similarly, an equivalent increment would result from each year of suboptimal treatment. Since the samples were not evaluated at the same timepoints, we ruled out the possibility that the total time under virologic control until sampling could affect the total amount of cell-associated HIV-1 DNA (Supplementary Figure 2*A* and 2*B*).

Ultrasensitive viral load quantification was used to compare pVL at the time of sampling (Table 1): residual low-level viraemia was detectable in 7 children (30%), 3 (21.4%) in the VET group and 4 (43.4%) in the ET group, with no significant differences between groups, neither in the frequency of detection nor in median levels.

HIV-1-specific antibody testing was performed to assess the children's serostatus. We analyzed the relationship between HIV-1 serostatus and age at initiation of treatment, time to virologic control and proviral reservoir size. As shown in Figure 2*B*, a higher proportion of VET children presented negative or indeterminate results: seven of the 14 children in the VET group (50%) vs only one of the 9 ET children (11.1%), probably as a result of early and sustained control of viral replication (Table 2 and Supplementary Table 1). However, this trend was not statistically significant (Fisher exact test *P*-value = .09), and no association was found between serological status and proviral reservoir size (Mann–Whitney test *P*-value = .20).

The potential role of an extremely small proviral reservoir as a correlate of protection against viral rebound after interruption of treatment remains controversial. Thus, we studied the effect of discontinuation on reservoir size by comparing the longitudinal dynamics of the latent reservoir in three children who

Table 1. Retrospective Study Cohort Characteristics

	Total 23 (100%)	Treatment Initiation		*P* Value
		Very Early (VET) 0–12 wk 14 (60.9%)	Early (ET) 12–54 wk 9 (39.1%)	
Subject characteristics				
Sex, No. (%)				
Female	15 (65.2)	8 (57.1)	7 (77.8)	.3998
HIV-1 subtype, No. (%)				
B	19 (82.6)	11 (78.6)	8 (88.9)	1.0000
No B	3 (13)	2 (14.3)	1 (11.1)	
Gestational age				
Median [IQR], wk	38 [36–39]	38 [36–39]	39 [38–41]	.1431
Received prophylaxis, No. (%)				
Yes	11 (47.8)	8 (57.1)	3 (33.3)	.4003
Zenith pVL				
Median [IQR], $\log_{10}$ cp/mL	5.8 [5.3–6.2]	5.6 [5.1–6.2]	6 [5.7–6.1]	.2560
Nadir $CD4^+$ T-cell count				
Median [IQR], cells/mm^3	663 [405–1125]	665 [386–1232]	663 [418–1058]	.9749
Median [IQR], %	26 [14–33]	31 [16–36]	15 [14–26]	.1382
Parameters at cART initiation				
Median age [IQR], wk	10 [6–28.4]	7.2 [2.8–9.6]	32.4 [27.3–35.6]	<.0001
CDC category				
N or A	16 (69.6)	13 (92.9)	3 (33.3)	.0091
B	2 (8.7)	0	2 (22.2)	
C	5 (21.7)	1 (7.1)	4 (44.4)	
$CD4^+$ T-cell count				
Median [IQR], cells/mm^3	1785 [1069–3120]	2733 [1455–3733]	1508 [932–1785]	.0507
Median [IQR], %	43 [24–48]	44 [42–50]	24 [16–35]	.0196
Immune category				
1	14 (60.9)	12 (85.7)	2 (22.2)	.0086
2	6 (26.1)	1 (7.1)	5 (55.6)	
3	3 (13)	1 (7.1)	2 (22.2)	
Initial cART regimen, No. (%)				
With protease inhibitors	18 (78.3)	9 (64.3)	9 (100)	.1157
Parameters at virologic control				
Time since cART initiation				
Median [IQR], yr	0.8 [0.5–4.2]	0.5 [0.4–3.8]	1.4 [0.7–5.3]	.2433
Median age [IQR], yr	1.3 [0.5–4.5]	0.6 [0.5–3.9]	2 [1.3–6.3]	.0725
Parameters at sampling				
Median age [IQR], yr	8 [5.1–10]	7.6 [4.3–9.6]	9.1 [6–10.4]	.3610
cART regimen, No. (%)				
with protease inhibitors	17 (73.9)	9 (64.3)	8 (88.9)	.3401
Time on cART				
Median [IQR], yr	7.9 [4.7–9.8]	7.5 [4.2–9.5]	8.6 [5.4–9.9]	.5495
Time under virologic control				
Median [IQR], yr	4.5 [3.3–6.9]	5.2 [2.9–7.4]	4.1 [3.7–4.8]	.8749

discontinued cART and nine children who continued cART. The 3 children who discontinued belonged to the VET group and had a median of 25 HIV-1 copies per million $CD4^+$ T cells (IQR: 15–68). However, this value increased to 199 copies (IQR: 123–778) after discontinuation despite virologic control for at least one year after reinitiation of cART (Table 3). Even though only a few patients were evaluated, longitudinal analysis revealed a statistically significant increase in the size of the proviral reservoir in this

Table 1 continued.

	Total 23 (100%)	Treatment Initiation		*P* Value
		Very Early (VET) 0–12 wk 14 (60.9%)	Early (ET) 12–54 wk 9 (39.1%)	
Ultrasensitive pVL, No. (%)				
Undetectable, No. (%)	16 (69.6)	11 (78.6)	5 (55.6)	.6244
Median [IQR] of detectable, cp/mL	10 [4.7–12]	10 [10–11]	5 [4–15]	.6600
$CD4^+$ T-cell count				
Median [IQR], cells/mm^3	1356 [1046–1668]	1258 [952–1649]	1416 [1179–1680]	.4310
Median [IQR], %	40 [36–44]	41 [36–43]	38 [37–51]	.7283

Virologic control was defined as 2 consecutive pVL determinations of <200 copies/mL after cART initiation. Values are shown as median [IQR] or number (%). The clinical classification of AIDS-defining events and immunologic categories were based on international guidelines. Prophylaxis with zidovudine, lamivudine, and nevirapine immediately followed by cART was considered as initiation of cART. Data were compared between infants initiating cART at less than 12 weeks of age (VET) and infants initiating cART between 12 and 54 weeks of age (ET) using the Mann–Whitney test and Fisher exact test for numerical or categorical variables, respectively.

Abbreviations: cART, combination antiretroviral therapy; CDC, Centers for Disease Control and Prevention; ET, early treatment; HIV-1, human immunodeficiency virus type 1; IQR, interquartile range; pVL, plasma viral load; VET, very early treatment.

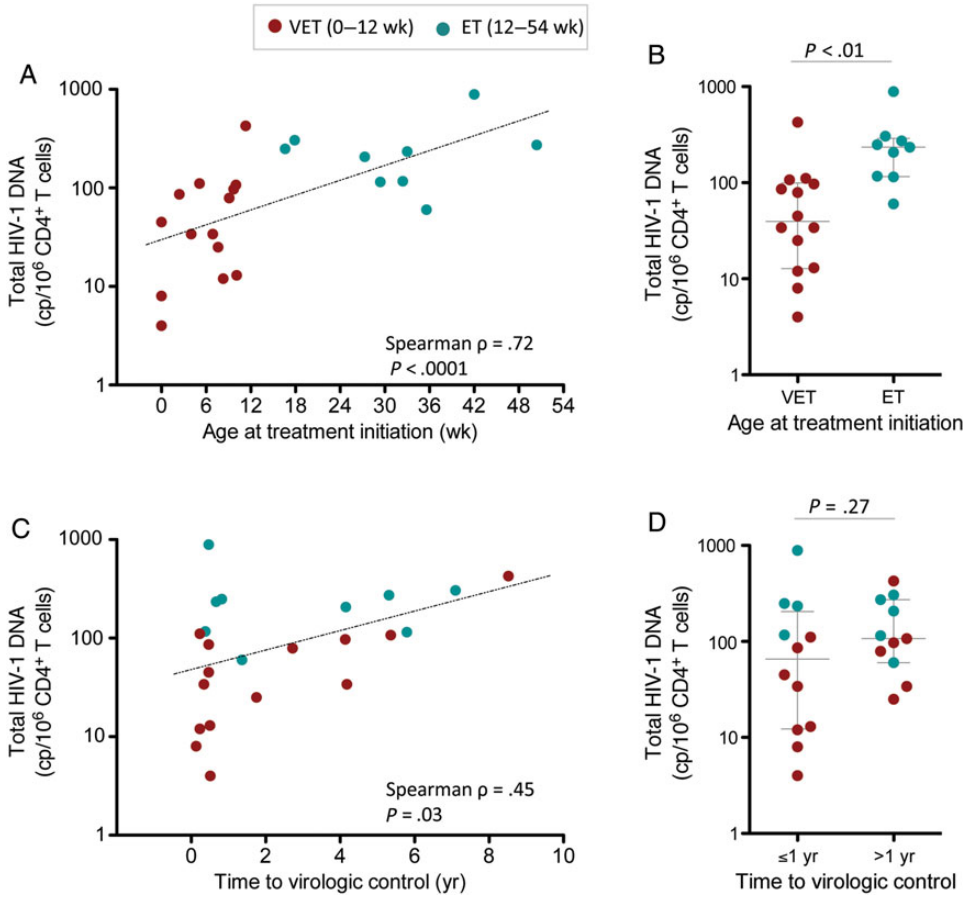

Figure 1. Association between human immunodeficiency virus type 1 (HIV-1) DNA copies/10^6 $CD4^+$ T cells and age at initiation of treatment (*A* and *B*) or time to virologic control after initiation of treatment (*C* and *D*). Median (interquartile range) levels are shown. The non-parametric Spearman correlation coefficient (ρ) and the associated 2-tailed *P* value (*A* and *C*) and 2-tailed *P* value of grouped comparisons (Mann–Whitney test) (*B* and *D*) are shown. Abbreviations: ET, early treatment; VET, very early treatment.

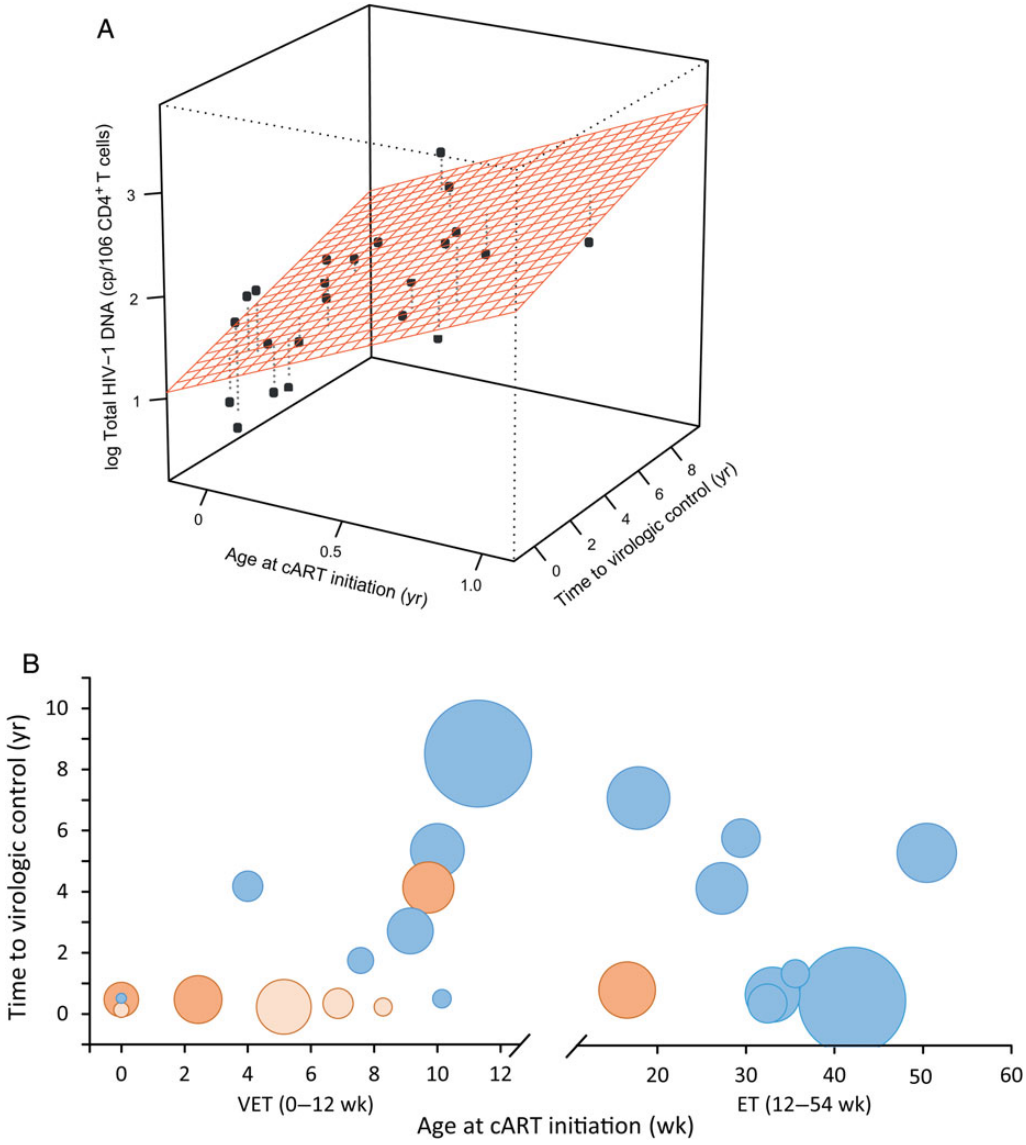

Figure 2. Influence of age at initiation of treatment and time to virologic control after initiation of treatment on proviral reservoir size and humoral profiles. *A*, Three-dimensional graph shows raw data (black circles) with the predicted multiple model (red plane). *B*, Bubble size represents human immunodeficiency virus type 1 (HIV-1) reservoir size (HIV-1 DNA copies/10^6 $CD4^+$ T cells). Measurements in infants who had fully seroconverted are in blue. Patients with negative or indeterminate results in the Inno-Lia assay are indicated in light or dark orange, respectively. Abbreviations: cART, combination antiretroviral therapy; ET, early treatment; VET, very early treatment.

group (slope = 0.29; $P = .0005$), whereas no significant longitudinal changes were found in individuals with complete adherence to cART (slope = –0.02; $P = .68$) (Figure 3*A*). Evaluation of each individual case revealed no correlation between serostatus or initial proviral reservoir size and the extent of reservoir replenishment or time to viral rebound.

Of particular interest is the case of patient (Pt03), whose plasma viraemia levels, $CD4^+$ T-cell counts, and proviral DNA are shown in Figure 3*B*. This patient initiated cART with zidovudine, lamivudine, and nevirapine immediately after birth and achieved viral suppression 6.5 months later. At 2 years of age, his proviral reservoir size was extremely low (3.8 total HIV-1 DNA copies/10^6 $CD4^+$ T cells), and at 2.2 years of age, treatment was discontinued for 3 weeks (Table 3 and Figure 3*B*). Unexpectedly, this brief treatment interruption led to viral rebound within the first week followed by a rapid increase in pVL up to 500 000 HIV-1 RNA copies/mL. Once cART was restored, pVL decreased rapidly, but the reservoir size increased approximately 50-fold and remained so for the next 3 years.

Table 2. Human Immunodeficiency Virus Type 1 Serostatus

HIV-1 Serostatus, No. (%)	Total 23 (100%)	Treatment Initiation		*P* Value
		VET (0–12 wk) 14 (60.9%)	ET (12–54 wk) 9 (39.1%)	
Negative	4 (17.4)	4 (28.6)	0	
Indeterminate	4 (17.4)	3 (21.4)	1 (11.1)	.086
Positive	15 (65.2)	7 (50)	8 (88.9)	

The result of the Inno-Lia HIV-1 I/II Score assay is shown as number (%). Data were compared between infants initiating combination antiretroviral therapy (cART) at <12 weeks of age (VET) and infants initiating cART between 12 and 54 weeks of age (ET) using the Fisher exact test.

Abbreviations: ET, early treatment; HIV-1, human immunodeficiency virus type 1; VET, very early treatment.

DISCUSSION

To date, the advantages of initiating cART within the first year in perinatally infected newborns were thought to maximize the potential benefits of limiting a latent reservoir size and enabling reservoir decay, which could probably increase the duration of remission and limit the capacity for reestablishment of viraemia in patients whose treatment is discontinued [20, 32–34]. Our study highlights the importance of very early initiation of cART, if possible within the first 12 weeks of life, and the benefit of optimal virologic control during the first years of life in order to limit the size of the viral reservoir. However, our study also casts doubt on the ability of a small viral reservoir to limit viral rebound after discontinuation (as observed in the case of the Mississippi baby).

In accordance with Persaud and colleagues [35], who highlighted the influence of age at virologic control on peripheral blood HIV-1 reservoir size in perinatally infected adolescents, our results for viral reservoir size in $CD4^+$ T cells also correlate significantly with age at virologic control (P-value <.005; Supplementary Figure 3*A*), namely, a 4-fold increase in cell-associated DNA in patients who achieved virologic control with more than 1 year of age (Supplementary Figure 3*B*). However, these results are markedly affected by the fact that 8 out of 9 patients who achieved virologic control during the first year of life started their cART before 12 weeks. We examined our findings in greater depth by independently analyzing age at initiation of cART and time to viral suppression and found that, although both parameters were associated with reservoir size, the effect of the age at initiation of treatment was stronger (Figure 1).

Our results show that the earlier cART is initiated, the smaller the size of the proviral reservoir in peripheral $CD4^+$ T cells in HIV-infected children. This finding is in line with those of a recent study that compared cell-associated HIV-1 DNA in perinatally infected infants initiating treatment before 12 weeks of age with patients initiating treatment during adolescence [36].

Table 3. Characteristics of Patients With Combination Antiretroviral Therapy Discontinuation

	Pt03	Pt08	Pt22
Subject characteristics			
Sex	Male	Male	Female
HIV-1 subtype	B	B	B
Gestational age, wk	38	30	38
Received prophylaxis	No	Yes	No
Zenith pVL, $\log_{10}$ cp/mL	4.0	5.0	6.5
Nadir $CD4^+$ T-cell count			
Cells/mm^3	2410	1466	1305
%	36	32	46
Parameters at cART initiation			
Age, wk	0.0	7.6	5.1
CDC category	A	A	A
$CD4^+$ T-cell count			
Cells/mm^3	ND	4133	2209
%	48.1	42	47
Immune category	1	1	1
Initial cART regimen	AZT/3TC/ NVP	ABC/3TC/ LPVr	AZT/ddI/ NVP
Parameters at virologic control			
Time since cART initiation, mo	6.1	21	2.8
Age, mo	6.2	22.7	3.9
Parameters of cART discontinuation			
Serostatus before TD	Positive	Positive	Negative
Duration of TD, wk	3	27	284
Highest pVL, $\log_{10}$ cp/mL	5.7	4.4	6.2
Total HIV-1 DNA, cp/10^6 $CD4^+$ T cells			
Before TD	3.8	25.42	111.0
After TD	199.1	46.1	1,356.7
Fold-change	52.4	1.8	12.2

Virologic control was defined as 2 consecutive pVL determinations of less than 200 copies/mL after cART initiation. The clinical classification of AIDS-defining events and immunologic categories were based on international guidelines. Prophylaxis with zidovudine, lamivudine, and nevirapine immediately followed by cART was considered as initiation of cART.

Abbreviations: 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; cART, combination antiretroviral therapy; CDC, Centers for Disease Control and Prevention; ddI, didanosine; HIV-1, human immunodeficiency virus type 1; LPVr, ritonavir-boosted lopinavir; ND, not determined; NVP, nevirapine; pVL, plasma viral load; TD, treatment discontinuation.

Moreover, our findings are consistent with those of a study of adult post-treatment controllers who initiated cART during acute HIV-1 infection [19], indicating that early treatment can prevent seeding of long-lived cellular reservoirs. Taken together, our results, which focus on a narrower time frame, clearly show the importance of treating infants immediately after birth to minimize seeding of long-lived cellular reservoirs.

The relevance of testing the HIV reservoir as a potential correlate of viral control after discontinuation has been the subject of intensive research in recent years, ever since the description

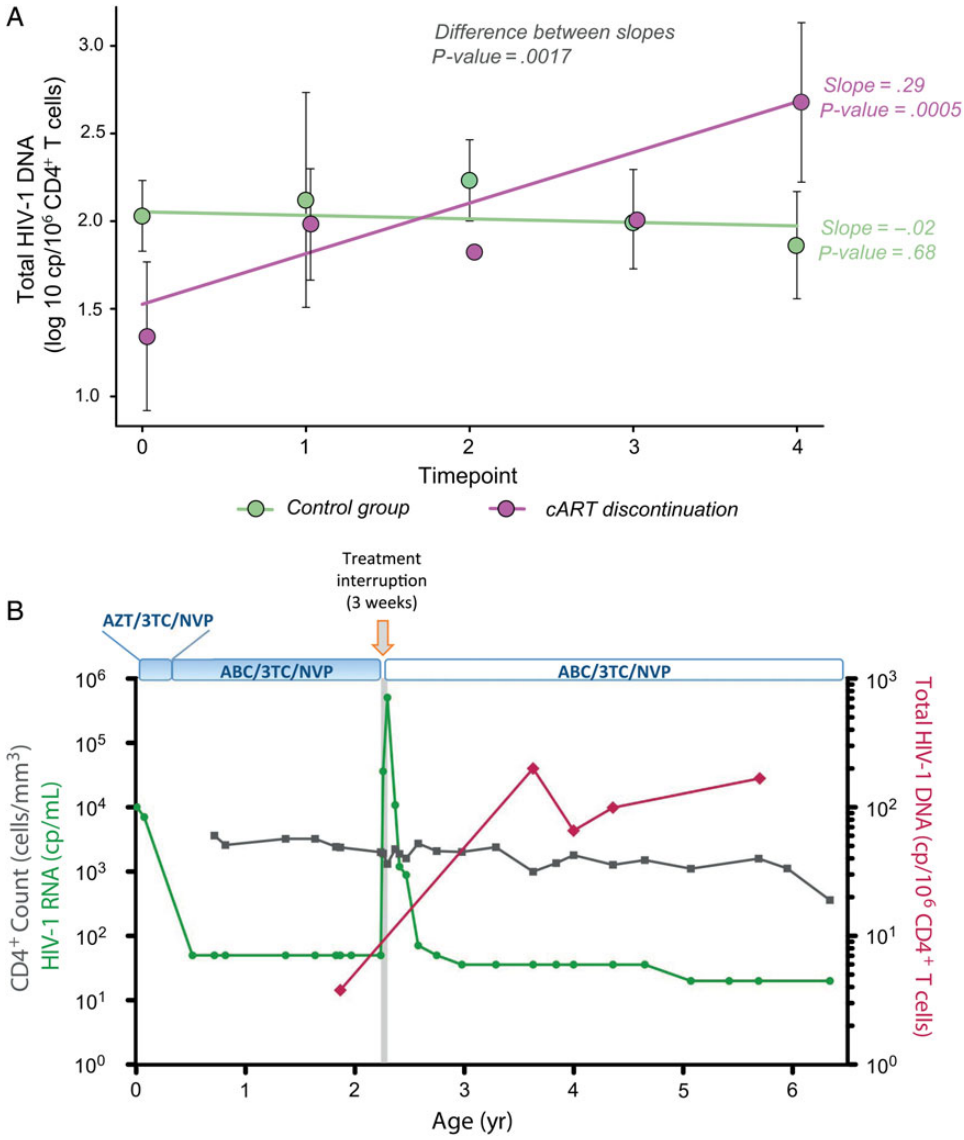

Figure 3. Reservoir size dynamics over time. *A*, Values of total human immunodeficiency virus type 1 (HIV-1) DNA from longitudinal samples are represented as mean ± standard error of the mean from patients who discontinued combination antiretroviral therapy (cART) (pink symbols) or did not discontinue cART (green symbols) after timepoint 0. The slope and *P* values were calculated using the linear mixed model. *B*, Longitudinal follow-up of Pt03. Plasma viral load (green) and CD4⁺ T-cell count (dark grey) were measured from birth to 6 years of age. Total HIV-1 DNA levels (red) were measured in 4 samples, 1 of which was taken before the 3-week discontinuation of treatment (light grey line). After reinitiation of cART, reservoir size was measured repeatedly over the following 3 years. Abbreviations: 3TC, lamivudine; ABC, abacavir; AZT, zidovudine; NVP, nevirapine.

of the case of the Mississippi baby [24]. Other cohorts of early-treated children with a very low or undetectable proviral reservoir have been established and proposed for treatment interruption programs. Both absence of detectable HIV-1 DNA and absence of low levels of replication-competent viruses in peripheral blood were recently described in a subgroup of HIV-1-infected children who initiated cART within 72 hours of birth, suggesting that early cART could significantly reduce HIV reservoir size [37]. However, definitive proof of functional cure can only be established after long-term virologic control following discontinuation of cART. Unfortunately, discontinuation in early-treated infected children has invariably led to plasma viral rebound [24, 38, 39]. Indeed, we also showed that despite a small proviral reservoir in the peripheral blood of 3 of the children in our study, plasma viraemia can rebound in only a few days, leading to a marked increase in total HIV-1 DNA in CD4⁺ T cells (Figure 3*A*). Although we observed no correlations between serostatus and the time to viral rebound in the children who stopped cART in our study, other immunological parameters, such as cytotoxic T lymphocyte responses or chronic

T-cell activation, could be relevant for remission of HIV and warrant further study.

The origin of viral rebound is unclear. Although it could originate in peripheral blood cells, a large proportion of the viral reservoir is actually located within the gut and other tissues that are not accessed in most studies [40]. On the other hand, replenishment of the reservoir might also become irreversible, as depicted by the sustained level of total HIV-1 DNA in Pt03 during the 3 years after discontinuation (Figure 3*B*). Although it seems reasonable to think that the reservoir should be as constrained as possible, we cannot conclude that a persistent increase in reservoir size after interruption would necessarily have long-term clinical consequences. Thus, before analytical treatment interruptions could be performed safely in vertically infected children, other reliable biomarkers to predict successful drug-free remission upon discontinuation need to be validated.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the Spanish HIV HGM BioBank, which is supported by Instituto de Salud Carlos III (ISCIII) and Paediatric Spanish AIDS Research Network Cohort.

Financial support. Work in the group of M. A. M.-F. was funded in part by the RD12/0017/0037 project as a component of the Plan Nacional R+D+I and cofunded by ISCIII-Subdirección General de Evaluación and the FEDER, RETIC PT13/0010/0028, Fondo de Investigacion Sanitaria (PI13/02016), "Fundación para la Investigación y la Prevención del Sida en España", Comunidad de Madrid (grant S-2010/BMD-2332), Pediatric European Network for Treatment of AIDS, and Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo 214RT0482. Centro de Investigación Biomédica en Red en Bioingeniería (CIBER), Biomateriales y Nanomedicina is an initiative funded by the VI National R&D&i Plan 2008–2011, Iniciativa Ingenio 2010, the Consolider Program, and CIBER Actions and by the ISCIII with assistance from the European Regional Development Fund. Work by the group of J. M.-P. is supported by the Spanish Secretariat of Research (grant SAF2013-49042-R) and the Spanish AIDS network "Red Temática Cooperativa de Investigación en SIDA" (RD12/0017).

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Prendergast A, Tudor-Williams G, Jeena P, Burchett S, Goulder P. International perspectives, progress, and future challenges of paediatric HIV infection. Lancet **2007**; 370:68–80.
2. Mofenson LM, Korelitz J, Meyer WA III, et al. The relationship between serum human immunodeficiency virus type 1 (HIV-1) RNA level, CD4 lymphocyte percent, and long-term mortality risk in HIV-1-infected children. National Institute of Child Health and Human Development Intravenous Immunoglobulin Clinical Trial Study Group. J Infect Dis **1997**; 175:1029–38.
3. McIntosh K, Shevitz A, Zaknun D, et al. Age- and time-related changes in extracellular viral load in children vertically infected by human immunodeficiency virus. Pediatr Infect Dis J **1996**; 15:1087–91.
4. Rich KC, Fowler MG, Mofenson LM, et al. Maternal and infant factors predicting disease progression in human immunodeficiency virus type 1-infected infants. Women and Infants Transmission Study Group. Pediatrics **2000**; 105:e8.
5. Comans-Bitter WM, de Groot R, van den Beemd R, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr **1997**; 130:388–93.
6. Erkeller-Yuksel FM, Deneys V, Yuksel B, et al. Age-related changes in human blood lymphocyte subpopulations. J Pediatr **1992**; 120(2 Pt 1): 216–22.
7. World Health Organization. Antiretroviral therapy of HIV infection in infants and children: towards universal access. Available at: http://www.who.int/hiv/pub/guidelines/paediatric020907.pdf. Accessed 28 June 2015.
8. Faye A, Le Chenadec J, Dollfus C, et al. Early versus deferred antiretroviral multidrug therapy in infants infected with HIV type 1. Clin Infect Dis **2004**; 39:1692–8.
9. Luzuriaga K, McManus M, Mofenson L, et al. A trial of three antiretroviral regimens in HIV-1-infected children. N Engl J Med **2004**; 350: 2471–80.
10. Violari A, Cotton MF, Gibb DM, et al. Early antiretroviral therapy and mortality among HIV-infected infants. N Engl J Med **2008**; 359: 2233–44.
11. Prendergast A, Mphatswe W, Tudor-Williams G, et al. Early virological suppression with three-class antiretroviral therapy in HIV-infected African infants. AIDS **2008**; 22:1333–43.
12. World Health Organization. Antiretroviral therapy of HIV infection in infants and children: towards universal access. Available at: http://www.who.int/hiv/pub/paediatric/infants2010/en/. Accessed 28 June 2015.
13. Panel on Antiretroviral Therapy and Medical Management of HIV-infected Children. Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection. Available at: http://aidsinfo.nih.gov/contentfiles/lvguidelines/pediatricguidelines.pdf. Accessed 28 June 2015.
14. Persaud D, Ray SC, Kajdas J, et al. Slow human immunodeficiency virus type 1 evolution in viral reservoirs in infants treated with effective antiretroviral therapy. AIDS Res Hum Retroviruses **2007**; 23:381–90.
15. Persaud D, Siberry GK, Ahonkhai A, et al. Continued production of drug-sensitive human immunodeficiency virus type 1 in children on combination antiretroviral therapy who have undetectable viral loads. J Virol **2004**; 78:968–79.
16. Aldhous MC, Raab GM, Doherty KV, Mok JY, Bird AG, Froebel KS. Age-related ranges of memory, activation, and cytotoxic markers on CD4 and CD8 cells in children. J Clin Immunol **1994**; 14:289–98.
17. Shearer WT, Rosenblatt HM, Gelman RS, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol **2003**; 112: 973–80.
18. Bunders MJ, van der Loos CM, Klarenbeek PL, et al. Memory $CD4^+$ $CCR5^+$ T cells are abundantly present in the gut of newborn infants to facilitate mother-to-child transmission of HIV-1. Blood **2012**; 120:4383–90.
19. Saez-Cirion A, Bacchus C, Hocqueloux L, et al. Post-treatment HIV-1 controllers with a long-term virological remission after the interruption of early initiated antiretroviral therapy ANRS VISCONTI Study. PLoS Pathog **2013**; 9:e1003211.
20. Persaud D, Gay H, Ziemniak C, et al. Absence of detectable HIV-1 viremia after treatment cessation in an infant. N Engl J Med **2013**; 369: 1828–35.
21. Ananworanich J, Schuetz A, Vandergeeten C, et al. Impact of multi-targeted antiretroviral treatment on gut T cell depletion and HIV reservoir seeding during acute HIV infection. PLoS One **2012**; 7:e33948.

22. Archin NM, Vaidya NK, Kuruc JD, et al. Immediate antiviral therapy appears to restrict resting CD4+ cell HIV-1 infection without accelerating the decay of latent infection. Proc Natl Acad Sci U S A **2012**; 109:9523–8.
23. Klein N, Sefe D, Mosconi I, et al. The immunological and virological consequences of planned treatment interruptions in children with HIV infection. PLoS One **2013**; 8:e76582.
24. Ananworanich J, Robb ML. The transient HIV remission in the Mississippi baby: why is this good news? J Int AIDS Soc **2014**; 17:19859.
25. Garcia-Merino I, de Las Cuevas N, Jimenez JL, et al. The Spanish HIV BioBank: a model of cooperative HIV research. Retrovirology **2009**; 6:27.
26. de Jose MI, Jimenez de Ory S, Espiau M, et al. A new tool for the paediatric HIV research: general data from the Cohort of the Spanish Paediatric HIV Network (CoRISpe). BMC Infect Dis **2013**; 13:2.
27. Revised classification system for human immunodeficiency virus infection in children less than 13 years of age. Available at: http://www.cdc.gov/mmwr/preview/mmwrhtml/00032890.htm. Accessed 28 June 2015.
28. Buzon MJ, Massanella M, Llibre JM, et al. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. Nat Med **2010**; 16:460–5.
29. Puertas MC, Salgado M, Moron-Lopez S, et al. Effect of lithium on HIV-1 expression and proviral reservoir size in the CD4+ T cells of antiretroviral therapy suppressed patients. AIDS **2014**; 28:2157–9.
30. Pollet DE, Saman EL, Peeters DC, et al. Confirmation and differentiation of antibodies to human immunodeficiency virus 1 and 2 with a strip-based assay including recombinant antigens and synthetic peptides. Clin Chem **1991**; 37(10 Pt 1):1700–7.
31. Team Rc. R: A language and environment for statistical computing. Available at: http://www.R-project.org/. Accessed 28 June 2015.
32. Ananworanich J, Puthanakit T, Suntarattiwong P, et al. Reduced markers of HIV persistence and restricted HIV-specific immune responses after early antiretroviral therapy in children. AIDS **2014**; 28:1015–20.
33. Jain V, Hartogensis W, Bacchetti P, et al. Antiretroviral therapy initiated within 6 months of HIV infection is associated with lower T-cell activation and smaller HIV reservoir size. J Infect Dis **2013**; 208: 1202–11.
34. Persaud D, Palumbo PE, Ziemniak C, et al. Dynamics of the resting $CD4^+$ T-cell latent HIV reservoir in infants initiating HAART less than 6 months of age. AIDS **2012**; 26:1483–90.
35. Persaud D, Patel K, Karalius B, et al. Influence of age at virologic control on peripheral blood human immunodeficiency virus reservoir size and serostatus in perinatally infected adolescents. JAMA Pediatr **2014**; 168: 1138–46.
36. Luzuriaga K, Tabak B, Garber M, et al. HIV type 1 (HIV-1) proviral reservoirs decay continuously under sustained virologic control in HIV-1-infected children who received early treatment. J Infect Dis **2014**; 210:1529–38.
37. Brophy J, Chun TW, Samson L, et al. Impact of early initiation of combination antiretroviral therapy on measures of virus in peripheral blood of vertically HIV-1-infected children. In: 20th International AIDS Conference. Melbourne, Australia, 2014.
38. Zanchetta M, Anselmi A, Vendrame D, et al. Early therapy in HIV-1-infected children: effect on HIV-1 dynamics and HIV-1-specific immune response. Antivir Ther **2008**; 13:47–55.
39. Giacomet V, Trabattoni D, Zanchetta N, et al. No cure of HIV infection in a child despite early treatment and apparent viral clearance. Lancet **2014**; 384:1320.
40. Yukl SA, Gianella S, Sinclair E, et al. Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy. J Infect Dis **2010**; 202: 1553–61.