Initiating antiretroviral treatment early in infancy has long-term benefits on the HIV reservoir in late childhood and adolescence

Véronique Avettand-Fenoel, Jérôme Lechenadec, Mariama Sadjo Diallo, Marine Fillion, Adeline Melard, Assia Samri, Catherine Dollfus, Stéphane Blanche, Albert Faye, Kahina Amokrane, et al.

Initiating antiretroviral treatment early in infancy has long-term benefits on the HIV reservoir in late childhood and adolescence.

Véronique AVETTAND-FENOEL[1,2,3,§], Jérôme LECHENADEC[3]*, Mariama Sadjo DIALLO[5*], Marine FILLION[2,3], Adeline MELARD[2,3], Assia SAMRI[5], Catherine DOLLFUS[6], Stéphane BLANCHE[2,7], Albert FAYE[2,8], Kahina AMOKRANE[2,9], Brigitte AUTRAN[5], Florence BUSEYNE[10,11§], Josiane WARSZAWSKI[3§], Pierre FRANGE[1,2,12], for the ANRS-EP59-CLEAC Study Group.

[1] AP-HP, Laboratoire de Microbiologie Clinique, Hôpital Necker-Enfants Malades, Paris, France

[2] Université de Paris, Faculté de Médecine, Paris, France

[3] INSERM U1016, CNRS, UMR8104, Institut Cochin, Paris, France

[5] Sorbonne Université, Inserm 1135, Centre d'immunologie et des maladies infectieuses, Cimi-Paris, F-75013 Paris, France

[6] AP-HP Sorbonne Université, Service d'Hématologie-Oncologie pédiatrique, Hôpital d'Enfants Armand Trousseau, Paris, France

[7] AP-HP, Service d'immuno-hématologie pédiatrique, Hôpital Necker-Enfants Malades, Paris, France

[8] APHP, Pédiatrie Générale et maladies infectieuses, Hôpital Robert Debré, Paris, France

[9] AP-HP, Laboratoire d'Immunologie et Histocompatibilité, Hôpital St-Louis, Paris, France

[10] Unité d'épidémiologie et physiopathologie des virus oncogènes, Institut Pasteur, Paris, France

[11] UMR CNRS 3569, Paris, France

[12] EHU 7328 PACT, Institut Imagine, Université de Paris, Paris, France.

*These authors contributed equally to the mansucript.

§ These authors contributed equally to the mansucript.

§ Corresponding author: Véronique Avettand-Fenoel

Laboratoire de Microbiologie clinique, 149 rue de Sèvres, 75015 Paris, France

veronique.avettand@aphp.fr (VAF)

Summary Initiating antiretroviral treatment early in infancy results in a smaller blood HIV reservoir until adolescence. Low HIV-DNA levels in children and adolescents correlated independently with age of treatment initiation, protective HLA and low cumulative viremia since cART initiation.

Abstract:

Background: Early combined antiretroviral therapy (cART) limits the total HIV-DNA load in children. However, data on its impact in older children and adolescents remain scarce. This study aims to compare HIV reservoirs in children (5-12 years) and adolescents (13-17 years) who started cART before 6 months (early (E-)group) or after 2 years old (late (L-)group).

Methods: The ANRS-EP59-CLEAC study prospectively enrolled 76 HIV-1 perinatally-infected patients who reached HIV-RNA<400 copies/mL less than 24 months after cART initiation, regardless of subsequent viral suppression (E-group: 27 children, 9 adolescents; L-group: 19 children, 21 adolescents). Total and integrated HIV-DNA were quantified in blood and in CD4+ T cell subsets. A substudy assessed HIV reservoir inducibility after *ex vivo* peripheral blood mononuclear cells (PBMCs) stimulation.

Results: Total HIV-DNA levels were lower in early- than late-treated patients (Children: 2.14 vs 2.87 log cp/million PBMCs, p<0.0001; Adolescents: 2.25 vs 2.74log, p<0.0001). Low reservoir was independently associated with treatment precocity, protective HLA and low cumulative viremia since cART initiation. The 60 participants with undetectable integrated HIV-DNA started cART earlier than the other patients (4 vs 54 months, p=0.03). In those with sustained virological control, transitional memory and effector memory CD4+T cells were less infected in the E-group than in the L-group (p=0.03 and 0.02, respectively). Viral inducibility of reservoir cells after normalization to HIV-DNA levels was similar between the groups.

Conclusions: Early cART results in a smaller blood HIV reservoir until adolescence, but all tested participants had an inducible reservoir. This deserves cautious consideration for HIV remission strategies.

Keywords: children, adolescents, HIV DNA, protective HLA, early ART

Introduction

The impact of combined antiretroviral therapy (cART) on the Human Immunodeficiency Virus (HIV) reservoir has been largely described in adults. Early cART limits circulating HIV reservoirs, which is essential for remission strategies [1,2]. To date, the effect of cART on the HIV reservoir in children infected by mother-to-child transmission has been described by a decrease in total HIV-DNA in peripheral blood mononuclear cells (PBMCs) [3-5], with immune benefits [6]. Indeed, the more rapidly viral suppression is achieved, lower is the reservoir [5,7-11,12,13]. In general, total HIV-DNA inversely correlates with the time spent with undetectable viremia [13], and very early initiation of antiretroviral therapy (in the first days or months of life) has been associated with undetectable HIV-DNA levels in blood CD4+T cells or negative serological tests in some children [8,10,13-17]. Nonetheless, few data on the long-term benefit of early cART initiation for children after the age of 2 and in adolescents are available.

In this context, the ANRS-EP59-CLEAC study aimed to determine the influence of the age at cART initiation, namely, <6 months of age ("Early group" - E) versus ≥24 months of age ("Late group" -L), on the blood HIV reservoir of a large group of well-characterized perinatally HIV-1-infected children and adolescents who achieved initial control of viral replication. Because poor adherence is frequent in pediatric patients and viral rebounds impact the HIV reservoir, the participants were not selected on the basis of sustained or transient viral control. We also aimed to investigate the influence of the age at cART initiation on the contribution of CD4+T cell subsets in the HIV reservoir and to determine whether PBMCs constitute an inducible blood HIV reservoir in a subset of patients with sustained virological control.

Materials and methods

Study population.

The CLEAC study included HIV perinatally infected patients between 5 and 17 years old who were diagnosed before 13 years and followed in the Paris area. The inclusion criteria were as follows: started antiretroviral treatment before 6 months or after 24 months of age and achieved initial virological success (HIV-RNA<400 copies/mL in the 24 months after cART initiation), regardless of subsequent viral suppression. The patients were divided into two groups: Children (5-12 years old) and Adolescents (13-17 years old). The subjects gave approval for participation if at an age to do so; otherwise, their parents or legal guardians provided informed written consent to participate in the study. Those for whom samples were collected for HIV diagnosis at birth and in the first week of life were considered to have in utero infection if the HIV genome was detected in the sample collected in the first 7 days of life and intrapartum infection if the HIV genome was detected after 7 days of life.

Blood samples were collected between June 2016 and January 2018. Using 50 mL of blood collected six months later, a substudy was performed to characterize the HIV reservoir among sorted PBMC subpopulations in children/adolescents weighing ≥45 kg and having continuous viral suppression (≥90% of the HIV-RNA measures <400 copies/mL) since first initiating cART.

HIV DNA quantification.

Total cell-associated HIV-DNA in blood and CD4+T cell subsets was quantified using ultrasensitive real-time PCR (Biocentric, France) [18-21]. The entire HIV-DNA extract was tested using two to four PCRs. The results are reported as either the actual HIV-DNA copy numbers/million PBMCs or as an estimated value calculated as 50% of the quantification

threshold value when the HIV-DNA was lower than the threshold. The thresholds varied according to the available cell numbers and were calculated for each assay (median 15 copies/million PBMCs, range 4-24) [20,21].

Integrated HIV-DNA was quantified using ultrasensitive *Alu*-PCR [22]. The thresholds varied according to the available cell numbers and were calculated for each assay (median 15 copies/million PBMCs, range 4-30).

Cumulative viremia.

Cumulative viremia was defined as the area under the curve of HIV-RNA load over time and was estimated as previously described [23]. To study the association of HIV-DNA with overall exposure to the virus independently of age and duration of infection, we calculated normalized cumulative viremia since cART and the time proportion exposed to viral loads above 400 copies/mL since birth.

Ultrasensitive HIV-RNA quantification was performed using 2 mL plasma from participants of the substudy [19].

Immunologic variables.

CD4+ and CD8+T cell subsets in fresh blood were quantified by flow cytometry [24]. Naïve cells were defined as CD45RA+CCR7+ and activated cells as HLA-DR+CD38+. *Cytomegalovirus* serology (ImmunoglobulinsG) was performed using a LiaisonXL (Diasorin, Italy). Second-field (four-digit)-resolution HLA-B genotyping was performed using Luminex reverse PCR sequence-specific oligonucleotides (Canoga Park, CA).

Cell sorting for the substudy.

Cryopreserved PBMCs were thawed, with viability above 80%, and depleted of CD8+ cells. Sorting was performed as described [20,21] with five laser beams FACSAria (Becton-Dickinson) and a BSL3 (CyPS platform). Resting (CD25-CD69-HLADR-) CD4+ T cells were subsorted into CD45RA+CCR7+CD27+ Naïve (TN), CD45RA-CCR7+CD27+ (TCM), CD45RA-CCR7-CD27+ transitional memory (TMT) and CD45RA-CCR7-CD27- Effector-memory (TEM) T cells. The cell numbers varied from 0.004 to 0.4 million cells among the subsets and patients, and the purity of the sorted subsets was >90%.

Amplification of HIV-1 from PBMCs to assess the presence of an inducible reservoir (substudy).

A fraction of the same PBMCs samples used for cell sorting was cultured in 10% FCS-RPMI1640 medium for 13 days after stimulation on Day 0 with anti-CD3/anti-CD28/IL-2 (Roche, 5 mg/mL). Viral production in the supernatants was measured using real-time PCR HIV-RNA (Biocentric, France). The viral production capacity is expressed as the ratio between the HIV-RNA copies in the supernatants and the level of HIV-DNA measured on Day 0 of culture.

Statistical analysis.

Wilcoxon, Kruskall-Wallis, Mann-Whitney and Spearman tests were employed. Results under the threshold were considered at half the threshold for statistical analyses. Univariate and multivariate linear regression models were generated, and univariate and multivariate analyses were performed to determine factors associated with HIV-DNA level. For each dependent variable, we first assessed the association with age at cART initiation in the two age groups (children and adolescents) as well as interactions between age at cART initiation

and age at inclusion. Factors associated with HIV-DNA levels were assessed for the entire group, as the interaction was not significant.

Results

We prospectively enrolled 76 participants: 27 children and 9 adolescents in the Early group and 19 children and 21 adolescents in the Late group. Their characteristics are presented in Table 1. All participants were on ART, with a good viro-immunological status (83% were virologically suppressed, and 92% had a CD4+T cell count>500 cells/µL).

Early treatment initiation was associated with low levels of total HIV-DNA in children and adolescents (Table 2). The median total HIV-DNA levels of the E-group were significantly lower than those of the L-group (2.17 vs 2.95 log copies/million PBMCs, respectively, p<0.0001). Moreover, an association between early treatment initiation and lower total HIV-DNA levels was observed in both age groups: medians [interquartile range (IQR)] 2.14 log [1.34-2.52] vs 2.87 log [2.82-3.08], respectively; p<0.0001 in children; medians [IQR] 2.25 log [1.29-2.74] vs 2.98 log [2.78-3.20], respectively; p=0.019 in adolescents (Figure 1).

Total HIV-DNA also correlated with some demographic and immune genetic parameters and virological history (Table 2). Compared to the adolescents, the children exhibited a trend of a lower total HIV-DNA load (medians 2.61 vs 2.86 log copies/million PBMCs, p=0.07). No difference according to the geographic origin of the mothers was observed. Interestingly, protective HLA alleles (HLA-B*27 and/or B*57:01 and/or B*57:03) were associated with significantly lower HIV-DNA (1.69 log) than deleterious (HLA-

B*35:02 and/or B*35:03 and/or B*53:01 and/or B*58:02) (2.97 log) and neither protective nor deleterious HLA alleles (2.69 log) (p=0.03).

Participants with a viremia <50 copies/mL at the time of the study had significantly lower HIV-DNA than the other subjects (p=0.0005). Overall, total HIV-DNA correlated positively with the duration of viral load greater than 400 copies/mL since birth (R=0.64, p<0.0001) and with normalized cumulative viremia since cART initiation (R=0.29, p=0.012).

Although the total HIV-DNA level did not correlate with the CD4+T cell count, CD4+/CD8+ ratio, or CD4+ nadir, it correlated negatively with the percentage of naïve cells among CD8+T cells (R=-0.45, p<0.0001) and positively with the percentage of activated HLA-DR+CD38+ memory CD8+T cells (R=0.29, p=0.0225). The total HIV-DNA load was significantly higher when the patient carried antibodies against *Cytomegalovirus* (p=0.008).

In order to determine the parameters independently associated with the total HIV-DNA level, we performed different multivariate analyses: a full model including the normalized cumulative viremia since cART initiation as a variable, and a reduced model without this variable to test the influence of current HIV-RNA<50 copies/mL which is highly correlated with cumulative viremia (median 2 vs 21 copies-year in patients with and without HIV-RNA<50 copies/mL, respectively, p <0.0001). According to the full model on the entire population, early cART and cumulative viremia were independently associated with lower HIV-DNA levels (respectively p<0.0001, p=0.0020) (Table 3). When restricting the analysis to the 63 patients with current HIV-RNA<50 copies/mL, early cART, cumulative viremia and protective HLA alleles were independently associated with lower HIV-DNA levels (Table S1). In the reduced model, we found early cART and current HIV-RNA<50 copies/mL to be independently associated with lower HIV-DNA levels (respectively

p<0.0001, p=0.0024). Similar results were observed when considering children only, with an additional association of HLA group with HIV-DNA levels (p=0.0083) in both full and reduced models (Table 3). As only one adolescent carried a protective HLA allele, we were not able to evaluate a potential association with HLA group in this age subgroup.

When normalized cumulative viremia since cART (not influenced by the age of treatment initiation) was replaced by the time proportion with HIV-RNA>400 copies/mL since birth (highly influenced by the age of treatment initiation) in the model for the entire population, early treatment, shorter cumulative duration of viremia >400 copies/mL and current HIV-RNA<50 copies/mL were associated with lower HIV-DNA levels (p=0.0104, 0.0047 and 0.0024, respectively) (Table S2). Globally, the different models showed that early cART, virologic history (cumulative viremia since cART or current HIV-RNA and shorter cumulative duration of viremia >400 copies/mL) and protective HLA alleles influenced the current level of total HIV-DNA.

By restricting the analysis to the E-group, we observed that protective HLA alleles and a low normalized cumulative viremia since cART were significantly associated with a low HIV-DNA level (Table S3). Moreover, by considering the E-group and children and adolescents with no detectable HIV-RNA at the time of enrollment, the 13 patients who started cART before two months of age had a significantly lower total HIV-DNA load than the 17 who started cART between two and four months of life (median 1.46 log vs 2.16 log, p=0.0069). No association for the L-group was detected (Table S4).

Integrated HIV-DNA. Regarding integrated HIV-DNA, the level was below the limit of detection for 59 participants and was between 1.58 and 3.88 log copies/million PBMCs for 17 participants (Table 1). The former group started continuous cART earlier than the latter group (at 4 vs 54 months of age, respectively, p=0.0316). In addition, there was a trend toward a

lower total HIV-DNA level when integrated HIV-DNA was not detected (2.70 vs 2.88 log copies/million PBMCs, p=0.09).

Contribution of CD4+T cell subsets to the HIV reservoir. In a substudy including nine participants (4 E-group, 5 L-group; 1 child, 8 adolescents) (median total HIV-DNA (range): 2.78 (1.53–3.21 log), we next assessed the contribution of CD4+T cell subsets to the HIV reservoir. The current viremia was <10 copies/mL in all but one subject (45 copies/mL). HIV-DNA was detected and quantified in all subsets, except in 3 TN and 1 TCM subsets with <20,000 cells studied. Overall, HIV-DNA levels were significantly lower in TN cells (median 2.65 copies/million cells) than in each subset of memory cells (TCM, 3.57 copies/million cells, p=0.031; TMT, 3.87 copies/million cells, p=0.028, TEM, 3.66 copies/million cells, p=0.008). When separating the E-group and L-group, TMT and TEM subsets were significantly less infected in the former group than in the latter group (3.44 vs 4.03 log, p=0.03 and 3.22 vs 3.96 log, p=0.02, respectively). No difference between groups for TN and TCM subsets was observed, some thresholds were high because of the small number of available cells (Figure 2).

We then calculated the contribution of each infected CD4+T subset to the total HIV reservoir by integrating the frequency of resting CD4+T cell subsets in blood and found that TN (median: 5%) contributed significantly less to the HIV reservoir than TCM (33%, p=0.0052), TMT (27%, p=0.0014) and TEM (23%, p=0.0027). Additionally, TN contributed significantly less than pooled memory cells for both the E- and L-groups (p=0.029 and 0.008, respectively). When considering each of the memory subsets separately, TN subsets contributed significantly less than TCM, TMT and TEM subsets in the L-group, but no significant difference was observed in the E-group (Figures 3). Moreover, there was no

difference between the E-group and the L-group with regard to the contribution of each subset.

Children and adolescents have an inducible blood HIV reservoir. For all 9 patients participating in the substudy, PBMCs were able to produce HIV-RNA after *ex vivo* activation (median: 2.74 log copies/mL supernatant, range: 2.28-5.17 log). Nonetheless, when the results were normalized by the number of HIV-DNA copies per well of culture, there was no difference between the E- and L-groups (medians: 1.48 vs 7.51 HIV-RNA copies/HIV-DNA at peak, respectively, p=0.56).

Discussion

This is the first large study to describe the HIV reservoir in children and adolescents and associate data on the ranges of total and integrated HIV-DNA levels, the inducibility of the HIV reservoir and the contribution of different CD4+T cell subsets to this reservoir.

Importantly, this is also the first large study describing the benefits of early cART initiation on the HIV reservoir in children after the age of 5 as well as in adolescents in which subjects were not selected based on criteria of sustained viral control since cART initiation. Low levels of total HIV-DNA were associated with treatment in the first six months of life, independent of current age. The benefit of early initiation of cART on the blood HIV reservoir was even observed in adolescents, who tend to have a high risk of poor treatment adherence. Interestingly, some early-treated children and adolescents achieved total HIV-DNA levels as low as those observed in adults with spontaneous or posttreatment HIV control [2]. This benefit of early treatment was also observed for another reservoir biomarker:

the level of stable integrated forms. Indeed, integrated HIV-DNA was more often below the threshold of quantification in participants receiving cART since a younger age.

Moreover, protective HLA alleles were independently associated with lower total HIV-DNA levels, even when analysis was restricted to patients treated early (Table S3). Interestingly, this association, previously described during natural history [19], was observed in children and adolescents after years of cART.

Total HIV-DNA levels were also independently associated with overall exposure to HIV (expressed as the cumulative viremia since cART initiation or the duration of detectable viremia since birth; the latter is highly influenced by the age of treatment initiation, but cumulative viremia under cART is not), specifying previous results [25,26]. Furthermore, the current viremia was independently associated with the total HIV-DNA load only when cumulative viremia was not included in the models, reflecting that current detectable viremia was often associated with previous periods of viremia since cART, except during specific periods such as adolescence. In fact, during adolescence, HIV-DNA was independently associated with current viremia and with the duration of detectable viremia since birth. Links between deleterious or protective HLA alleles and the level of total HIV-DNA were found in children and adolescents even after years of effective cART. In addition, high HIV-DNA levels were associated with seropositivity for *Cytomegalovirus*, which could reflect either enhanced HIV seeding of cells activated by CMV-infection or the higher CMV prevalence in L-group than in E-group. High HIV DNA levels were associated with lower naïve CD8+T cell percentages and higher levels of activated memory CD8+T cells. Additional analyses are consistent with active HIV replication driving memory CD8 T cell expansion and activation (manuscript in preparation). This correlation of total HIV-DNA with key immune parameters

of HIV pathogenesis underlines its clinical relevance in children and adolescents, as extensively described in adults [2] and untreated children [27].

Nevertheless, despite this early treatment and low reservoir, the HIV reservoir was inducible in all children and adolescents evaluated, as previously shown in children [28]. This inducibility is congruent with recent data describing some rare intact HIV proviruses that persist in children seven to nine years after initiation of antiretroviral therapy in the first year of life [29]. Interestingly, the ability to reactivate was linked to the HIV-DNA level, as previously described in adults [30], and did not differ between the E- and L-groups after normalization to HIV-DNA levels. In this study, reduced viral inducibility of cells in patients treated since the time of primary infection compared with those treated later was not observed. Further larger studies are needed to confirm these results and assess the infectiosity of the induced virus.

In participants with long-term viral suppression while on cART, TN were less infected than memory CD4+T cells and contributed less to the HIV reservoir than each of the explored memory CD4+T cell subsets (TCM, TMT and TEM cells), as previously described in adults treated since the time of primary infection or the chronic stage [31,32]. Regardless, relative protection of TN with high proliferative ability and a long half-life was observed in both the E- and L-groups. TMT and TEM cells were less infected in the E-group than in the L-group. Palmer recently showed that TEM cells in adults harbor proviruses genetically identical to viral sequences derived from pre- and on-therapy plasma samples and that these TEM are capable of encoding infectious HIV-1 [33]. Such data are lacking in children and adolescents; however, considering the data for adults, the lowest level of infection of TEM subsets in early-treated participants might explain the observed benefits on the HIV reservoir. No

difference between the two groups was observed for the other subsets, but there were only a few participants in this substudy.

In conclusion, this study reports extended viral characterization of the blood HIV reservoir (total and integrated HIV-DNA, contribution of different resting CD4+T cell subsets, inducibility of reservoir) in children and adolescents treated early or late after perinatal HIV infection and not selected for the control of viremia afterwards. These data emphasize that initiating cART early in infancy and achieving viral suppression in the first months of life limit the HIV reservoir in children, and this effect is also observed in the long term in adolescents. The findings reinforce the clinical benefit of a very early diagnosis and effective therapy in children. Early-treated patients may be good candidates for HIV remission strategies to limit cART duration. Nevertheless, normalized levels of HIV reservoir inducibility were similar in the E- and L-groups, and this observation deserves further and cautious consideration.

Acknowledgments

We thank the children and adolescents who participated in the study. We thank M. Hasan, S. Novault and P-H. Commere from the Cytometry core facility at Institut Pasteur and the Import/export facility at Institut Pasteur.

Disclaimer

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, writing of the report, or decision to submit for publication.

Funding

The ANRS CLEAC study was supported by the ANRS (French National Agency for Research on AIDS and Viral Hepatitis).

Conflict of interest

Authors have no conflict of interest with this study.

The ANRS CLEAC study group:

Hôpital Armand Trousseau, Paris: Mary-France Courcoux, Catherine Dollfus, Marie-Dominique Tabone, Geneviève Vaudre ; Hôpital Bicêtre, Le Kremlin-Bicêtre: Corinne Fourcade, Josiane Warsazawski, Jérôme Lechenadec, Olivia Dialla, Laura Nailler, Lamya Ait Si Selmi, Isabelle Leymarie, Thierry Wack, Alexandre Hoctin, Razika Feraon-Nanache; Centre hospitalier intercommunal de Créteil: Isabelle Hau ; Hôpital Delafontaine, Saint-Denis: Cécile Gakobwa ; Hôpital Necker-Enfants Malades, Paris: Véronique Avettand-Fenoël, Stéphane Blanche, Marine Fillion, Pierre Frange, Nizar

Mahlaoui, Adeline Mélard, Florence Veber, Marie-Christine Mourey; Maternité Port-Royal, Paris: Valérie Marcou ; Hôpital Robert Debré, Paris: Albert Faye, Martine Lévine, Sandrine Richard ; Pitié-Salpêtrière, Paris : Brigitte Autran, Assia Samri, Mariama Diallo ; Hôpital Saint-Louis : Sophie Caillat-Zucman, Kahina Amokrane, Rayna Ivanova-Derin, Centre hospitalier intercommunal de Villeneuve-Saint-Georges: Anne Chacé ; Institut Pasteur Paris: Florence Buseyne, Thomas Montange, Damien Batalie, Ingrid Fert, Asier Saez-Cirion, Valérie Monceaux, Daniel Scott-Algara ; ANRS : Lucie Marchand, Delphine Lebrasseur, Axel Levier.

References

1. Hocqueloux L, Avettand-Fenoel V, Jacquot S, et al. Long-term antiretroviral therapy initiated during primary HIV-1 infection is key to achieving both low HIV reservoirs and normal T cell counts. Journal of Antimicrobial Chemotherapy **2013**; 68(5): 1169-78.
2. Avettand-Fenoel V, Hocqueloux L, Ghosn J, et al. Total HIV-1 DNA, a Marker of Viral Reservoir Dynamics with Clinical Implications. Clin Microbiol Rev **2016**; 29(4): 859-80.
3. De Rossi A, Walker AS, De Forni D, Gibb DM. Biphasic decay of cell-associated HIV-1 DNA in HIV-1-infected children on antiretroviral therapy. AIDS **2002**; 16(14): 1961-3.
4. Saitoh A, Powell CA, Fenton T, et al. Longitudinal analysis of lymphocyte ratios and HIV-1 intracellular DNA levels in children. J Infect Dis **2004**; 189(7): 1216-20.
5. Moragas M, Distefano M, Mecikovsky D, et al. Impact of the time to achieve viral control on the dynamics of circulating HIV-1 reservoir in vertically infected children with long-term sustained virological suppression: A longitudinal study. PLoS One **2018**; 13(10): e0205579.
6. Garcia-Broncano P, Maddali S, Einkauf KB, et al. Early antiretroviral therapy in neonates with HIV-1 infection restricts viral reservoir size and induces a distinct innate immune profile. Sci Transl Med **2019**; 11(520).
7. 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(12): 1483-90.
8. 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(12): 1138-46.
9. van Zyl GU, Bedison MA, van Rensburg AJ, Laughton B, Cotton MF, Mellors JW. Early Antiretroviral Therapy in South African Children Reduces HIV-1-Infected Cells and Cell-Associated HIV-1 RNA in Blood Mononuclear Cells. J Infect Dis **2015**; 212(1): 39-43.
10. Martinez-Bonet M, Puertas MC, Fortuny C, et al. Establishment and Replenishment of the Viral Reservoir in Perinatally HIV-1-infected Children Initiating Very Early Antiretroviral Therapy. Clin Infect Dis **2015**; 61(7): 1169-78.
11. Uprety P, Patel K, Karalius B, et al. Human Immunodeficiency Virus Type 1 DNA Decay Dynamics With Early, Long-term Virologic Control of Perinatal Infection. Clin Infect Dis **2017**; 64(11): 1471-8.
12. Kuhn L, Paximadis M, Da Costa Dias B, et al. Age at antiretroviral therapy initiation and cell-associated HIV-1 DNA levels in HIV-1-infected children. PLoS One **2018**; 13(4): e0195514.

13. Tagarro A, Chan M, Zangari P, et al. Early and Highly Suppressive Antiretroviral Therapy Are Main Factors Associated With Low Viral Reservoir in European Perinatally HIV-Infected Children. J Acquir Immune Defic Syndr **2018**; 79(2): 269-76.
14. Kfutwah AK, Tejiokem MC, Ateba FN, et al. Seronegativation in early treated HIV-infected infants: frequency and potential implications on care and follow-up in a resource-limited country. J Acquir Immune Defic Syndr **2011**; 58(2): e43-6.
15. Bitnun A, Samson L, Chun TW, et al. Early initiation of combination antiretroviral therapy in HIV-1-infected newborns can achieve sustained virologic suppression with low frequency of CD4+ T cells carrying HIV in peripheral blood. Clin Infect Dis **2014**; 59(7): 1012-9.
16. de Souza MS, Pinyakorn S, Akapirat S, et al. Initiation of Antiretroviral Therapy During Acute HIV-1 Infection Leads to a High Rate of Nonreactive HIV Serology. Clin Infect Dis **2016**; 63(4): 555-61.
17. Brice J, Sylla M, Sayon S, et al. Qualitative and quantitative HIV antibodies and viral reservoir size characterization in vertically infected children with virological suppression. J Antimicrob Chemother **2017**; 72(4): 1147-51.
18. Avettand-Fenoel V, Chaix ML, Blanche S, et al. LTR real-time PCR for HIV-1 DNA quantitation in blood cells for early diagnosis in infants born to seropositive mothers treated in HAART area (ANRS CO 01). J Med Virol **2009**; 81(2): 217-23.
19. Avettand-Fenoel V, Bayan T, Gardiennet E, et al. Dynamics in HIV-DNA levels over time in HIV controllers. J Int AIDS Soc **2019**; 22(1): e25221.
20. 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 Pathogens **2013**; 9(3).
21. Descours B, Avettand-Fenoel V, Blanc C, et al. Immune responses driven by protective human leukocyte antigen alleles from long-term nonprogressors are associated with low HIV reservoir in central memory CD4 T cells. Clin Infect Dis **2012**; 54(10): 1495-503.
22. Tremeaux P, Lenfant T, Boufassa F, et al. Increasing contribution of integrated forms to total HIV DNA in blood during HIV disease progression from primary infection. EBioMedicine **2019**.
23. Zoufaly A, Stellbrink HJ, Heiden MA, et al. Cumulative HIV viremia during highly active antiretroviral therapy is a strong predictor of AIDS-related lymphoma. J Infect Dis **2009**; 200(1): 79-87.
24. Blanche S, Scott-Algara D, Le Chenadec J, et al. Naive T Lymphocytes and Recent Thymic Emigrants Are Associated With HIV-1 Disease History in French Adolescents and Young Adults Infected in the Perinatal Period: The ANRS-EP38-IMMIP Study. Clinical Infectious Diseases **2014**; 58(4): 573-87.
25. Avettand-Fenoel V, Blanche S, Le Chenadec J, et al. Relationships between HIV disease history and blood HIV-1 DNA load in perinatally infected adolescents and young adults: the ANRS-EP38-IMMIP study. J Infect Dis **2012**; 205(10): 1520-8.
26. Boullé C, Rouet F, Fassinou P, et al. HIV-1 DNA concentrations and evolution among African HIV-1-infected children under antiretroviral treatment (ANRS 1244/1278). J Antimicrob Chemother **2014**; 69(11): 3047-50.
27. Scott-Algara D, Rouzioux C, Blanche S, et al. In untreated HIV-1-infected children, PBMC-associated HIV DNA levels and cell-free HIV RNA levels are correlated to distinct T-lymphocyte populations. J Acquir Immune Defic Syndr **2010**; 53(5): 553-63.

28. 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(3): 381-90.
29. Katusiime MG, Halvas EK, Wright I, et al. Intact HIV Proviruses Persist in Children Seven to Nine Years after Initiation of Antiretroviral Therapy in the First Year of Life. J Virol **2020**; 94(4).
30. Noel N, Pena R, David A, et al. Long-term Spontaneous control of HIV-1 relates to low frequency of infected cells and inefficient viral reactivation. J Virol **2016**.
31. Chomont N, El-Far M, Ancuta P, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nat Med **2009**; 15(8): 893-900.
32. Cheret A, Bacchus-Souffan C, Avettand-Fenoel V, et al. Combined ART started during acute HIV infection protects central memory CD4+ T cells and can induce remission. J Antimicrob Chemother **2015**; 70(7): 2108-20.
33. Lee E, von Stockenstrom S, Morcilla V, et al. The impact of antiretroviral therapy duration on the HIV-1 infection of T-cells within anatomic sites. J Virol **2019**.

Figure legends

Figure 1. Total HIV-1 DNA levels (log_{10} copies/10^6 PBMCs) in children and adolescents according to the time of treatment initiation. Early-treated patients (E) are indicated by blue symbols and late-treated patients (L) by red symbols. Circles represent children and squares adolescents. Filled symbols, numbers of cell HIV-DNA copies; open symbols, levels below the threshold of detection calculated for each assay according to the number of cells available.

Figure 2: Total HIV-DNA levels in blood resting CD4+ T-cell subsets. Total HIV-DNA was quantified in sorted resting CD25+CD69+-HLA-DR CD4+ T-cell subsets (TN: naïve CD4+ T cells, TCM: central memory CD4+ T cells, TMT: transitional memory T cells, TEM: effector memory T cells) by ultrasensitive real-time polymerase chain reaction, and the result is expressed as cell HIV-DNA copies/10^6 cell subsets. Four patients from the early group (E: circles) were compared with 5 patients from the late group (L: squares). Filled symbols, numbers of cell HIV-DNA copies; open symbols, levels below the threshold of detection calculated for each assay according to the number of cells available. The Mann-Whitney test was used to compare groups. Same results were obtained when levels below the detection limit were set as the detection limit or half of the detection limit.

Figure 3. Contribution of CD4+ T-cell subsets to total cell HIV-DNA in children/adolescents with long-term viral suppression while on cART in the E- and L-groups. Only significant differences are shown. The Mann-Whitney test was used to compare groups. TN: naïve CD4 T cells, TCM: central memory CD4 T cells, TMT: transitional memory T cells, TEM: effector memory T cells.

Table 1. Patient characteristics

	Early (n=36)		Late (n=40)		All (n=76)
	Children (n=27)	**Adolescents (n=9)**	**Children (n=19)**	**Adolescents (n=21)**	
Age at first cART (months) (median [IQR])	2 [0;3]	2 [0;2]	54 [49;80]	92 [55;136]	25 [2;78]
Male sex % (n)	33 (9)	44 (4)	42 (8)	67 (14)	46 (35)
Geographic origin % (n)					
Europe	11 (3)	11 (1)	15 (3)	19 (4)	14 (11)
Sub-Saharan Africa	74 (20)	78 (7)	74 (14)	76 (16)	75 (57)
Other	15 (4)	11 (1)	11 (2)	5 (1)	11 (8)
Place of birth % (n)					
France	93 (25)	100 (9)	26 (5)	29 (6)	59 (45)
Sub-Saharan Africa	0 (0)	0 (0)	63 (12)	57 (12)	32 (24)
Other	7 (2)	0 (0)	11 (2)	14 (3)	9 (7)

Age (years) at evaluation	9	15	8	15	11
(median [IQR])	[6;11]	[14;16]	[7;10]	[13;15]	[8;14]
HLA % (n)					
Protective	15 (4)	0 (0)	5.3 (1)	5 (1)	8 (6)
Deleterious	18.5 (5)	44 (4)	26.4 (5)	47.5 (10)	31.5 (24)
Protective + deleterious	3.5 (1)	0 (0)	5.3 (1)	0 (0)	2.5 (2)
None	63 (17)	56 (5)	63 (12)	47.5 (10)	58 (44)
On cART % (n)	100 (27)	100 (9)	100 (19)	100 (21)	100 (76)
Current HIV RNA < 50 copies/mL % (n)	89 (24)	67 (6)	89 (17)	76 (16)	83 (63)
CD4+ T-cell count/µL (median [IQR])	951 [725;1320]	840 [713;1078]	1009 [745;1527]	740 [622;1092]	856 [622;1092]
Total HIV-DNA (Log) (median [IQR])	2.14 [1.25;2.52]	2.25 [1.29;2.73]	2.87 [2.82;3.09]	2.98 [2.78;3.20]	2.74 [2.20;3.09]
Integrated HIV-DNA % positive (n)	18.5 (5)	11.1 (1)	26.3 (5)	29 (6)	22 (17)

CMV seropositivity % (n)	70.4 (19)	77.8 (7)	89.5 (17)	95.2 (20)	82.9 (63)
CMV IgG titer (UA/mL) (median [IQR])	60.6 [2.5;100.0]	102.0 [73.3;109.0]	94.6 [57.4;118.0]	104.0 [74.6;124.0]	98.7 [73.9;116.5]

Protective HLA alleles: HLA-B*27 and/or B*57:01 and/or B*57:03; Deleterious HLA: HLA-B*35:02 and/or B*35:03 and/or B*53:01 and/or B*58:02.

Table 2: Univariate analysis of parameters associated with total HIV-1 DNA level in PBMCs

	N	Median [IQR] or Pearson correlation coefficient	Regression coefficient	p value
Treatment initiation				
Early	36	2.17 [1.27;2.61]	-0.90	<0.0001
Late	40	2.95 [2.80;3.15]	Reference	
Demographics				
Sex				
Male	35	2.84 [2.37;3.20]	0.23	0.16
Female	41	2.66 [1.93;3.04]	Reference	
Age				
13-17 years	30	2.86 [1.93;2.94]	0.32	0.07
5-12 years	46	2.61 [1.98;2.94]	Reference	
Sub-Saharan African Origin				
No	19	2.68 [2.20;2.82]	0.05	0.80
Yes	57	2.84 [2.21;3.11]	Reference	
Immuno-genetics				
HLA				
Protective	6	1.69 [0.98;2.95]	0.28	0.03
Deleterious	24	2.97 [2.36;3.19]	-0.72	
Protective + deleterious	2	2.3 [1.73;2.87]	Reference	
None	44	2.69 [2.17;2.95]	-0.18	

Virological status				
Current HIV RNA ≥ 50 copies/mL				
Yes	13	3.19 [2.84;3.23]	0.62	0.0005
No	63	2.67 [1.93;2.95]	Reference	
Normalized cumulative viremia since cART	76	0.29	0.0113	0.012
Time proportion with HIV RNA >400 copies/mL since birth to period previous to inclusion	76	0.64	1.58	<0.0001
Immunologic status				
Current CD4 T cell count	76	-0.07	-0.0001	0.57
Current CD4/CD8 ratio	76	-0.19	-0.2	0.11
CD4 nadir while on treatment	76	-0.06	-0.00013	0.56
Proportion of time with CD4 <25% since cART	76	0.15	0.49	0.21

Protective HLA alleles: HLA-B*27 and/or B*57:01 and/or B*57:03; Deleterious HLA: HLA-B*35:02 and/or B*35:03 and/or B*53:01 and/or B*58:02.

Table 3: Multivariate linear regression models to determine factors associated with HIV-DNA level, including the normalized cumulative viremia since cART.

		CHILDREN						ADOLESCENTS						WHOLE POPULATION					
		FULL MODEL			REDUCED MODEL			FULL MODEL			REDUCED MODEL			FULL MODEL			REDUCED MODEL		
		Regression Coefficient	Confident Interval	P Value	Regression Coefficient	Confident Interval	P Value	Regression Coefficient	Confident Interval	P Value	Regression Coefficient	Confident Interval	P Value	Regression Coefficient	Confident Interval	P Value	Regression Coefficient	Confident Interval	P Value
Age Group	13-17	.		.	.		.	Ref		.	Ref		.	-0.01	-0.27;0.26	0.955	-0.08	-0.36;0.19	0.551
	05-12	Ref		.	Ref		.	.		.	.		.	Ref		.	Ref		.
Treatment Gi	Late	0.88	0.58;1.17	**<.0001**	0.80	0.47;1.13	**<.0001**	0.94	0.49;1.39	**0.0003**	0.93	0.47;1.38	**0.0003**	0.92	0.68;1.17	**<.0001**	0.88	0.62;1.13	**<.0001**
	Early	Ref		.	Ref		.	Ref		.	Ref		.	Ref		.	Ref		.
HIV RNA <50	No	0.05	-0.48;0.60	0.840	0.53	0.01;1.05	**0.046**	0.46	-0.11;1.03	0.109	0.62	0.12;1.11	**0.017**	0.25	-0.12;0.63	0.179	0.55	0.20;0.90	**0.002**
	Yes	Ref		.	Ref		.	Ref		.	Ref		.	Ref		.	Ref		.
HLA Group	Deleterious	0.20	-0.15;0.56	**0.008**	0.19	-0.21;0.59	**0.032**	-0.13	-0.59;0.34	0.642	-0.05	-0.49;0.40	0.761	0.03	-0.24;0.30	0.100	0.07	-0.22;0.36	0.140
	Protective	-0.75	-1.22;-0.28	.	-0.72	-1.25;-0.19	.	0.37	-0.79;1.54	.	0.38	-0.79;1.55	.	-0.55	-0.99;-0.11	.	-0.52	-0.99;-0.05	.
	Both	-0.05	-0.75;0.65	.	-0.14	-0.93;0.65	.	.		.	.		.	-0.07	-0.81;0.66	.	-0.15	-0.93;0.63	.
	None	Ref		.	Ref		.	Ref		.	Ref		.	Ref		.	Ref		.
Normalized cumulative viremia since cART		0.01	0.01;0.02	**0.001**	.		.	0.01	-0.01;0.03	0.262	.		.	0.01	0.01;0.02	**0.002**	.		.

Protective HLA alleles: HLA-B*27 and/or B*57:01 and/or B*57:03; Deleterious HLA: HLA-B*35:02 and/or B*35:03 and/or B*53:01 and/or B*58:02.

Figure 1

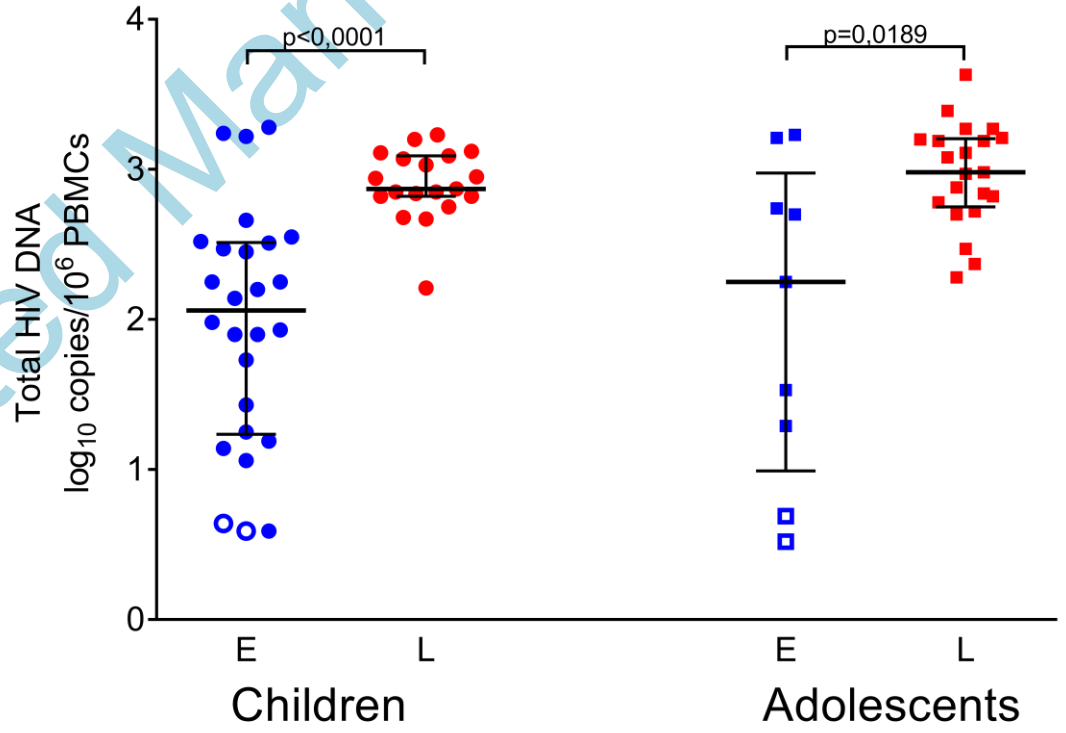

Figure 2

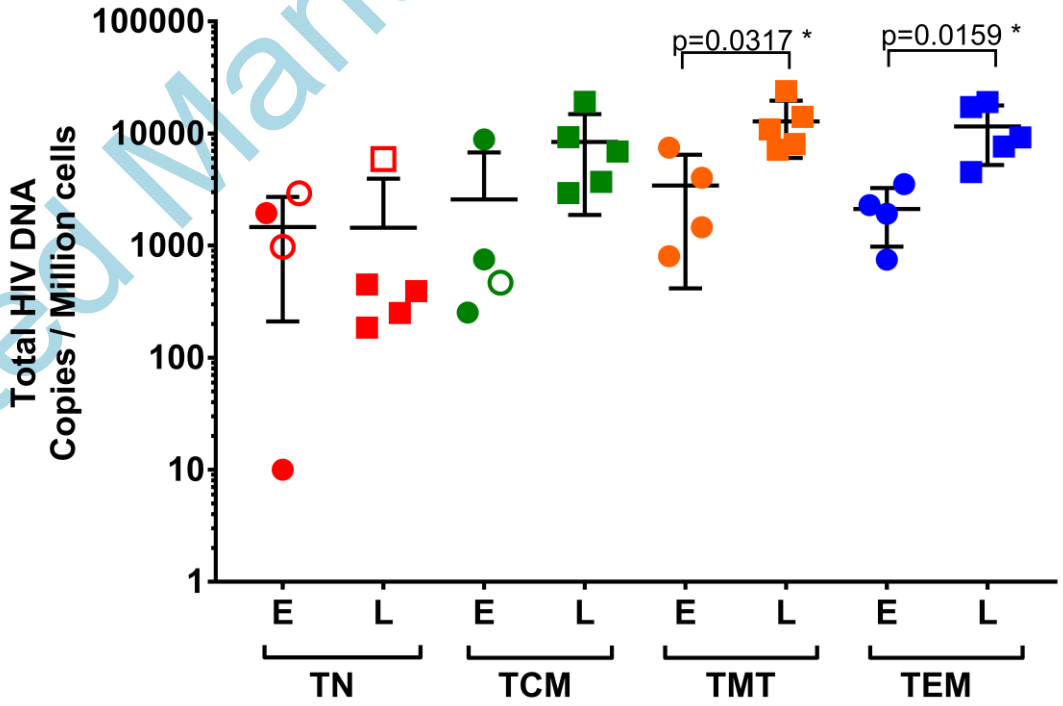

Figure 3