

Purifying Selection in Human Immunodeficiency Virus-1 *pol* Gene in Perinatally Human Immunodeficiency Virus-1-Infected Children Harboring Discordant Immunological Response and Virological Nonresponse to Long-Term Antiretroviral Therapy

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Abstract

Background: Biological monitoring of antiretroviral treatment (ART) in human immunodeficiency virus (HIV)-infected pediatric population remains challenging. The aim of the present study was to assess the long-term HIV-1 genetic diversity in *pol* gene in HIV-

1-infected children in virological failure under antiretroviral regimen adapted according to the successive World Health Organization (WHO) guidelines for resource-constrained settings.

Methods: HIV-1 diversity in *pol* gene was assessed in HIV-1-infected children and adolescents born from HIV-infected mothers (median age at follow-up: 13.8 years) in virological failure (VF⁺) despite long-term regimen recommended by the WHO. The numbers of nonsynonymous substitutions per potential nonsynonymous site (dN) and of synonymous substitutions at potential synonymous sites (dS) in HIV-1 *pol* gene and the dN/dS ratios were used to estimate the selective pressure on circulating HIV-1.

Results: The immunological responses to ART basically corresponded to: 1) Full therapeutic failure with immunological (I⁻) and virological nonresponses in one-quarter (24.6%) of study children ((I⁻, VF⁺) subgroup); 2) Discordant immunovirological responses with paradoxical high CD4 T cell counts (I⁺) and high HIV-1 RNA load in the remaining cohort patients (75.4%) ((I⁺, VF⁺) subgroup). The mean dS was 1.8-fold higher in (I⁺, VF⁺) than (I⁻, VF⁺) subgroup (25.9 ± 18.4 vs. 14.3 ± 10.8). In the (I⁺, VF⁺) subgroup, the mean dS was 1.6-fold higher than the mean dN. Finally, the mean dN/dS ratio was 2.1-fold lower in (I⁺, VF⁺) than (I⁻, VF⁺) subgroup (0.6 ± 0.3 vs. 1.3 ± 0.7), indicating purifying selection in the immunovirological discordant (I⁺, VF⁺) subgroup and positive selection in the immunovirological failure (I⁻, VF⁺) subgroup.

Conclusions: Children and adolescents in immunovirological therapeutic failure harbor positive selection of HIV-1 strains favoring diversifying in *pol*-encoded amino acids. In contrast, children with persistent discordant immunovirological responses show accumulation of mutations and purifying selection in *pol* gene sequences, indicating limited genetic evolution and likely suggesting genetic adaptation of viruses to host functional constraints.

Keywords: Children; Adolescents; Virological failure; Immunovirological dissociation; Synonymous mutations; Nonsynonymous mutations; Purifying selection; HIV-1 *pol* gene

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Introduction

Nearly 90% of human immunodeficiency virus (HIV)-infected children reside in sub-Saharan Africa [1]. Treating HIV-infected children in resource-limited settings remains tricky. Antiretroviral treatment (ART) in African pediatric populations remains associated with high rates of treatment failure ranging from 19% to 53% [2].

HIV-1 RNA load and CD4 T cell count constitute complementary surrogate biological markers to monitor ART in pediatric acquired immunodeficiency syndrome (AIDS) [3-5]. According to the World Health Organization (WHO) recommendations, the threshold for virological failure (VF⁺) in HIV-infected children and adolescents treated by ART was set at 1,000 copies/mL [4, 6-8]. In practice, the correlation between immunological and virological failures in pediatric cohorts appeared often minimal with frequent immunological and virological discordant responses during prolonged use of antiretroviral drugs [5, 9-11]. Beyond the classical quantitative surrogate markers, including HIV-1 RNA load and CD4 T cell count, the understanding of genetic evolution of circulating HIV-1 strains in HIV-infected children taking long-term ART and in virological failure could provide relevant insights to improve and adapt the management of ART-experienced children exhibiting immunological and virological discordant responses.

An observational cohort of HIV-infected children was prospectively established since 2007 at the Complexe Pédiatrique of Bangui, the main health care clinic for HIV-infected children of the Central African Republic [12-14]. The aim of the present study was to assess the long-term HIV-1 genetic diversity in *pol* gene in HIV-1-infected children in virological failure under antiretroviral regimen adapted according to the successive WHO guidelines for resource-constrained settings [3, 4, 6].

Materials and Methods

Study population

A total of 220 HIV-1-infected children and adolescents born from HIV-1-infected mothers attending the Complexe Pédiatrique of Bangui were prospectively recruited in 2009 and further followed up until 2013 for 39 months in a descriptive observational cohort study, assessing their outcome under ART, as previously reported elsewhere [13, 14]. The median age of the children was 13.8 years (interquartile range (IQR): 8 - 17), and the sex ratio (male/female) was 0.85 (101/119). The majority of the children and adolescents (n = 198, 90%) received first-line regimen according to the revised WHO recommendations of 2013 [4], for a mean duration in first-line of 65.6 months (range: 18.8 - 69.9). The remaining children and adolescents (n = 22, 10%) received second-line regimen for a mean duration of 78.2 months (range: 13.3 - 88.3). The most prescribed treatment was stavudine (d4T)/lamivudine (3TC)/nevirapine (NVP) combination. Only a minority (12.7%

(28/220)) of children was under protease inhibitor (PI)-based regimen.

Virological and immunological categorization of children under ART

Plasma HIV-1 RNA load and CD4 T cell measurements were carried out, as previously described [14]. In brief, plasma HIV-1 RNA loads were measured using the Amplix[®] platform developed by Biosynex (Strasbourg, France), which integrates a fully automated station for nucleic acid extraction with a real-time polymerase chain reaction (PCR) amplification station, using lyophilized Amplix HIV-1 RNA quantitative reagents (Biosynex). The assay detects HIV-1 groups M and O and several circulating recombinant forms (CRFs). The CD4 T lymphocyte count was performed using the Apogee Auto40 flow cytometer from Apogee Flow Systems laboratories (Hemel Hempstead, London, UK).

Successful immunological response to therapy (I⁺) was defined as increase in CD4 T cells count according to the age at follow-up visit (i.e., CD4 T cells count > 750/μL for children less than 5 years and CD4 T cells count > 500/μL for children and adolescents older than 5 years) [3, 4].

Finally, children under ART in virological failure (VF⁺) according to the WHO threshold (HIV-1 RNA load > 1,000 copies/mL) were classified into two categories according to their immunovirological responses: (I⁺, VF⁺) for immunological responders but virological nonresponders; and (I⁻, VF⁺) for both immunological and virological nonresponders.

Detection of drug resistance mutations

Detection of drug resistance mutations (DRMs) was carried out in plasmas from randomly selected children with HIV-1 RNA load above 1,000 copies/mL after 39 months of follow-up, as reported [14]. Randomization for DRMs genotyping consisted in the selection of one out of three children from the (I⁺, VF⁺) subgroup and one out of two children in the (I⁻, VF⁺) subgroup.

Overtime genetic variability of HIV-1 RNA *pol* gene sequences

To evaluate whether, and to what extent, the genetic variability correlates with positive selection for amino acid changes, the accumulation rate of synonymous (S) and nonsynonymous (N) substitutions per potential synonymous and nonsynonymous site was analyzed in each patient by pairwise comparisons of HIV-1 sequences between inclusion and follow-up. The genetic variability overtime in the two subgroups of virological failure (I⁺, VF⁺) and (I⁻, VF⁺) was estimated using aligned sequences according to open-reading frame and codons by pairwise comparisons of all HIV-1 *pol* gene sequences obtained at baseline and after 39 months of prospective follow-up from selected children and adolescents of each subgroup, correspond-

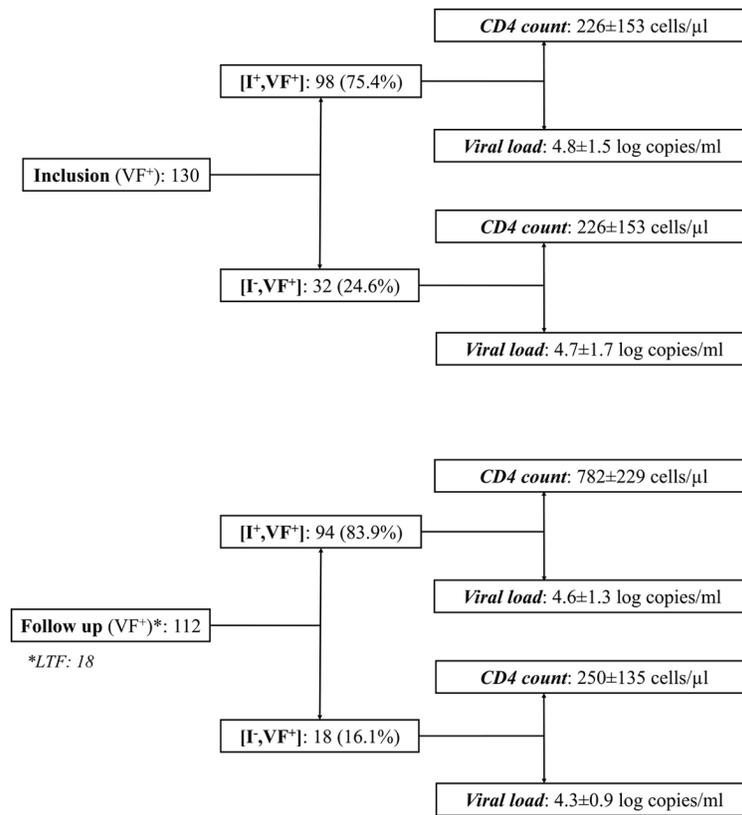


Figure 1. Distribution of study children in virological failure (VF⁺) at inclusion and after the follow-up period, according to their immunological (CD4 T cell count) and virological (circulating viral load) responses to antiretroviral treatment. Results are expressed as mean ± standard deviation. I⁺: immunological responders; I⁻: immunological nonresponders; LTF: lost-to-follow up; CD4: cluster of differentiation 4.

ing to the appraisal of 433 paired nucleotide positions. Analyses were conducted according to the Nei-Gojobori model [15], using the Jukes-Cantor correction for multiple substitutions implemented in the Molecular Evolutionary Genetics Analysis version 6 (MEGA6) program package (www.meagasoftware.net), as described [16]. All ambiguous positions or gap were removed for each sequence pair. For each positions, synonymous (dS) and nonsynonymous (dN) nucleotide differences between each pair of HIV-1 *pol* gene sequences at baseline and follow-up, corresponding to the genetic distance (d) between paired sequences, were determined, and the nonsynonymous/synonymous ratio rates ($\omega = dN/dS$) were further calculated. The ω ratios are important indicators of selective pressure at the protein level, with $\omega = 1$ meaning neutral mutations, $\omega < 1.0$ purifying selection and $\omega > 1.0$ diversifying positive selection favoring diversity at the amino acid level [17-19].

After validation, all nucleotide sequences were submitted to the European Nucleotide Archive at GenBankEMBL-EBI, with the following accession numbers: LT577626-LT577673; LT578170-LT578280 and LT726745-LT726792.

Statistical analyses

Means and standard deviations (SD) were calculated for quan-

titative variables using the software R (version 3.5.2). Average genetic distances were compared within each subgroup by the nonparametric Wilcoxon’s test for paired data, and between subgroups using the Welch’s *t*-test assuming unequal variances between subgroups.

Ethics statement

The study was formally approved by the Scientific Committee of Faculte des Sciences de la Sante (“FACSS”) of Bangui, Central African Republic, and was conducted in compliance with the ethical standards of the responsible institution on human subjects as well as with the Helsinki Declaration.

Results

Longitudinal immunovirological outcomes of the cohort children after 39 months of follow-up

The Figure 1 depicts the distribution of study children at inclusion and after the follow-up period, according to their immunological and virological responses to ART. At baseline in

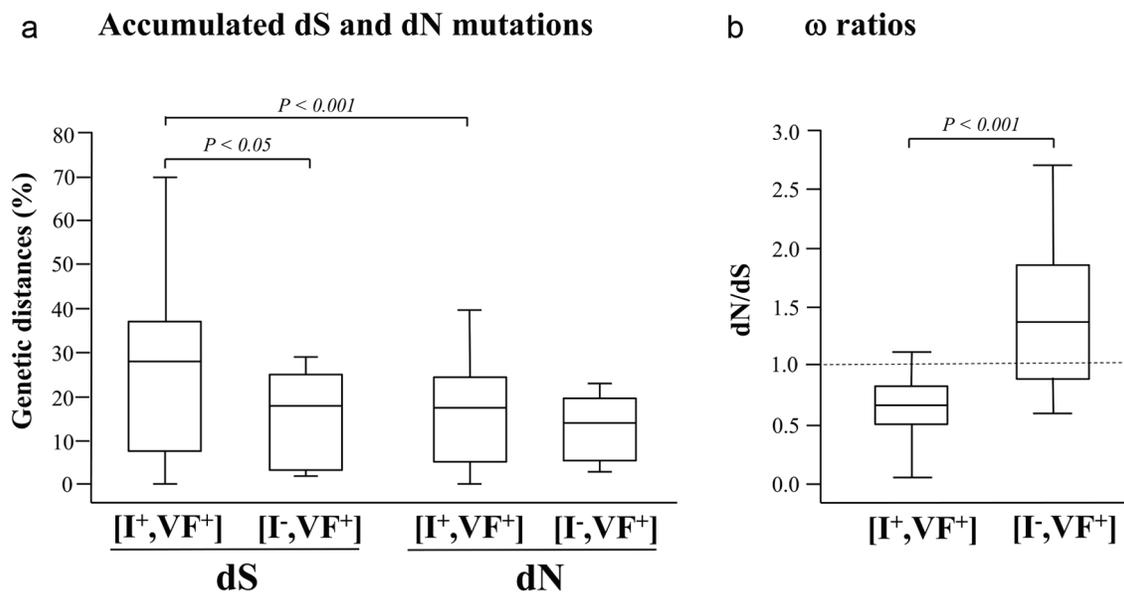


Figure 2. Estimates of codon-based evolutionary divergences between pairs of HIV-1 *pol* gene nucleotide sequences during a 39 months period of prospective follow-up of children and adolescents attending the Complexe Pediatrique of Bangui, Central African Republic. The genetic distances (expressed in percentage) of synonymous substitutions per 100 potential synonymous sites (dS) and of nonsynonymous substitutions per 100 potential nonsynonymous site (dN) accumulated during the follow-up period in circulating HIV-1 *pol* gene (a), as well as the (dN/dS) ω ratios (b) are shown for both (I⁺, VF⁺) and (I⁻, VF⁺) subgroups. For each distribution, the blank box represents the interquartile ranges, the horizontal bar corresponds to the median and the vertical bars and hats indicate the 10th and 90th percentiles. The dotted line identifies the neutral selection ratio ($\omega = 1$). Comparisons used Wilcoxon's test for paired data and Welch's *t*-test between subgroups. P values are given above indentations.

2009, 130 (55.1%) children and adolescents among the 245 cohort children were in virological failure (VF⁺). Among them, 32 (24.6%) were immunological and virological nonresponders (I⁻, VF⁺), with a very low mean CD4 T cells count (mean: 226 ± 153 cells/ μ L) and high viral load (mean: 4.7 ± 1.7 log copies/mL), while the majority ($n = 98$; 75.4%) were immunological responders but virological nonresponders (I⁺, VF⁺) with normal CD4 T cell count (mean: 809 ± 280 cells/ μ L) and elevated viral load (mean: 4.8 ± 1.5 log copies/mL).

After 39 months, only 18 (8.2%) children were lost to followed up, including three (1.3%) in the discordant (I⁺, VF⁺) subgroup and 15 (6.8%) in the immunovirological non-responder subgroup. A total of 112 children and adolescents were in virological failure (VF⁺), including 18 (16.1%) immunological and virological nonresponders (I⁻, VF⁺), with low CD4 T cells count (mean: 250 ± 135 cells/ μ L) and high viral load (mean: 4.3 ± 0.9 log copies/mL) and 94 (83.9%) immunological responders but virological nonresponders (I⁺, VF⁺), with high CD4 T cell count (mean: 782 ± 229 cells/ μ L) and viral load (mean: 4.6 ± 1.3 log copies/mL).

Temporal variation of genetic diversity in HIV-1 RNA *pol* gene from circulating HIV-1 strains according to immunovirological subgroups of virological failure

Paired genotyping for DRMs in HIV-1 *pol* gene at baseline and after follow-up was obtained in viruses from 32 selected children in the (I⁺, VF⁺) subgroup and from nine selected children

in the (I⁻, VF⁺) subgroup.

After 39 months of follow-up, 40 (97.5%) (including 31/32 (96.8%) in (I⁺, VF⁺) subgroup and 9/9 (100%) in (I⁻, VF⁺) subgroup) of the 41 genotypes from the children cohort in virological failure harbored genotype profiles of resistance to WHO-recommended first-line therapeutic regimen with a high frequency of natural polymorphisms in the protease gene sequences in over 90% of sequenced viruses (not shown). The mutation V82A, identified as being more frequent in patients with a good immunological response despite virological failure [20] was observed in viruses from five children belonging to the subgroup (I⁺, VF⁺). Most sequenced viruses remained susceptible to the majority of the WHO-recommended antiretroviral drugs (not shown).

Variation in genetic variability overtime between baseline and follow-up involved 64 HIV-1 *pol* gene nucleotide sequences in the (I⁺, VF⁺) subgroup and 18 in the (I⁻, VF⁺) subgroup (Fig. 2). The number of nonsynonymous substitutions per potential nonsynonymous site (dN), the number of synonymous substitutions at potential synonymous sites (dS) and the dN/dS ratios were assessed to estimate the selective pressure during the follow-up period on circulating HIV-1 RNA from each randomly selected children or adolescents. No difference in mean dN could be observed between (I⁺, VF⁺) and (I⁻, VF⁺) subgroups for all pairwise comparisons of HIV-1 *pol* sequences between inclusion and follow-up (15.9 ± 11.7 in (I⁺, VF⁺) subgroup vs. 13.2 ± 7.2 in (I⁻, VF⁺) subgroup; $P > 0.05$). In contrast, mean dS was 1.8-fold higher in (I⁺, VF⁺) than (I⁻, VF⁺) subgroups (25.9 ± 18.4 in (I⁺, VF⁺) subgroup vs.

14.3 ± 10.8 in (I⁻, VF⁺) subgroup; P < 0.05). In the (I⁺, VF⁺) subgroup, the mean dS was 1.6-fold higher than the mean dN (P < 0.01). Finally, the mean ω ratio was 2.1-fold lower in (I⁺, VF⁺) than (I⁻, VF⁺) subgroups (0.6 ± 0.3 in (I⁺, VF⁺) subgroup vs. 1.3 ± 0.7 in (I⁻, VF⁺) subgroup; P < 0.001), indicating purifying selection in the immunovirological discordant (I⁺, VF⁺) subgroup and positive selection in the immunovirological failure (I⁻, VF⁺) subgroup.

Discussion

Immunological surrogate marker (CD4 T cell count) and HIV-1 genetic diversity in *pol* gene were evaluated longitudinally in perinatally HIV-1-infected children and adolescents born from HIV-infected mothers, in virological failure despite long-term WHO-recommended ART and followed up for 39 months at the Complexe Pediatrique of Bangui. The immunological responses to ART corresponded basically to two categories, including full therapeutic failure with immunological and virological nonresponses in nearly one-quarter of children always associated with high levels of DRMs, and discordant immunovirological responses in the majority (about 75%) of remaining children. Outstandingly, the high prevalence of children and adolescents harboring discordant immunovirological responses with paradoxical high CD4 T cell counts and high HIV-1 RNA load demonstrates *a posteriori* minimal correlation between immunological and virological biological markers.

The subgroup of children and adolescents in full therapeutic failure (I⁻, VF⁺) in whom plasma HIV-1 RNA load was elevated overtime and CD4 T cell counts remained persistently low after the follow-up period represented one-quarter of children and adolescents in virological failure, in keeping with generally high rate of ART failure in African pediatric cohorts [2, 21-26].

The subgroup of children and adolescents who exhibited discordant response to treatment with immunological response and virological nonresponse (I⁺, VF⁺) represented the majority of cohort children in virological failure. Although these patients failed to control HIV-1 viral replication after the follow-up period and thus were all in virological failure with viruses harboring high rates of DRMs (> 97%), their CD4 T cell counts have remained persistently above normal levels according to age of children (i.e., CD4 T cells count > 750/μL for children less than 5 years and CD4 T cells count > 500/μL for children and adolescents older than 5 years, as defined by the WHO) [3, 4]. Increased CD4 T cell count, higher than the WHO-threshold of immunological success without suppressing viral load demonstrating discordant response, was previously reported [7, 26-30]. It has been suggested that different mechanisms may be involved in this phenomenon. Firstly, the recovery of thymic function and higher thymic output under ART favor immune restoration in HIV-1-infected children [27, 28, 31-33]. Secondly, partial virus suppression under certain drugs regimen (mainly including PI) may reduce CD4 T cell turnover and activation [24, 34]. Thirdly, the resistance mutation V82A (in protease) previously reported in discordant patients in virological failure but with persistent high levels of

CD4 counts [20], was observed in a minority of children in the (I⁺, VF⁺) subgroup. Fourthly, discordant responses during ART may be related to enhance HIV-directed immune responses, diminished cellular activation, and preservation of non-syncytium-inducing (CCR5-tropic) viruses, as previously reported [30]. Fifthly, the accumulation of high level of DRMs overtime could have provided impaired viral fitness [28, 35-40], which would substantially modify the viral pathogenic potential [41]. Finally, persistent normal or elevated CD4 T cell count associated with high circulating HIV-1 RNA load may procure selective advantages for the virus as well as for the discordant children, who appeared unexpectedly tolerant to the virus.

The evolutionary patterns of HIV-1 *pol* gene diversity were further evaluated in the two categories of children and adolescent in virological failure by pairwise comparisons of the numbers of synonymous (silent) and nonsynonymous (amino acid altering) nucleotide substitutions in HIV-1 *pol* gene sequences between inclusion and follow-up. Amino acid sites in viral proteins are expected to be under different selective pressures. Thus, the genetic distances of synonymous amino acids (e.g., dS) reflect mutation rates and generation times, whereas the distances of nonsynonymous (e.g., dN) may be affected by selective factors, especially immune pressure [42], reflecting the adaptive evolution against the background rate of evolution [43]. Remarkably, the evolutionary patterns of HIV-1 *pol* gene diversity in the study cohort differed between the two categories of virological failure, with different underlying ω (dN/dS) ratios, pointing to the existence of different viruses and host factors contributing to the evolution of HIV-1 quasispecies *in vivo*. These findings likely denote the lack of genetic homogeneity between circulating HIV-1 RNA from the two groups of children in virological failure, with concordant (I⁻, VF⁺) or discordant (I⁺, VF⁺) immunological response. Different evolutionary patterns mainly in HIV-1 *env* gene were previously reported during the natural history of HIV-1 infection as well as during ART [29, 42, 44-46].

Within children and adolescents in full therapeutic failure, the mean ω ratio of viral strains between baseline and follow-up was above 1, suggesting diversifying positive selection in HIV-1 *pol* gene [19]. This observation contrasts with natural history of HIV-1 infection in the absence of ART during which the rate of synonymous substitution in HIV-1 *pol* gene generally exceeds that of nonsynonymous substitution, indicating that *pol* gene is naturally subject to purifying selection [44]. Positive selection of HIV-1 *pol*-encoded amino acids overtime in patients in virological failure under ART may affect viral proteins conformations and/or biological functions, possibly to escape to antiretroviral drug pressure. Despite the low CD4 T cell counts in the (I⁻, VF⁺) subgroup, another hypothesis is that positive selection in *pol* gene may occur under the selective pressure of changes in the dynamics of the HIV-specific immune response. Indeed, positive selection in HIV-1 *env* gene with increase in dN/dS ratio in the V3 loop and increase of dN/dS ratio above 1 in genes coding for cytotoxic T lymphocytes epitopes in HIV-1 variants has been hypothesized to be caused by immune-mediated selection for sequence diversity in order to avoid immune recognition [44, 47, 48].

Within children and adolescents with persistent discordant immunological response during virological failure under

prolonged ART, the mean ω ratio of viral strains between baseline and follow-up was below 1, suggesting purifying selection overtime in HIV-1 *pol* gene sequences in the (I⁺, VF⁺) subgroup [19, 42]. In addition, viral strains in (I⁺, VF⁺) subgroup accumulated synonymous mutations overtime. These findings indicate neutral or relatively limited genetic evolution and likely suggest genetic adaptation of the viruses to host functional constraints, similarly to the natural selection on HIV-1 *pol* gene in the absence of ART, which is basically subject to purifying selection [44], suggesting that genetic drift rather than deterministic pressure is likely to play a substantial role in viral evolution in immunovirological discordant children and adolescents. Furthermore, our observations about the accumulation of synonymous substitutions in circulating HIV-1 RNA *pol* gene sequences with their purifying selection overtime in the (I⁺, VF⁺) subgroup also support the hypothesis that persistent replication of HIV-1 strains with high level of DRMs and probably modified viral fitness could be largely independent of HIV-specific immune response. Finally, faster progression to AIDS was previously correlated with higher rate of synonymous substitutions [46], and similar hypothesis in the pediatric cohort at the Complexe Pediatrique of Bangui warrants further investigation.

The discordant immunovirological responses in our study in perinatally HIV-infected children are reminiscent of the pediatric non-progressor children described by the group of Phillip Goulder in South Africa [49]. These children expressed two cardinal immunological features of non-pathogenic simian immunodeficiency virus (SIV) infection in sooty mangabeys, low immune activation despite high viremia and low CCR5 expression on long-lived central memory CD4 T-cells [49].

In conclusion, our observations provide important information on immunological outcome of perinatally infected children and adolescents in virological failure despite receiving lifelong WHO-recommended ART. Thus, very particular host-pathogen interactions occur in HIV-infected children and adolescents born from HIV-infected mother harboring sustained HIV replication under long-term ART. In the (I⁺, VF⁺) subgroup of children and adolescents in full therapeutic failure, the positive selection is strong, suggesting negligible genetic drift and deterministic evolution under therapeutic and host factors. In contrast, in the (I⁺, VF⁺) subgroup of children and adolescents of discordant immunological response and virological failure, the viral evolution would likely proceed according to a stochastic model of genetic drift.

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the performing of this study.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Informed Consent

Informed written consent was obtained from mothers for themselves and on behalf of their respective child.

Author Contributions

RSMB, CDMK, JCG, and LB conceived and designed the experiments. CDMK, LB, and RSMB analyzed the data. PR and RSMB carried out resistance genotypes and phylogenetic analyses. GG and JCG contributed to patients' data collection and ethical committee approval. CC, CDMK, DV, HP, JFDS, LB, MAJ, PR, RSMB and SM wrote the paper. All authors read and approved the final version of the manuscript.

Data Availability

The data supporting the findings of this study have been deposited in the European Nucleotide Archive at GenBankEMBL-EBI (available at: <http://www.ebi.ac.uk/ena/data/view/>) and can be accessed with the following accession numbers: LT577626-LT577673; LT578170-LT578280 and LT726745-LT726792.

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