

# Predictors of Virologic Response to Antiretroviral Therapy (ART) and Characterization of Immune Repopulation in HIV-1-Infected Children with Different Virologic Response to ART

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## Abstract

**Background:** Peripheral immune repopulation despite virologic failure is a phenomenon often observed in children under ART. The aim of this study was to investigate the predictors of virologic failure and the immune repopulation in children with or without virologic response to ART.

**Methods:** 30 HIV-1-infected children, pre-treated with reverse transcriptase inhibitors (RTI), who entered therapy with RTI and protease inhibitors (PI) were analyzed. At baseline, HIV-1 DNA and intracellular HIV-1 mRNAs were quantified by real-time PCR. Sequence analyses of RT and PR regions of plasma viral isolates were performed in all children at baseline and 14-24 months after ART initiation in children with detectable HIV-1 RNA in plasma. CD4 and CD8 T subsets were investigated at baseline and after 14-24 months by flow cytometry evaluating the expression of CD45RA, CD27 molecules and "maturation/activation" CD38 marker.

**Results:** 13 children (VR) showed a virologic response to ART (HIV-1 RNA below 50 copies/ml plasma within 6 months) and thereafter persistent undetectable viremia, while 17 had virologic failure (VNR). At baseline, HIV-1 mRNA/HIV DNA was significantly higher in VNR than VR; 4 VR and 8 VNR had viral isolates with RTI-resistance mutations. During ART, 9 of 17 VNR developed viruses with PI-resistance mutations. All CD4 cell subsets increased significantly in VR children; among the VNR children only those with PI-resistance mutations had a significant increase in CD4, mainly contributed by naive CD4 cells. CD38CD4 cells increased in both groups, and the increase was correlated with the increase in naive CD4 cells. Notably, CD38CD8 T cells decreased in VR children, but remained high in VNR group.

**Conclusions:** Our data suggest that the high mRNA/DNA ratio and RTI-resistance mutations at baseline impair subsequent therapy effectiveness, promoting development of resistance to PI. PI-resistance mutations appeared to reduce viral fitness, allowing CD4 T-cell immunorepopulation by naive CD4 cells. Increase in CD38CD4 cells in both VR and VNR infants, and its relationship with increase in naive CD4 cells, support the notion that CD38 in CD4 cells is a marker of cell immaturity rather than cell activation. Persistence of viremia in VNR impaired the expansion of central memory and effector/memory CD4 cells, and sustained the persistence of high levels of "activated" CD38CD8 cells.

## INTRODUCTION

Highly active antiretroviral therapy (HAART) efficiently reduces HIV-1 load to undetectable levels and increases the number of circulating CD4 T cells in children and adults. Immune reconstitution in children differs from that in adults. While there is an initial rapid increase in memory CD4 T cells followed by a slower and smaller increase in naive CD4 T cells in adults (1), in HAART-treated children the immune repopulation mainly occurs with naive CD4 T cells, due to a more efficient thymopoiesis (2, 3). In addition, HAART-treated children show a high rate of virological failure that could be caused by the development of antiretroviral drugs resistance via mutations in the reverse transcriptase (RT) and protease (PR) regions of the pol gene. Moreover, some children show a discordant response to HAART, with a significant increase in CD4 T cell counts, despite the persistence of detectable plasma viremia (4, 5). The aim of this study was to investigate the predictors of virologic failure and the immune repopulation in children with and without virologic response to therapy.

## PATIENTS AND METHODS

**Patients.** This study included 30 HIV-1-infected children, all born to HIV-1-seropositive mothers and followed at the Pediatric Department of Padova University since birth. All patients underwent a HAART regime consisting of a combination of two nucleoside or non-nucleoside reverse transcriptase inhibitors (RTI) and one protease inhibitor (PI). All were naive for PI and 19 children had been treated with nucleoside RTI before HAART. CD4 T cell counts and plasma HIV-1 RNA levels were followed over time. Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood by centrifugation on Ficoll-Paque gradient and then cryopreserved until use.

**Quantification of HIV-1 mRNA.** Total cellular RNA was extracted from PBMC using Trizol (Invitrogen), unspliced HIV-1 RNA (HIV-1 uRNA) and multiply-spliced HIV-1 RNA (HIV-1 mRNA) encoding *tat/rev* were quantified by real-time PCR as previously described (6).

**HIV-1 DNA quantification.** Quantitative determination of HIV-1 DNA was performed on lysed PBMC by real-time PCR, as previously described (3).

**Quantification of HIV-1 RNA in plasma.** Plasma HIV-1 RNA levels were determined using the Amplicor HIV-1 Monitor Test (Roche) according to the manufacturer's instructions.

**HIV-1 genotyping analysis.** Analyses of drug resistance mutations were performed at baseline in all 30 children and after HAART initiation (mean period 16 months, range 9-24 months) in virological non-responders. Sequence analyses were performed for protease (PR) and reverse transcriptase (RT) regions of the HIV-1 *pol* gene. HIV-1 RNA was extracted from plasma samples, retranscribed and amplified by nested PCR using the following primers designed from the isolate HIVP22 (GenBank accession number: K02083): outer protease primers PR1-forward (nt 2268-2292) and PR2-reverse (nt 2645-2621); inner protease primers PR3-forward (nt 2294-2313) and PR4-reverse (nt 2636-2617); outer RT primers RT1-forward (nt 2529-2560) and RT2-reverse (nt 3358-3330); inner RT primers RT3-FW forward (nt 2672-2695) and RT4-RV reverse (nt 3044-3021); RT4-FW forward (nt 2664-2987) and RT4-RV reverse (nt 3343-3320). PCR products were purified by enzymatic treatment (ExoSAP-IT, Amersham Biosciences) and sequenced using the Big Dye Terminator v1.1 cycle sequencing ready reaction kit (Applied Biosystems) and ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The HIV Drug Resistance Database (<http://hivdb.stanford.edu>) was utilized to discriminate reverse transcriptase and protease mutations with a major role in conferring a high (primary mutations) or moderate (secondary mutations) level of resistance to antiretroviral drugs.

**Flow-cytometry quantification of CD4 and CD8 T lymphocyte subpopulations.** Analyses of T lymphocyte subpopulations were performed at baseline and after HAART initiation (mean period 21 months, range 11-30 months). T lymphocyte subsets were stained for four-colors flow cytometry using the following labeled monoclonal antibodies (mAbs): anti-CD3 (FITC), anti-CD4 (PerCP), anti-CD8 (PerCP), anti-CD27 (PE), anti-CD45RA (APC), anti-CD38 (PE) and appropriate isotypic controls (mouse IgG1-PE and mouse IgG2b-APC) were used (Becton-Dickinson). Acquisition was performed in a FACScan/ultra-cytometer using the CELLQuest Software (Becton-Dickinson). 50,000 events were collected, and the percentage of CD45RA, CD27 and CD38 positive cells was calculated within the CD3+CD4+ or CD3+CD8+ gate. The CD45RA and CD27 expression was used to identify CD4 and CD8 T cell subsets as follows: CD45RA+CD27+ (naive), CD45RA+CD27- (central memory), CD45RA-CD27- (effector/memory CD4 cells, and pre-terminally differentiated effector CD8 T cells) and CD8+CD45RA+CD27- (terminally differentiated cytotoxic effector).

**Statistical Analysis:** Differences in characteristics among infants were analyzed using non-parametric tests. The Mann-Whitney U test was used to compare data between groups, and the Wilcoxon's non-parametric test was used to compare data within groups. Correlations between different parameters were analyzed using the non-parametric Spearman's test.

## RESULTS

### Virological characteristics at baseline.

Children were classified in two groups according their virologic response to therapy: 13 were virological responders (VR), i.e. demonstrated a decrease in plasma HIV-1 RNA load to undetectable levels (<50 copies/ml of plasma) within 6 months after HAART initiation and thereafter remained persistently aviremic, and 17 were virological non-responders (VNR), i.e. with detectable HIV-1 RNA in plasma during the entire study period.

Characteristics of the children at baseline are shown in Table 1. VR and VNR groups did not differ in terms of plasma HIV-1 RNA, CD4 T cell count, or CD4 T cell percentage (Table 1). All children were naive for PI, and only 4 VR but 15 VNR children were treated with nucleoside RTIs before starting HAART for a mean period of 4 and 17 months, respectively (Table 1).

HIV-1 DNA load tended to be higher in VNR than VR, although the difference was not significant (Figure 1). Intracellular HIV-1 uRNA could be considered as a marker of viral replication and HIV-1 mRNA a marker of newly infected cells. HIV-1 uRNA, HIV-1 mRNA and the HIV-1 uRNA/HIV-1 DNA ratio tended to be higher in VNR than VR, and the HIV-1 mRNA/HIV-1 DNA ratio was significantly higher in VNR than VR ( $P=0.039$ ) (Figure 1).

Table 1. Characteristics of the studied population at baseline

	Total	VR	VNR
N patients	30	13	17
Male	18	11	7
Female	12	2	10
Mean age at onset (years)	5.9 ± 4	6.1 ± 3.5	5.7 ± 4.3
Mean age at baseline (years)	12.6 ± 4.2	13.1 ± 4.1	12.1 ± 4.2
HIV-1 RNA			
undetectable (log copies/ml)	4.9 ± 0.9	4.7 ± 0.6	5.1
detectable (log copies/ml)	4.9 ± 0.9	4.8 ± 0.9	5.0
(range)	(2.4-6.2)	(3.3-6.2)	(2.7-6.2)
CD4 cells			
naive (cells/mm <sup>3</sup> )	219	216	220
total (cells/mm <sup>3</sup> )	840	(94.11)	(84.28)
CD8 cells			
naive (cells/mm <sup>3</sup> )	11	11	11
total (cells/mm <sup>3</sup> )	19	4	15
N virological responders	13	13	0
N virological non-responders	17	0	17
N children naive for PI	26	11	15

VR = virological responders children;  
VNR = virological non-responders children

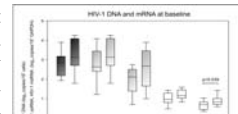


Figure 1: Median and percentiles of HIV-1 DNA, HIV-1 uRNA, HIV-1 mRNA, HIV-1 mRNA/HIV-1 DNA ratio and HIV-1 mRNA/HIV-1 DNA ratio in VR and VNR children at baseline.

### Drug resistance mutations.

At baseline, 4/4 VR and 8/15 VNR children pre-treated with nucleoside RTI had viruses with RTI-resistance mutations (Table 2). During HAART, 9 VNR children developed both RTI- and PI-resistance mutations, 5 only RTI-resistance mutations and 3 maintained wild-type isolates. Notably, 6 of 9 children who acquired PI-resistance mutations during HAART had RTI-resistance mutations at baseline (Table 2).

Table 2. Drug resistance mutations

Group	Pre-treated children	Drug resistance mutation at baseline	Drug resistance mutation in RTI region at follow-up	Drug resistance mutation in PI region at follow-up
VNR	1			
	2	M41I, L74V, M184V, T215Y	M46I, L43P, A71T, V97I, V92A, I84V, I80M	
	3	nd	M184V	L10I, D30N, M46I, L43P, V77I
	4	M184V	M184V	V75I, M40I, L43P, A71V, V82A
	5	M184V, T215Y	M184V, T215Y	L10I, D30N, L28P, A71V, V97I, S88D
	6		M41I, E44D, D67N, I69K, K219P, Y90A, V116L, Y24C, M184V, L210W, T215Y	
	7	M184V, L210W, T215Y	M41I, M184V, L210W, T215Y	L10I, D30N, L43P, H60N, A71E, V77I, S88D
	8	M41I, M184V, L210W, T215Y	M41I, M184V, V75A, L210W, T215Y	D30N, L43P, V77I, V82A
	9	M41I, E44D, D67N, M184V, L210W, T215Y	M41I, E44D, D67N, L210W, T215Y	K20I, S46I, L52G, V77I, L50M, L10I, D30N, T215Y
	10	M41I, E44D, D67N, M184V, L210W, T215Y	M41I, E44D, D67N, L210W, T215Y	L10I, D30N, M46I, L43P, P33M
	11	M41I, E44D, D67N, M184V, L210W, T215Y	M41I, E44D, D67N, L210W, T215Y	
	12	M41I	M41I, M184V, L210W, T215Y	
	13	M41I, E44D, D67N, V10I, M184V, L210W, T215Y	M41I, E44D, D67N, I69K, V10I, V116I, M184V, L210W, T215Y	
	14	ND	M184V, T215Y	M40I, M46I, L43P, I30M, I93I
	15	ND	L28P, V75I, M184V, T215E, K219P	
	NT untreated children			

### Changes in T cell subsets during HAART in virological responders and virological non-responders children.

At baseline, VR and VNR groups did not show significant differences in the total number of CD4 and CD8 T cell counts. However, while values of all CD4 T cell subsets were very similar between VR and VNR, cell count levels of pre-effector CD45RA-CD27-CD8+, effector CD45RA+CD27-CD8+, and activated CD38+CD8+ T cells were higher in VNR than VR (Figure 2).

During HAART, there was a significant increase in CD4 T cell count in both VR (from 291 to 834 cells/mm<sup>3</sup>,  $P=0.002$ ) and VNR groups (from 285 to 499 cells/mm<sup>3</sup>,  $P=0.026$ ), but the increase was higher in the VR than VNR group ( $P=0.04$ ). In addition, CD4 T cell subsets increased in a different manner in VR and VNR groups. Indeed, in VR the increase was significant in naive (from 155 to 445 cells/mm<sup>3</sup>,  $P=0.03$ ), central memory (from 109 to 280 cells/mm<sup>3</sup>,  $P=0.01$ ), and CD38+ cells (from 69 to 161 cells/mm<sup>3</sup>,  $P=0.03$ ) (Figure 2B), while only naive CD4 T cells increased significantly in VNR children (from 166 to 287 cells/mm<sup>3</sup>,  $P=0.05$ ) (Figure 2C). Interestingly, there was a positive correlation between the increase of naive CD4 and CD38+CD4+ T cells in both VR ( $r=0.68$ ,  $P=0.03$ ) and VNR groups ( $r=0.75$ ,  $P=0.008$ ).

Although the total CD8 T cell count number did not significantly change during HAART in either group (Figure 2D), CD8 T cell subsets varied differently in VNR and VR. After therapy naive CD8 T cells increased significantly in both VR (from 199 to 309 cells/mm<sup>3</sup>,  $P=0.006$ ) and VNR (from 164 to 301 cells/mm<sup>3</sup>,  $P=0.001$ ), while CD38+CD8+ T cells decreased only in the VR group (from 163 to 59 cells/mm<sup>3</sup>,  $P=0.009$ ) and remained high in VNR children (Figure 2E and F).

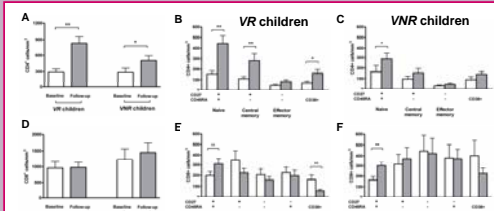


Figure 2: T cell repopulation in virological responder and virological non-responder children. The expression of CD27, CD45RA and CD38 was determined by flow cytometry in virological responder (VR) and virological non-responders (VNR) children. CD4 and CD8 T cell count (A and D) in VR and VNR children. CD4 and CD8 T cell subsets in VR (B and E) and VNR children (C and F) at baseline (white bars) and after HAART (grey bars). Mean and standard error (bar) are shown. \*Indicates  $P<0.05$ . \*\*Indicates  $P<0.01$ .

### T cell subsets in PI-susceptible and PI-resistant children.

CD4 T cell subsets were studied in children who had maintained a wild-type viremia in the PR region (PI-s) and in children who developed viruses with PI-resistance (PI-r) (Table 2). At baseline, PI-r children had lower CD4 T cell counts than PI-s children. However, during HAART CD4 T cells increased in PI-r children (from 102 to 483 cells/mm<sup>3</sup>,  $P=0.03$ ), while did not significantly change in PI-s children (Figure 3A). The immunorepopulation in PI-r children occurred mainly in naive (from 66 to 276 cells/mm<sup>3</sup>,  $P=0.05$ ) and CD38+ (from 36 to 148 cells/mm<sup>3</sup>,  $P=0.03$ ) CD4 T cell subsets (Figure 3C). In contrast, the levels of CD4 T cell subsets in PI-s children did not show any significant increase during HAART (Figure 3B).

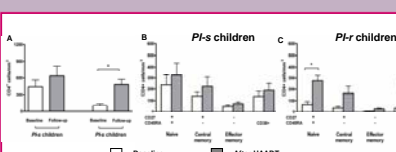


Figure 3: CD4 T cell repopulation in PI-susceptible and PI-resistant HIV-infected children. CD4 T cell subsets were determined by flow cytometry in PI-susceptible (PI-s) and PI-resistant HIV-infected children (PI-r). CD4 T cell counts in PI-s (A) and PI-r (B) CD4 T cell subsets in PI-s (B) and PI-r (C) at baseline (white bars) and after HAART (grey bars). Mean and standard error (bar) are shown. \*Indicates  $P<0.05$ . \*\*Indicates  $P<0.01$ .

## CONCLUSION

Our data suggest that non-potent regimes with one or two nucleoside RTIs before HAART and the consequent onset of drug resistance mutations may induce a high level of viral replication, as indicated by the high HIV-1 mRNA/HIV-1 DNA ratio at baseline. These baseline conditions impair the subsequent therapy effectiveness, promoting the onset of drug-resistant mutations. Furthermore, RTI-resistance mutations at baseline enhanced the onset of PI-resistance mutations during HAART, probably due to mistakes introduced by polymerase during retranscription (7).

During HAART, the immune reconstitution was different in VR and VNR children. CD4 T cell repopulation was stronger in VR than VNR children and distributed in all cell subsets. Only naive CD4 and CD8 T cells increased significantly in both groups, and this may indicate an increase of thymic function/output regardless of the viral load. In VR children, the increases in the other cell subsets may indicate a functional switch from naive to memory cells. In contrast, the persistence of viremia in VNR children impaired the expansion of memory CD4 T cell subsets, since these cells are susceptible to HIV-1 infection. The increase in CD38+CD4+ T cells observed in the VR group, and the relationship between CD38+CD4+ and naive CD4 T cells in both the VNR and VR groups suggest that CD38 is a marker of cell immaturity rather than cell activation in CD4 T cell compartment; thus its expression could be used as a marker to evaluate immune reconstitution after therapy (8). In contrast, CD38 in CD8 T cells decreased only in VR children whereas they remained high in VNR, thus indicating that CD38 in CD8 cell compartment might be an activation marker that reflects the chronic antigenic stimulation by persistence of HIV-1 antigens.

Of interest, among the VNR children the increase of CD4 T cells mainly occurred in the subgroup of children who developed PI-resistance mutations. It has been demonstrated that viruses with developed PI-resistance mutations have reduced fitness (9), this weakness may then allow for CD4 T cell immunorepopulation.

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