

**ANTIRETROVIRAL THERAPY, VIRAL LOAD, AND T LYMPHOCYTES
RATE IN HIV-1 INFECTED CHILDREN AFTER GENOTYPING DRUG
RESISTANCE ASSESSMENT.**

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ABSTRACT

Studies related to Human Immunodeficiency Virus type 1-infected children are of a special meaning due to multiple covariates such as timing of transmission, viral phenotypes, immunological patterns, viral dynamics progression and clinical evolution of disease. With antiretroviral therapy becoming more widely available, HIV resistance identification and monitoring of disease remains of great importance in infected children. The major HIV-1 infection markers usually used for monitoring viral infection and disease course are CD4+ T cell counts or percentages and HIV viral load. Both of them are helpful indicating when to start therapy and evaluating its efficacy. Also, their association with genotyping tests identifying viral resistant mutations may help clinicians for the most adequate clinical conduct. In the present study, we assessed HIV-1 viral load and CD4+ and CD8+ T lymphocyte rates for the immunological status evaluation of 25 antiretroviral-treated children or at the beginning of therapy, managing therapeutic regimens according to genotyping results. The management of highly active antiretroviral therapy (HAART) according to viral resistance in our group of pediatric patients allowed an increase in CD4+ T cell counts and/or percentage in almost all children, showing an improvement in their HIV-associated immunodeficiency status. Important viral burden declines were observed in 24 children, most of them multi-drug resistant, with HIV RNA undetectable levels reached in 12 of them. In particular, HAART introduction allowed a more significant viral load reduction for those pediatric patients who were drug treatment-naïve, initiating antiretroviral therapy as they were enrolled at this study.

INTRODUCTION

Human Immunodeficiency Virus type 1 infection occurs as a dynamic process showing a continuous rate of viral particles production and clearance and is characterized by CD4+ T cell depletion with progressive immune dysfunction (Ho et al. 1995, Saag et al. 1991). The association of this process with HIV-1 high genomic mutation rate (it has been estimated one viral mutation for each virus replication cycle) results in viral mutants referred as quasispecies, which are able to escape from effectors immune mechanisms. Also, they may present mutations in genes coding for protease and reverse transcriptase enzymes leading to antiretroviral drug resistance (St. Clair et al. 1991, Molla et al. 1996).

Studies related to HIV-infected children are of most importance as they present special immunological patterns, showing extremely high viral loads and rapid evolution to AIDS in case of no therapy. In vertically infected children, there is a distinct viral dynamics progression compared with the pattern observed in infected adults. Primary viremia usually takes place early in life, when the immunological system is not fully competent, and viremia peaks can be reported as soon as at two months of life reaching very high levels, frequently over 1,000,000 HIV RNA copies/mL, and decrease only slowly over time. (Shearer et al. 1997, Melvin et al. 1999) Decline rates are reported even in the absence of therapy and are estimated to occur at 0.6 log₁₀/year in the first two years of life and at 0.3 log₁₀/year up to five years of life.

The major HIV-1 infection markers usually used for monitoring viral infection and disease course are CD4+ T cell counts and viral load. CD4 cell counts are used to assess immune status, susceptibility to opportunistic infections, need for antiretroviral therapy and opportunistic infection prophylaxis, and for defining AIDS. The plasma HIV-1 RNA viral load estimates the damage to the immune system. Both of them are

helpful indicating not only when to start antiretroviral therapy but also in the therapy efficacy evaluation.

A more consistent monitoring of disease progression in infants and children under 5 years old is the percentage of CD4+ cells as it tends to show less age-related variability than CD4+ cell counts. It has been found that in all ages a CD4+ cell percentage less than 15% poses a greater risk of disease progression and death (Palumbo et al. 1998). However, it is important to keep in mind that both the CD4+ cell percentage and the viral load value must be considered together to improve the ability to predict disease progression and death. Confirmed laboratorial results of viral load declines of are of important clinical and biological meanings if were observed alterations higher than five times ($0.7\log_{10}$) for children below two years old and at least three times ($0.5\log_{10}$) for those above this age (Ministério da Saúde 2007).

HIV drug-resistant strains emergence is related to therapeutic failure and has impaired the management of antiretroviral treatment possibilities. In this matter, genotyping test is of undeniable meaning, helping clinicians for the most adequate clinical conduct. Its association with viral load levels, CD4+ T cell rate, and clinical parameters may determine important therapeutic alterations for keeping or improving clinical and immunological patient conditions.

The aim of this study was to evaluate the values of HIV-1 viral load and CD4+ and CD8+ T lymphocytes related to therapy according to genotyping test results, as a parameter of immunological status evaluation in HIV-infected children on a long-term antiretroviral therapy or initiating it.

MATERIAL AND METHODS

Study subjects.

Twenty-five HIV-1 infected children were enrolled in this open label, uncontrolled study. They were regularly followed-up and received care at the Materno-Infantil Department, Hospital Universitário Pedro Ernesto, Rio de Janeiro State University, Brazil. Twelve children were male and 13 female, aging 1 to 15 years old. All of them were HIV vertically infected and were classified in AIDS-defining clinical categories according to Centers for Disease Control and Prevention (CDC 1994) criteria adaptation and laboratorial parameters. Inclusion criteria were to be under antiretroviral therapy or initiating it and presenting plasma viral loads preferentially equal or higher than 1.000 HIV RNA copies/mL. Parents or legal guardians gave written informed consent prior to enrollment in the study, which was approved by the Institutional Ethical Committee.

Clinical categories.

Among the 25 children enrolled at this study, 12 were classified in the C3 clinical category, 2 were classified in the C2 category and the remaining 2 were classified in the C1 category indicating, in all of them, the presence of severe clinical signals and/or symptoms with, respectively, severe immunological alteration, moderate immunological alteration and no immunological alteration. Three children were classified in the B1 clinical category, three in the B2 category and one child was in the B3 category, presenting moderate clinical signals and/or symptoms. One from the remaining two children was classified in the A2 category showing mild clinical signals and/or symptoms and a moderate immunological alteration and the other child was classified in the N3 clinical category, without important clinical signals or symptoms but showing a severe immunological alteration.

Antiretroviral therapy.

21 children were on a long-term antiretroviral therapy before genotyping test resistance evaluation. Therapeutic regimens included different possibilities: the association of two drugs, AZT + ddI or AZT + 3TC was given to three children. In the remaining patients, the highly active antiretroviral therapy (HAART) was given according to the following association: 1) AZT, 3TC associated with NFV or ABC or RTV or EFV or NVP or Kaletra (LPV+RTV); 2) AZT, ddI associated with NVP or Kaletra; 3) AZT, TDF, 3TC, RTV; 4) 3TC, d4T associated with APV or NFV or Kaletra; 5) 3TC, ABC, Kaletra. Four children had their HAART treatment initiated at the time of their inclusion in the study.

Viral load and CD4, CD8 measurements.

Five-milliliter blood samples were collected from each patient at the Central Laboratory of Hospital Universitário Pedro Ernesto for plasma viral load and T lymphocyte subsets determination. Plasma viral loads were measured using either commercial kits, the NucliSens HIV-1 QT (NASBA, bioMerieux Inc., Durham, North Carolina) with a lower limit of quantification at 80 copies of RNA/mL or the Quantiplex HIV-1 RNA 3.0 Assay (bDNA, Bayer Diagnostics, Walpole, Massachusetts) with a lower limit of quantification at 50 copies of RNA/mL, according to manufacturer's instructions. T cell lymphocyte subpopulations were stained for flow cytometry quantification of CD4+ and CD8+ cells performed in the FACSCalibur, Becton-Dickinson (FACSCount™) cytometer, as previously described (de Wolf et al. 1988).

HIV-1 genotyping.

Patients' plasma samples presenting HIV-1 viral load values lower than 1,000 copies/mL were previously concentrated by centrifugation at 15,000 rpm 30 minutes

and then were processed as follows: HIV RNA was extracted using commercial kit (QIAamp Viral RNA purification, QIAGEN Inc., Valencia, CA). Viral RNA was retrotranscribed in cDNA and amplified by a single-tube rt-PCR using commercial HIV-1 genotyping kit (TruGene™, Bayer HealthCare, Tarrytown, NY) resulting in a 1.3 kb amplicon covering HIV-1 protease gene (codons 1-99) and the major part of HIV-1 reverse transcriptase gene (codons 39-244). Sequencing reactions were generated from the amplified cDNA by CLIP™ sequencing (OpenGene™, Bayer HealthCare) under thermal cycling (94°C 5 min, 30 cycles 94°C 20 s, 56°C 20 s, 70°C 1 min 30 s, 1 cycle 70°C 5 min). Each sequencing reaction was loaded on an automated DNA sampler and run on 6% acrylamide gel electrophoresis (MicroGene Clipper™, Bayer HealthCare). The assays were base called with GeneObjects (GeneObjects, Bayer HealthCare), aligned and assembled together with GeneLibrarian (GeneLibrarian, Bayer HealthCare). The sequences were compared to a database of wild type HIV-1 sequences (HxB2, GenBank K03455) to find out which mutations were present in the HIV-1 RNA. The classification of mutations associated with decreased drug sensitivity was established according to the consensus statement on antiretroviral drug resistance testing.

RESULTS

HIV-1 genotyping and viral resistance.

All children included in this study presented antiretroviral resistance mutations and/or polymorphisms in HIV-1 reverse transcriptase and protease genes. Important mutations conferring viral resistance to drugs included in the therapeutic regimen of each child were reported in 18 from the 25 children. Among the seven children presenting no viral resistance, four children were drug naive (numbers 18, 19, 21 and

30) and reported no relevant mutations, and in the remaining three, on antiretroviral treatment, reverse transcriptase and protease polymorphisms were detected showing no evidence of viral resistance. The major polymorphisms detected were F77L and V118I in the reverse transcriptase gene, and M36I, L63P, A71V, V77I, and L89M in the protease gene.

Nucleoside analogue reverse transcriptase inhibitors (NRTIs) and non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs) resistance mutations and the viral resistance observed in all children are indicated in table 1. Viral resistance to protease inhibitors (PIs) and viral resistance mutations are indicated in table 2.

The management of therapy was done according to genotyping test results, with therapeutic regimen alterations done in 16 from the 18 antiretroviral-resistant children, while on the period of study observation. Therapeutic regimen modification was also done for one child among the seven who showed no antiretroviral resistance evidence.

Table 1. HIV-1 reverse transcriptase gene resistance mutations and nucleoside analogue and non nucleoside analogue reverse transcriptase viral resistance.

Child	RT resistance mutations	NRTIs and NNRTIs resistance (possible resistance)
1	M41L, D67N, K70R, V118I, T215F, K219Q	AZT, ddI, ddC, d4T, ABC, TDF
2	M41L, D67N, V118I/V, T215Y	AZT, (ddI), (ddC), d4T, (ABC), (TDF)
3	M184V	(ddC), 3TC/FTC
5	M41L, E44D, M184V, T215Y	(AZT), (ddC), 3TC/FTC, (d4T), ABC, (TDF)
6	A62V, K65R, M184V, T215Y	(AZT), (ddI), ddC, 3TC/FTC, (d4T), ABC, TDF
7	M41L, E44D, D67N, M184V, L210W, T215Y	(AZT), ddI, ddC, 3TC/FTC, (d4T), ABC, TDF
8	M41L, E44D, D67N, T69D, K70R, A98G, M184V, T215F, K219Q	AZT, ddI, ddC, 3TC/FTC, d4T, ABC, TDF, (NVP), (DLV), (EFV)
9	M41L, E44D, D67N, T69D, V118I, M184V, L210W, T215Y	(AZT), ddI, ddC, 3TC/FTC, (d4T), ABC, TDF
10	M41L, D67N, K103N, M184V, H208Y	(AZT), (ddC), 3TC/FTC, (d4T), (ABC), (TDF), NVP, DLV, EFV
11	M41L, E44D, D67N, T69D/N, M184V, T215Y	(AZT), ddI, ddC, 3TC/FTC, (d4T), ABC, (TDF)
12	F77L	No evidence of resistance
13	V118I	No evidence of resistance
14	M41L, K70R, V75M, F77L, K101E, V118I, M184V, G190A, T215F, K219Q	(AZT), ddI, ddC, 3TC/FTC, d4T, (ABC), TDF, NVP, (DLV), (EFV)
15	M184V	3TC/FTC
17	M41L, M184V, T215Y	(AZT), (ddI), (ddC), 3TC/FTC, (d4T), ABC, (TDF)
18	No relevant mutations detected	No evidence of resistance
19	No relevant mutations detected	No evidence of resistance
20	D67N, K101E, G190A, K219Q	(AZT), (d4T), NVP, (DLV), (EFV)
21	No relevant mutations detected	No evidence of resistance
23	A62V, K70R, A98G, V118I, M184V, H208Y, T215C/F, K219Q	(AZT), 3TC/FTC, (d4T), (DLV)
24	M41L, K103N, M184V, T215Y	(AZT), (ddI), 3TC/FTC, (d4T), (ABC), (TDF), NVP, DLV, EFV
25	M41L, T69N, K70R, K101E, V106A, G190A, T215F, K219Q	AZT, ddI, d4T, TDF, NVP, DLV, (EFV)
26	No relevant mutations detected	No evidence of resistance
27	M41L, D67N, V118I, L210W, T215Y	AZT, ddI, d4T, (ABC), TDF
30	No relevant mutations detected	No evidence of resistance

Table 2. HIV-1 protease gene resistance mutations and viral resistance to protease inhibitors.

Child	PR resistance mutations	PIs resistance (possible resistance)
1	L63P	No evidence of resistance
2	L10F, D30N, L33F, M36L, N88D	NFV, (ATV)
3	L10I, M36I, L63P, L90M	SQV, (IDV), (RTV), NFV, (ATV)
5	L10F, D30N, M36V, L63P	NFV, (ATV)
6	L10I, K20R, D30N, M36V, L63P, A71V, N88D	NFV, ATV
7	D30N, L33F, I54L, L63P, A71T, I84V, N88D, L90M	SQV, IDV, RTV, NFV, APV/FPV, (LPV/r), ATV
8	L63P	No evidence of resistance except for ATV: insufficient evidence
9	G16E, K20I, L33V, M36I, M46I, G73S, L90M	SQV, (SQV/r), IDV, (IDV/r), RTV, NFV, APV/FPV, (APV/r or FPV/r), ATV
10	M36I, L63P	No evidence of resistance
11	L10V, M36I, M46I, I54V, L63P, V82S	SQV, (SQV/r), IDV, IDV/r, RTV, NFV, APV/FPV, APV/r or FPV/r, (LPV/r), ATV
12	M36I	No evidence of resistance
13	M36I, V77I, L89M	No evidence of resistance
14	L10I, K20I, M36I, M46I, I50V, I54V, L63P, A71V, V82A, L89I	SQV, SQV/r, IDV, IDV/r, RTV, NFV, APV/FPV, APV/r or FPV/r, LPV/r, ATV
15	L10I, I13V, D60E, L63P, A71V, G73S, V77I, L90M	SQV, (SQV/r), IDV, (IDV/r), RTV, NFV, (APV/FPV), (APV/r or FPV/r), (LPV/r), (ATV), (ATV/r)
17	K20I, M46I, L63P, T74S, V77I, L90M	SQV, (SQV/r), IDV, (IDV/r), RTV, NFV, APV/FPV, (APV/r) or (FPV/r), (ATV)
18	L63P, A71V, V77I	No evidence of resistance
19	L63P	No evidence of resistance
20	K20R, D60E, L63P	No evidence of resistance
21	M36I, L89M	No evidence of resistance
23	I13V, L24I, L33F, M46L, I54V, L63P, V77I, V82A, L89I	SQV, (SQV/r), IDV, IDV/r, RTV, NFV, APV/FPV, APV/r or FPV/r, LPV/r, ATV, ATV/r, (TPV/r)
24	L63P	No evidence of resistance
25	L63P, A71T	No evidence of resistance
26	L63P	No evidence of resistance
27	M46L, A71V	IDV, (IDV/r), (RTV), APV/FPV, (APV/r or FPV/r)
30	M36I, L63P	No evidence of resistance

Viral load, CD4 and CD8 measurements and therapy.

HIV-1 viral load determinations and CD4+ and CD8+ T cell measurements were done for each child at varied intervals, during different followed-up periods ranging from 9 months to 33 months (average 17.4 months), except for one child without return to the clinical visits, followed-up only for 3 months. Laboratorial data evaluation for viral load and T lymphocyte subsets, referred to child's inclusion in the study, were defined as those most close to blood drawn date for genotyping test evaluation.

In order to normalize the data comparison, viral load drop evaluation was calculated in a logarithmic scale, considering the difference between viral load initial value, equivalent to the child inclusion in the study, and the consecutive value registered after the antiretroviral regimen modification. Adopted significant differences were those equal to $0.7 \log_{10}$ for pediatric patients aged up to two years old and $0.5 \log_{10}$ for those older than two years old.

The clinical classification for each child enrolled at this study was considered that related to the clinician evaluation done at the child inclusion date, irrespectively to its clinical course evolution. This classification, the viral load, CD4 and CD8 rates and the therapeutic regimen modifications are indicated in table 3. The evaluation of these same clinical and laboratorial data for the group of children with no therapeutic alterations is indicated in table 4.

Table 3. Clinical and laboratorial data from children on HAART therapy presenting drug regimen modification.

Child	Age	Clinical category	Therapy		Viral load		CD4	CD8	CD4/CD8	
			Date	Regimen	Date	Copies/mL	Log	Cells/ μ L (%)	Cells/ μ L (%)	CD8
1	6y	C3	2005 Feb	AZT, ddI	2005 Apr	5,300	3.724	435 (12.5)	2,226 (63.97)	0.19
			2005 Aug	AZT, 3TC, NVP	2005 Sep	<80	<1.903	714 (22.04)	1,808 (55.8)	0.39
			2006 Feb	AZT, 3TC, NVP	2006 Feb	3,100	3.491	532 (24.4)	1,049 (48.12)	0.50
			2006 Jul	AZT, 3TC, NVP	2006 Jun	4,500	3.653	577 (17.07)	2,016 (59.64)	0.28
			2006 Oct	AZT, 3TC, NVP	2006 Set	3,500	3.544	509	1,536	0.33
2	12y	C3	2005 Apr	AZT, 3TC, Kaletra	2005 May	83,000	4.919	547 (26.68)	1,155 (56.34)	0.47
			2005 Jul	AZT, 3TC, Kaletra	2005 Jul	270,000	5.431	307 (23.0)	422 (32.0)	0.72
			2005 Sep	3TC, Kaletra, EFV	2005 Aug	140,000	5.146	424 (25.54)	753 (45.36)	0.56
			2005 Dec	3TC, Kaletra, EFV	2005 Dec	1,300	3.114	480 (25.53)	1,022 (54.36)	0.46
			2006 Mar	3TC, Kaletra, EFV	2006 Mar	1,900	3.279	568 (26.54)	865 (40.42)	0.65
			2006 Jul	3TC, Kaletra, EFV	2006 Jul	1,300	3.114	580 (32.22)	668 (37.11)	0.86
			2007 May	3TC, Kaletra, EFV	2007 Apr	19,757	4.296	543	1,107	0.49
			2007 Oct	3TC, Kaletra, EFV	2007 Jun	80,891	4.908	652	1,644	0.40
3	10y	C3	2005 Mai	AZT, 3TC, NFV	2005 Feb	530,000	5.724	78 (4.02)	1,299 (66.96)	0.06
			2005 Jul	ABC, 3TC, Kaletra	2005 Aug	3,400	3.531	165 (6.6)	1,741 (69.64)	0.09
			2006 Jan	ABC, AZT, Kaletra	2006 Feb	73,000	4.863	457 (13.68)	2,359 (70.63)	0.19
			2006 Mai	ABC, AZT, Kaletra	2006 Jun	190,000	5.279	376 (16.35)	1,516 (65.91)	0.24
5	11y	C3	2005 Aug	AZT, 3TC, NFV	2005 Aug	24,000	4.380	485 (18.0)	1,826 (69.0)	0.26
			2005 Oct	AZT, 3TC, NFV	2005 Oct	14,000	4.146	596 (23.0)	1,602 (63.0)	0.37
			2005 Dec	AZT, 3TC, NFV	2005 Dec	19,000	4.278	439 (17.0)	1,626 (66.0)	0.26
			2006 Mar	AZT, ddI, Kaletra	2006 Apr	110	2.041	567 (20.25)	1,758 (62.79)	0.32
			2006 Aug	AZT, ddI, Kaletra	2006 Sep	580	2.763	711	2,036	0.34
			2007 Sep	AZT, 3TC, Kaletra	2007 Aug	183	2.262	864 (30.0)	877 (47.0)	0.99
			2007 Nov	AZT, 3TC, Kaletra	2007 Oct	81	1.908	651 (27.0)	1,199 (51.0)	0.54
			2008 Mar	AZT, 3TC, Kaletra	2008 Mai	<50	<1.698	708 (24.0)	1,565 (54.0)	0.45
6	4y	C3	2005 Oct	3TC, d4T, NFV	2005 Sep	22,000	4.342	787 (38.0)	899 (43.0)	0.87
			2006 Mar	3TC, d4T, NFV	2006 Jan	20,000	4.301	895 (32.0)	1,318 (47.0)	0.67
			2006 Aug	3TC, d4T, Kaletra	2006 Mar	21,000	4.322	1,081 (38.2)	1,262 (44.59)	0.85
			2006 Oct	3TC, d4T, Kaletra	2006 Oct	<80	<1.903	1,132	1,554	0.72

Table 3. Clinical and laboratorial data from children on HAART therapy presenting drug regimen modification (continuation).

Child	Age	Clinical category	Therapy		Viral load		CD4	CD8	CD4/	
			Date	Regimen	Date	Copies/mL	Log	Cells/ μ L (%)	Cells/ μ L (%)	CD8
8	10y	B1	2005 Dec	AZT, 3TC, ABC	2005 Aug	18,000	4.255	667 (37.0)	783 (44.0)	0.85
			2006 Jan	AZT, 3TC, ABC	2005 Nov	16,000	4.204	417 (25.74)	518 (31.98)	0.80
			2006 Mar	AZT, 3TC, ABC	2006 Feb	11,000	4.041	628 (29.9)	935 (44.52)	0.67
			2006 Aug	AZT, 3TC, Kaletra	2006 Jun	<80	<1.903	1,074	1,372	0.78
			2006 Oct	AZT, 3TC, Kaletra	2006 Sep	<80	<1.903	979	1,144	0.85
9	10y	C3	2005 Dec	AZT, 3TC, NFV	2005 Sep	2,000	3.301	474 (22.0)	1,205 (57.0)	0.39
			2006 Feb	AZT, 3TC, NFV	2006 Jan	1,400	3.146	452 (21.0)	1,147 (54.0)	0.39
			2006 Jun	AZT, 3TC, Kaletra	2006 Jun	<80	<1.903	531	1,423	0.37
			2006 Aug	AZT, 3TC, Kaletra	2006 Aug	140	2.146	520 (19.26)	1,514 (56.07)	0.34
			2006 Oct	AZT, 3TC, Kaletra	2006 Oct	100	2.000	624	1,381	0.45
10	7y	B3	2005 Dec	AZT, 3TC, ABC	2005 Nov	5,400	3.732	902 (38.55)	1,160 (49.57)	0.77
			2006 Apr	AZT, 3TC, ABC	2006 Feb	5,700	3.756	861 (35.88)	1,058 (44.08)	0.81
			2006 Jun	AZT, 3TC, ABC	2006 Mar	12,000	4.079	941 (35.51)	1,274 (48.08)	0.73
			2006 Oct	AZT, 3TC, ABC	2006 Aug	15,000	4.176	851 (38.68)	965 (43.86)	0.88
			2007 Jan	AZT, 3TC, Kaletra	2007 Mai	<50	<1.698	1,107	1,209	0.92
			2007 Aug	AZT, 3TC, Kaletra	2007 Aug	<50	<1.698	1,232	2,335	0.53
			2007 Dec	AZT, 3TC, Kaletra	2007 Dec	<50	<1.698	1,502 (42.0)	1,080 (34.0)	1.39
			2008 Apr	AZT, 3TC, Kaletra	2008 Apr	81	1.908	1,560 (44.0)	1,108 (33.0)	1.41
13	5y	A2	2006 Jun	AZT, 3TC	2006 Feb	4,300	3.633	1,419 (47.78)	952 (32.05)	1.49
			2006 Oct	AZT, 3TC	2006 Sep	1,200	3.079	703	640	1.10
			2007 Jul	AZT, 3TC, EFV	2007 Aug	<50	<1.698	1,097 (51.0)	535 (28.0)	2.05
14	8y	C3	2005 Aug	EFV, TDF, 3TC, RTV	2005 Oct	620,000	5.792	347 (16.0)	1,265 (61.0)	0.29
			2005 Dec	AZT, TDF, 3TC, RTV	2006 Jul	72,000	4.857	40 (4.44)	541 (60.11)	0.07
			2006 Oct	TDF, 3TC, Kaletra	2006 Sep	190,000	5.279	43	949	0.05
15	10y	C2	2006 Mar	AZT, 3TC, NFV	2006 Mar	13,000	4.114	578 (30.26)	935 (48.95)	0.61
			2006 Jul	AZT, 3TC, NFV	2006 Jun	9,000	3.954	415 (24.85)	1,011 (60.54)	0.41
			2006 Nov	AZT, 3TC, NFV	2006 Dec	9,000	3.954	509	1,169	0.44
			2007 Mar	AZT, 3TC, Kaletra	2007 Apr	<50	<1.698	451	977	0.46
			2007 Jul	AZT, 3TC, Kaletra	2007 Sep	<50	<1.698	656 (26.0)	818 (42.0)	0.80

Table 3. Clinical and laboratorial data from children on HAART therapy presenting drug regimen modification (continuation).

Child	Age	Clinical category	Therapy		Viral load		CD4 Cells/ μ L (%)	CD8 Cells/ μ L (%)	CD4/ CD8	
			Date	Regimen	Date	Copies/mL				Log
17	4y	C1	2006 Mar	3TC, d4T, NFV	2006 Feb	220,000	5.342	939 (18.48)	2,031 (39.98)	0.46
			2006 Aug	3TC, d4T, NFV	2006 Sep	100,000	5.000	1,086	2,358	0.46
			2007 Feb	3TC, d4T, NFV	2007 Mar	30,234	4.480	781	2,775	0.28
			2007 Apr	3TC, d4T, NFV	2007 May	212,779	5.328	829	1,785	0.46
			2007 Oct	AZT, 3TC, EFV	2008 Feb	44,564	4.649	678 (16.0)	2,672 (63.0)	0.25
20	11y	N3	2006 Aug	AZT, 3TC, EFV	2006 Apr	57,000	4.756	473 (11.68)	3,180 (78.52)	0.15
			2006 Nov	AZT, 3TC, EFV	2006 May	60,000	4.778	464	3,201	0.14
			2007 Aug	AZT, 3TC, Kaletra	2007 Aug	105,708	5.024	264 (12.0)	1,400 (63.0)	0.19
			2008 Feb	AZT, 3TC, Kaletra	2008 Jan	38,359	4.584	468 (14.0)	2,249 (67.0)	0.21
23	12y	C2	2007 May	AZT, 3TC, RTV	2007 May	3,569	3.553	962	1,642	0.59
			2007 Sep	TDF, 3TC, NFV	2007 Aug	<50	<1.698	877 (32.0)	960 (38.0)	0.91
			2007 Dec	AZT, 3TC, RTV	2008 Apr	253	2.403	1,504 (35.0)	1,728 (37.0)	0.87
			2008 Mar	TFV, 3TC, NFV	2008 Aug	-	-	1,074	1,184	0.91
24	10y	B2	2007 May	AZT, 3TC, NVP	2007 May	3,786	3.578	802	1,465	0.55
			2007 Sep	AZT, 3TC, Kaletra	2008 Feb	<50	<1.698	1,308 (33.0)	1,693 (46.0)	0.77
			2008 Mar	AZT, 3TC, Kaletra	2008 Jul	-	-	1,158	1,604	0.72
25	4y	B2	2007 Jun	AZT, ddI, NVP	2007 Jul	5,036	3.702	650 (34.0)	1,097 (57.0)	0.59
			2007 Sep	ABC, 3TC, Kaletra	2007 Nov	< 50	<1.698	635 (26.0)	972 (35.0)	0.65
			2008 Feb	ABC, 3TC, Kaletra	2008 Jul	-	-	774 (36.83)	607 (30.06)	1.28
27	6y	B1	2007 Aug	AZT, ddI	2007 Aug	1,960	3.292	944	1,281	0.74
			2007 Oct	AZT, ddI	2007 Nov	<50	<1.698	785 (29.0)	1,559 (40.0)	0.50
			2007 Dec	AZT, 3TC, EFV	2008 May	<50	<1.698	1,083 (30.0)	1,649 (39.0)	0.66

Table 4. Clinical and laboratorial data from children on HAART therapy presenting no drug regimen modification.

Child	Age	Clinical category	Therapy		Viral load		CD4	CD8	CD4/CD8	
			Date	Regimen	Date	Copies/mL	Log	Cells/ μ L (%)	Cells/ μ L (%)	CD8
7	8y	C3	2005 Oct	d4T, 3TC, APV	2005 Jul	38,000	4.580	1,053 (22.4)	2,607 (55.47)	0.40
			2005 Dec	d4T, 3TC, APV	2005 Nov	34,000	4.531	1,061 (23.0)	2,624 (58.0)	0.40
			2006 Mar	d4T, 3TC, APV	2006 Feb	24,000	4.380	1,228 (22.78)	2,958 (54.88)	0.41
			2006 May	d4T, 3TC, APV	2006 Jul	17,000	4.230	1,466 (25.72)	3,134 (54.98)	0.46
11	10y	C3	2005 Dec	AZT, 3TC, RTV	2005 Oct	1,500	3.176	546 (26.25)	1,076 (51.73)	0.50
			2006 Apr	AZT, 3TC, RTV	2006 Feb	11,000	4.041	499 (20.28)	1,541 (62.64)	0.32
			2006 Oct	AZT, 3TC, RTV	2006 Sep	20,000	4.301	413	1,122	0.37
			2007 May	AZT, 3TC, RTV	2007 May	15,134	4.180	523	1,472	0.36
			2007 Jul	AZT, 3TC, RTV	2007 Jul	49,220	4.692	423	1,033	0.41
12	5y	C1	2006 Feb	AZT, 3TC, NFV	2006 Feb	8,700	3.940	1,343 (49.38)	982 (36.10)	1.37
			2006 Aug	AZT, 3TC, NFV	2006 Set	2,400	3.380	967	961	1.01
			2007 Jun	AZT, 3TC, NFV	2007 Aug	<50	<1.698	1,084 (53.0)	551 (25.0)	1.97
18	12mo	C3	2006 May	AZT, ddI, Kaletra	2006 Mar	2,000,000	6.301	435 (10.56)	3,001 (72.84)	0.14
			2006 Jun	AZT, ddI, Kaletra	2006 Jun	160,000	5.204	958	4,411	0.21
19	2y	C3	2006 Jun	AZT, ddI, NVP	2006 May	830,000	5.919	171	2,428	0.07
			2006 Aug	AZT, ddI, NVP	2006 Jul	99	1.996	563 (9.71)	2,933 (50.57)	0.19
			2006 Nov	AZT, ddI, NVP	2006 Aug	570	2.756	591 (12.85)	2,517 (54.72)	0.23
			2007 Feb	AZT, ddI, NVP	2007 Jan	<50	<1.698	969	1,573	0.62
			2007 Apr	AZT, ddI, NVP	2007 May	<50	<1.698	856	1,724	0.50
			2007 Sep	AZT, ddI, NVP	2007 Aug	<50	<1.698	1,397 (15.0)	2,807 (40.0)	0.50
			2007 Nov	AZT, ddI, NVP	2007 Dec	<50	<1.698	658 (15.0)	1,628 (35.0)	0.40
21	5y	B2	2006 Oct	No drugs	2006 Jun	<80	<1.903	353 (14.12)	1,294 (51.76)	0.27
			2007 Jan	No drugs	2006 Jul	350	2.544	585 (17.21)	2,379 (69.97)	0.25
			2007 Jun	AZT, 3TC, EFV	2007 May	<50	<1.698	326	1,129	0.29
			2007 Oct	AZT, 3TC, EFV	2007 Jul	<50	<1.698	298 (16.0)	591 (44.0)	0.50
			2008 Feb	AZT, 3TC, EFV	2008 Feb	-	-	367 (20.0)	695 (33.0)	0.53
26	16y	C3	2007 Aug	AZT, 3TC, Kaletra	2007 Jul	313,000	5.495	140 (15.0)	538 (57.0)	0.26
			2007 Dec	AZT, 3TC, Kaletra	2008 Mar	33,400	4.520	108 (9.0)	-	-
			2008 Aug	AZT, 3TC, Kaletra	2008 Aug	188,000	5.270	29 (7.0)	269 (64.0)	0.11
30	12mo	B1	2008 Jan	AZT, 3TC, NVP	2007 Aug	>500,000	>5.698	1,961 (27.0)	2,534 (32.0)	0.77
			2008 Feb	AZT, 3TC, NVP	2007 Oct	-	-	1,440 (18.0)	3,361 (39.0)	0.43
			2008 Apr	AZT, 3TC, NVP	2008 Jan	482	2.683	1,866 (32.0)	2,206 (38.0)	0.85
			2008 May	AZT, 3TC, NVP	2008 May	<50	<1.698	2,051 (42.0)	1,240 (28.0)	1.65

In the group of the 17 children with drug regimen alteration done according to genotyping results, the management of HAART resulted in viral load declines observed as soon as four weeks after the therapeutic modification. Among them, 16 showed significant values with viral load drops ranging from 0.513 to 2.439 \log_{10} (average 1.752 \log_{10}). The lowest rate registered in this group (0.513 \log_{10}) was reported in a C3 clinical category 8 years old child who presented unfavorable clinical evolution and died. This child presented an important multi-drug resistance profile, elevated RNA levels and very low CD4 counts, showing CD4/CD8 ratios as low as 0.05. One patient among the 17, classified at N3 clinical category and followed-up for a 20 months period, showed a minor viral load decline (0.172 \log_{10}), below the 0.5 \log_{10} limit. Twelve from the 16 children presented important viral load declines reaching RNA levels below the lower test limit, and two children presented viral load rebounds to the initial levels after they were receiving antiretroviral therapy lasting for more than 11 months.

Five children among 8 with no drug regimen alteration, showed viral load declines higher than 0.5 \log_{10} (average 2.481 \log_{10}) and one child presented a non-significant viral load drop (0.225 \log_{10}). Only polymorphisms or no relevant HIV-1 reverse transcriptase and protease mutations were detected in all the 6 children. The remaining 2 children, showing antiretroviral resistance, presented non-significant viral load declines (0.350 \log_{10}) or viral load rises with 1.516 \log_{10} RNA rate difference. In summary, we observed that initiating or keeping antiretroviral regimen allowed the viral load reduction in 7 among the 8 children included in this group. In addition, RNA levels reached low levels, below the lower test limit in 4 of them, while they were on the study observation period.

CD4+T lymphocytes and HIV-associated immunodeficiency.

CD4+ T lymphocyte values used for HIV-associated immunodeficiency classification showed that, irrespectively to the child age, 8 from 25 children presented CD4 percentages lower than 15%, characterizing severe immunodeficiency. Seven children reported these low rates at the time of their first laboratorial evaluation as they were enrolled in this study and CD4 decreasing percentages were observed in a child during the followed-up period. Therapeutic regimen modification or its maintenance promoted increases in CD4 percentages in 4 among the 8 children, and in the remaining 4 we observed the following results: an important CD4 drop reaching 4.44% was observed in the child who died; in another child, followed-up for a three month period, we could merely report a single CD4 percentage not allowing comparisons, and in 2 children we observed CD4 percentages lower than 15% for the entire followed-up period, coincident with elevated viral load levels.

In the remaining 17 children, CD4 evaluation was done according to the child age range, using CD4 percentages for children aging from 12 months to 59 months, and T CD4+/mL cell counts for those aging or older than 5 years old. Four children were included in the age group ranging from 12 months to 59 months, showing HIV-associated immunodeficiency level as follows: mild (1 child), advanced (1 child) and non-significant (2 children). In this group, alterations or the maintenance of the antiretroviral regimen resulted in CD4 percentage increases in the three children presenting mild or non-significant immunodeficiency levels, promoting the change of the mild level to non-significant one or the maintenance of the non-significant level during the entire followed-up study. The forth child, presenting CD4 percentages equal to 18.48 and 16.0, compatible with HIV-associated advanced immunodeficiency, showed only minimum decreases or raises in the viral load rates, with values ranging

from 4.480 log₁₀ to 5.342 log₁₀. In the 13 children group, aging or older than 5 years old, the HIV-associated immunodeficiency level distribution according to CD4 cell counts was seen as follows: advanced (1 child), mild (3 children), and non-significant (9 children). In the 4 children presenting advanced or mild immunodeficiency, alterations or the maintenance of the antiretroviral regimen resulted in a level change to the non-significant status, with an increase in the CD4 T cell counts. Seven children among the 9 who presented non-significant immunodeficiency level maintained the same immunodeficiency level, showing CD4 cell counts raises or only minor fluctuations. However, 2 children presenting HIV-associated non-significant immunodeficiency level had their immunodeficiency status changed to the mild level. One of them, to whom some antiretroviral regimen modification was done, showed viral load decreases reaching undetectable levels, and the second one who had no therapy regimen alterations, reported increasing HIV-1 viral loads.

Although CD4+ T lymphocyte counts or percentages had indicated in the majority of the children an improvement of their HIV-associated immunodeficiency level, CD8+ T lymphocyte counts constantly remained elevated or presented only minor fluctuations, rendering CD4/CD8 ratios lower than 1 in 20 from the 25 children included in this study.

DISCUSSION

The advent of highly active antiretroviral treatment (HAART) in the HIV-infected children and adults therapy has resulted in a significant longer life expectancy and an important delay in disease progression. (Gortmaker et al. 2001). The availability and use of HAART has promoted marked declines in mortality and morbidity rates reported in recent years (Gona et al. 2006; McConnell et al. 2005). However, it is hard

to reach complete virus suppression in HIV-infected people due to multiple factors such as poor adherence to medication, drug intolerability, drug interactions or at inadequate levels. The virological response strongly depends on the previous drug history, reflecting previous selection of drug-resistant viral mutants as a result of suboptimal therapy.

Despite initial promising results, lasting for several years in many children, treatment failure has been frequently reported requiring reassessment of treatment options. HIV genotypic resistance determinations may be helpful, monitoring drug resistance at the time of therapy failure or prior to therapy initiation (Hirsch et al. 2000). Information on patterns of resistance is important for decisions on how to combine drugs to achieve an optimum antiviral effect. In the present study, we observed an important decline of viral burden in 17 children, most of them multi-drug resistant, who had alternative therapeutic options based on HIV-1 resistance genotype test results. Undetectable levels were reached in 12 of them. In 7 from the remaining 8 children, with 6 showing no evidence of drug resistance, the introduction of HAART or keeping the previous antiretroviral therapy regimen allowed a significant reduction of viral load, mainly for those pediatric patients who were drug treatment naïve initiating therapy as they were enrolled at this study.

With regard to virus-specific CD4 T cells, it is well established their critical role in antiviral immunity, keeping effective cytotoxic T lymphocyte responses (Sherdlock & Shen 2003; Janssen et al. 2003). In untreated HIV-infected people, HIV infection develops with early and massive depletion of these cells (Wahren et al. 1987; Douek et al. 2002) which can contribute to the lack of effective immune control of virus replication and to the progression of HIV-associated disease. In pediatric HIV-infected patients under 5 years old, the percentage of CD4+ T cells lends to more consistent

monitoring of disease as it tends to show less age-related variability when compared to cell counts. So, definitions have been formulated based on child age for immune suppression providing guidance for the monitoring of disease progression.

The management of HAART according to viral resistance in our group of pediatric patients allowed an increase in CD4+ T cell counts and/or percentage in almost all children, showing an improvement in their HIV-associated immunodeficiency status. However, 20 of the 25 HIV-infected children presented elevated rates of CD8+T lymphocytes, irrespective of HAART success. It is well known that HIV infection influences CD8+ T cells, causing their immune activation and their cellular expansion (Paul et al. 2005). It is also established that this cell population present a constitutive antiviral capacity that could account for viral replication control in some rare HIV-infected adult individuals who have persistently undetectable plasma viral load in the absence of therapy, known as HIV controllers (Sáez-Cirión et al. 2007). HIV disease is characterized by chronic immune activation and lymphocyte activation is the hallmark of disease progression. (Giorgi et al. 1999) The persistence of viral load, even at undetectable or low ratios, can result in sustained levels of CD8+ T cells and it has been suggested that a state of chronic immune activation contributes to the exhaustion of immune functions and to the loss of CD4+ T lymphocytes, leading to disease progression (Derdeyn & Silvestri 2005).

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