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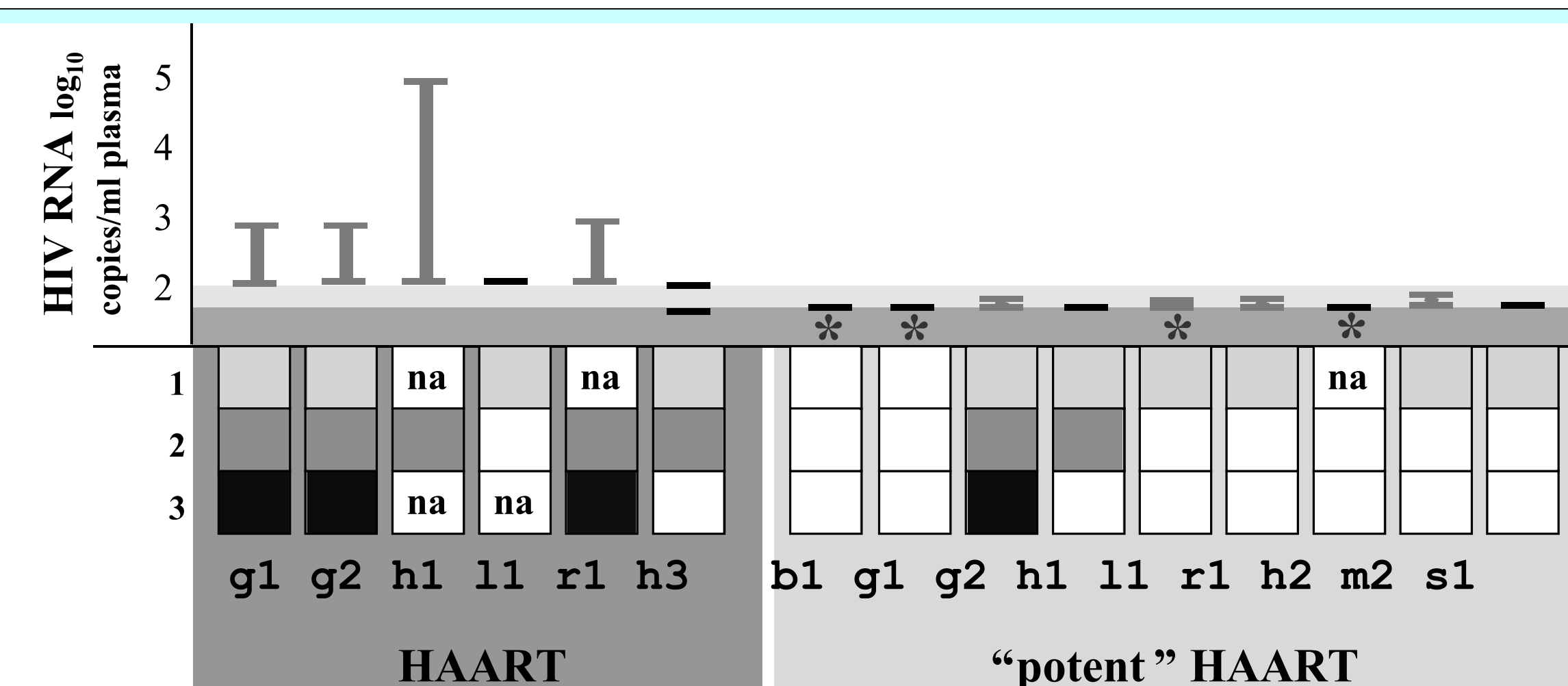
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**Objective:** To evaluate HIV-1 replication and selection of drug-resistant viruses during "effective" HAART, defined by plasma HIV-1 RNA levels below the limits of detection.

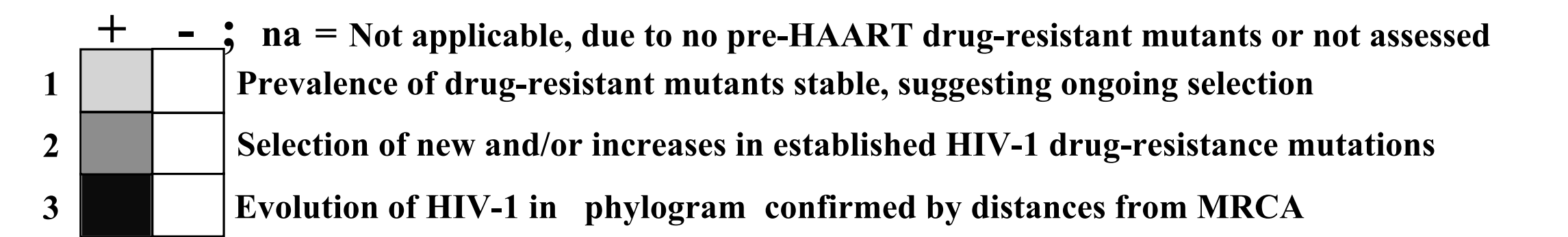
**Methods:** At intervals before and during a median of 4.5 years (range 1.8-5.8 yrs) of effective HAART, HIV-1 levels in CD4+ blood cells were quantified and multiple viral sequences were analyzed in ten nucleoside analog-experienced children. Ten or more PR, RT and env amplicons derived from specimens diluted to single viral molecule templates were directly sequenced. Phylogenetic relationships, distances from the most recent common ancestor (MRCA) of the quasispecies and changes in the frequency of drug-resistance mutations were assessed.

**Results:** The initial HAART regimen was with two classes of antiretrovirals (nucleoside reverse transcriptase inhibitors [NRTI] and one protease inhibitors [PI]) in six children. More potent HAART, with three classes of antiretrovirals or four drugs was given to nine children, five following an initial two class regimen. Rebound of plasma HIV-1 RNA to >500 copies/ml was observed in four, all during the less potent HAART, with selection of new resistance mutants observed in three. During the more potent HAART, the median plasma HIV-1 RNA was <50 copies/ml in all children—nevertheless, full cycles of viral replication were evident in most of these children, by viral evolution (n = 1), increased genetic distances from the MRCA (n = 1), and selection of drug-resistance mutants (n = 4). Lower absolute levels, and decreases of > 1 log<sub>10</sub> of HIV-1 DNA in CD4+ cells after a year of HAART occurred among children with less evidence of viral replication (P = 0.007). A statistically significant decrease in the genetic distance to the MRCA occurred in at least one viral gene during potent HAART of four children, indicating selective die-off of recently infected cells. Low-level plasma virus (<250 copies/ml) grouped phylogenetically with blood viruses found early in the course of infection of two children, suggesting that the persisting ancestral virus was not uniformly defective. However, plasma virus (<101 copies/ml) from one child also demonstrated selection of mutations associated with high-level drug resistance.

**Conclusions:** Evidence of continuing HIV-1 replication can be found in many children during "effective" HAART. An increasing frequency of specific drug-resistance mutants over time was a more sensitive indicator of ongoing viral replication than phylogenetic analysis. Rebound of plasma viremia was associated with less potent HAART. No to minimal evidence of viral replication was associated with a significant drop and lower absolute PBMC DNA levels after starting HAART, suggesting that HIV-1 DNA load might be a useful in monitoring residual low-level viral replication during HAART.



**I** Range of HIV-1 RNA in plasma  
 All values below limit of detection of HIV-1 RNA assay  
 \* > 1 log<sub>10</sub> decrease in PBMC DNA post-HAART



**Table 1. Summary of Subjects' Characteristics and HIV-1 Evolution**

Subject	Age at start of HAART (years old)	Years of HAART (Dates of HAART)	Decrease DNA load >1 log <sub>10</sub> from pre- to post-HAART	New drug-resistance mutations during HAART	Decrease (negative value indicates increase) in genetic distance from most recent common ancestor (MRC A) during HAART			Frequency of plasma HIV-1 RNA 50-250 (fold) one or more >250) copies/ml by year HAA RT								
					env	rt	pro	2-class HAART								
B1	5.9	4.0 (1/97-11/01)	+	-	0.009	0.800	0.070	0	0	0	0	0	0	0	0	0
G1	9.1	5.6 (4/96-11/01)	+	++	0.020	<0.001	0.030	1	0	1	0	0	0	0	0	0
G2	11.2	5.6 (4/96-11/01)	-	++	-0.001	-0.001	<-0.001	0	3	3	3	3	0	0	0	0
H1	1.7	3.5 (6/97-12/00)	-	-	0.444	0.860	0.090	3	1	0	0	0	0	0	0	0
H2	0.2	4.2 (9/97-11/01)	+	-	0.290	0.222	0.385	1	1	0	0	0	0	0	0	0
H3	14.7	3.6 (5/96-12/99)	-	-	0.110	-0.350	0.990	0	0	0	0	0	0	0	0	0
L1	6.2	4.8 (2/97-11/01)	-	-	0.040	0.640	0.120	0	1	0	0	0	0	0	0	0
M2	5.5	4.3 (7/97-11/01)	-	-	0.850	0.129	-0.056	0	1	1	0	0	0	0	0	0
R1	2.3	5.3 (8/96-11/01)	-	-	0.055	0.720	0.490	3	0	0	1	0	0	0	0	0
S1	3.0	1.8 (7/97-3/99)	-	-	0.690	0.440	0.740	1	0	0	0	0	0	0	0	0

^ = when taking <3 drugs; did not persist when changed to 4 drugs  
 - = last value, after change to lopinavir/ritonavir had > 1 log<sub>10</sub> drop, however not confirmed

**Figure 1. Summary of Virologic Study**  
 Subjects are group by intensity of HAART therapy. "HAART" signifies treatment with two classes of antiretrovirals, two nucleoside analogs and one protease inhibitor, except for l1 who received a 2-drug regimen of stavudine and zalcitabine. "Potent HAART" signifies treatment with three classes of drugs or a four-drug combination; most children received one or two nucleoside analogs, one or two protease inhibitors and in all but g1 a non-nucleoside analog. The range of plasma HIV-1 RNA levels is indicated as well as the lower limit of the assay used; a decrease in HIV-1 DNA in CD4+ cells of >1 log<sub>10</sub> within 1-2 years of HAART is indicated (\*); and findings on sequence analyses are indicated in the boxes.

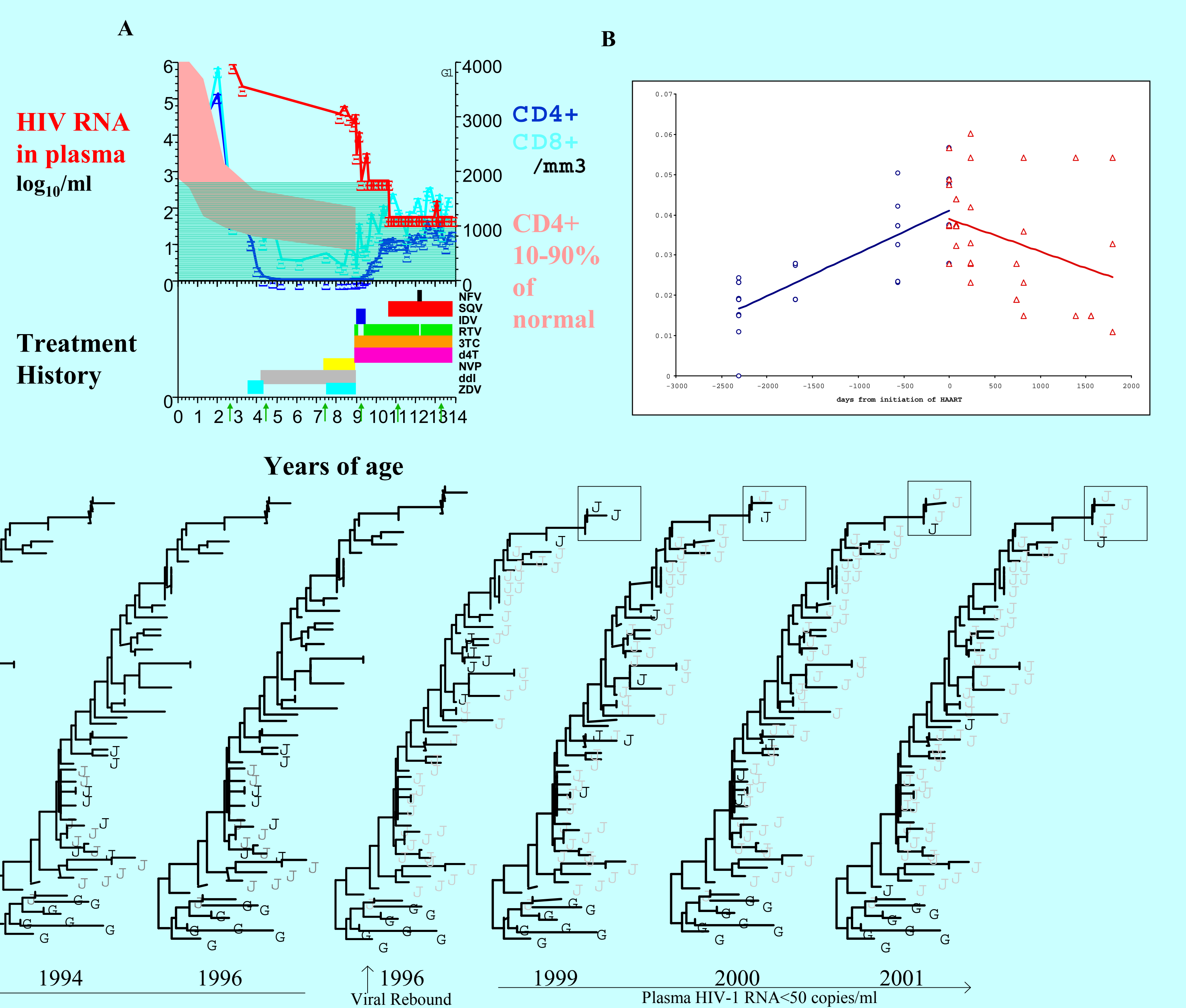
**Table 1. Summary of Subjects' Characteristics and HIV-1 evolution**  
 Plasma HIV-1 RNA levels were determined two to eight times per year after falling below the limit of detection. These values are depicted for each year of HAART. No sustained viral rebound was detected among subjects adhering to "potent" HAART. The change of distances to MRCA of sequences after HAART is also analyzed, a significant decrease in the distance to the MRCA occurred in at least one viral gene during HAART of four children, indicating selective die-off of recently infected cells.

**Conclusion:** Less viral evolution, less selection of drug-resistant mutants and fewer episodes of viremia were observed during potent HAART, and marked decreases of HIV-1 DNA in PBMC occurred among children with little or no genetic indicators of viral replication.

**Figure 3. Clinical History and Viral Sequence Analysis of Subject g1**

The clinical and laboratory course, including drug history, plasma HIV-1 RNA levels, CD4 and CD8 cell numbers, is shown in Panel A. The neighbor joining phylogenetic analysis for HIV-1 encoding protease (PR) is shown in Panel B, and genetic distances of these same sequences from the inferred most recent common ancestor (MRCA) in Panel C.

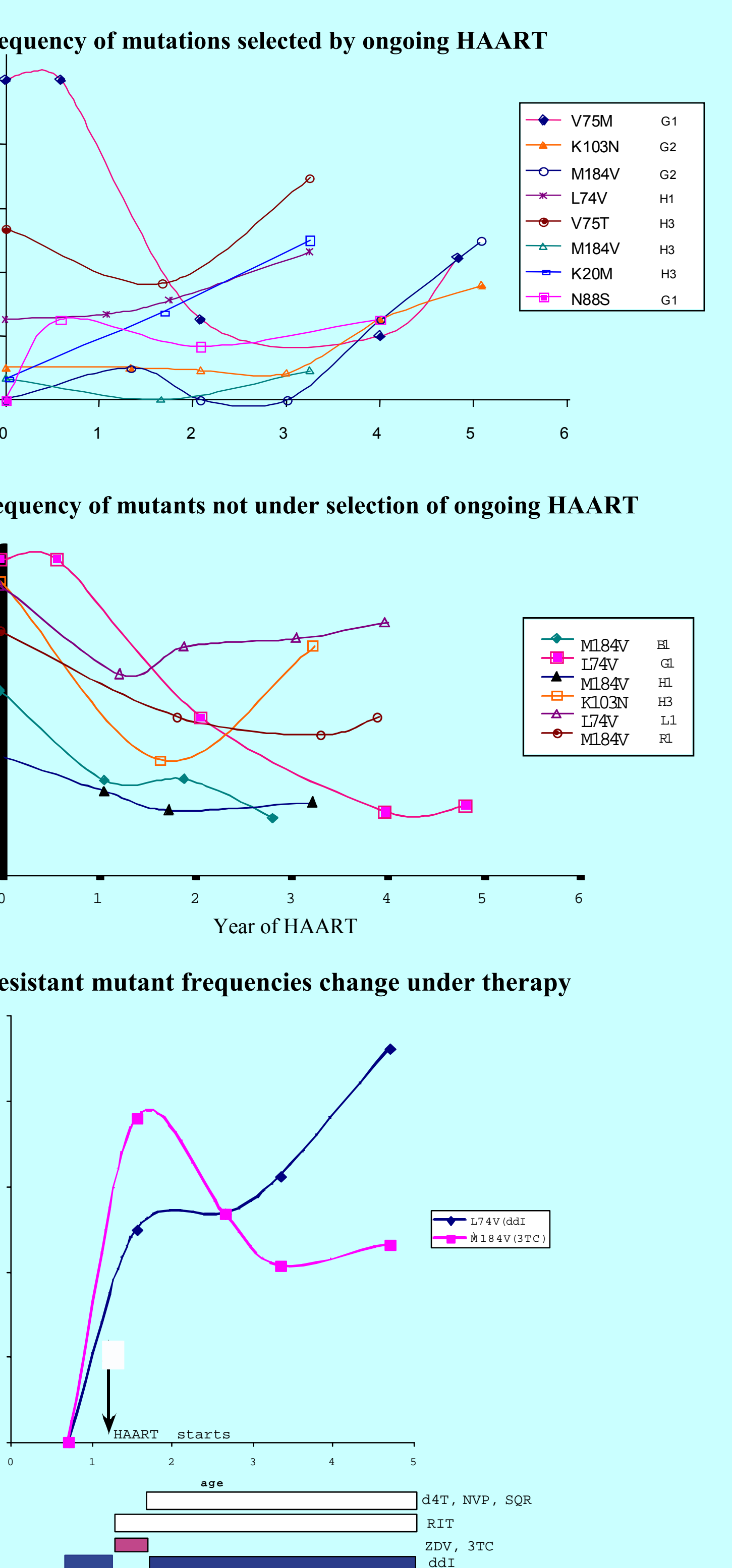
The pre-HAART specimens demonstrated time-ordered evolution. A mutant conferring resistance to protease inhibitors (N88S, shown in box) was selected during early HAART. Subsequently, there was a gradual loss of viral sequences that grouped with all but the earliest time-point, except for a few drug-resistant variants (N88S) that persisted but did not increase in prevalence.



**Figure 2. Frequency of Drug Resistant Mutants During HAART**

The proportions of drug-resistant mutants from children with plasma HIV-1 RNA levels <50 copies/ml are shown. Mutants in Panel A were selected at an increasing rate (P = 0.0001) by the ongoing HAART regimen, while mutants in Panel B were not selected and decreased in frequency (P = 0.0013). In Panel C data are shown from a single subject h1 who took lamivudine in the past but was studied during HAART including didanosine. The frequency of mutants varies over time, with the selection of the L74V mutation associated with resistance to didanosine and a decrease in the frequency of M184V associated with resistance to lamivudine. (The effective population size did not change much under HAART in all patients in this figure, data not shown)

**Conclusion:** Evolution occurs as a result of successive change in the kinds and frequencies of genes. Ongoing viral replication was detected in seven subjects by measuring the change in frequency of resistant variants under HAART. Detection of frequency shift of resistance with therapy was a more sensitive indicator of ongoing viral replication than phylogenetic analysis (evolution was only detected phylogenetically in two subjects).



**Figure 4. Clinical History and Viral Sequence Analyses of Subject g2**

The clinical and laboratory course, including drug history, plasma HIV-1 RNA levels, CD4 and CD8 cell numbers, is shown in Panel A. The neighbor joining phylogenetic analysis for HIV-1 encoding protease (PR) is shown in Panel B (circled symbols represent plasma viral sequences), and genetic distances of these same sequences from the inferred most recent common ancestor (MRCA) in Panel C.

Wild-type and drug-resistant viruses were detected in the plasma (circled symbols) after several years of HAART, plasma virus was progressively comprised of an increasing frequency of highly-drug resistant mutants. The substitution of lopinavir/ritonavir for zalcitabine / saquinavir after 4.8 years of HAART was associated with the cessation of low-level viremia, in addition, the HIV-1 DNA in CD4+ cells decreased by >1 log<sub>10</sub>.

