Effect of Human Immunodeficiency Virus (HIV) Type 1 Envelope Subtypes A and D on Disease Progression in a Large Cohort of HIV-1–Positive Persons in Uganda

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The effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression was investigated in 1045 adults in Uganda. At enrollment and every 6 months, a clinical history, examination, and laboratory investigations that included CD4 cell counts were done. HIV-1 envelope subtype was assessed mainly by peptide serology supplemented by heteroduplex mobility assay and DNA sequencing. A multivariate analysis of survival was performed to assess the prognostic value of HIV-1 subtype on death. A marginal general linear model also determined the effect of subtype on CD4 cell count during follow-up. Subtype D was associated with faster progression to death (relative risk, 1.29; 95% confidence interval, 1.07–1.56; $P = .009$) and with a lower CD4 cell count during follow-up ($P = .001$), compared with subtype A, after adjusting for CD4 cell count at enrollment. In Africa, envelope subtype D is associated with faster disease progression, compared with subtype A.

Human immunodeficiency virus (HIV) type 1 is diverse. To date, 10 different subtypes (A–J) have been described globally [1]. These subtypes differ from each other by at least 25%–35% of the amino acid sequences in the envelope proteins [2]. In Uganda, multiple HIV-1 subtypes have been described [3–16]; however, in large studies, subtypes A and D were the most prevalent [10, 12, 15]. Recombinant viruses and dual infections have also been reported [12, 14, 17], but it is unclear whether there are regional differences in the distribution of these subtypes in Uganda. Moreover, the dynamics of these subtypes over time has not been well described. Demographic characteristics of persons infected with the 2 principal HIV-1 subtypes, A and D, were similar in one study and did not differ significantly between early 1994 and late 1997 [15].

The biologic relevance of HIV-1 subtypes is of great interest—especially in relation to vaccine development, pathogenesis, and transmission [18–23]. Information on HIV-1 subtypes and disease progression is beginning to emerge. Two studies investigated the role of HIV-1 subtype and disease progression prospectively from time of infection. One study [20] found that persons infected with non-A subtypes, mainly subtypes C, D, and G, were 8 times more likely to develop AIDS than were persons infected with subtype A, suggesting that HIV-1 subtypes may differ in rates of progression to AIDS. We recently reported in a study of 164 HIV-infected participants (117 with known dates of seroconversion) that those infected with subtype D had faster disease progression than those with subtype A, although this did not reach statistical significance for most indicators of progression [24].

Other prospective studies have used seroprevalent cohorts to study CD4 cell decline and clinical progression in different populations infected with different HIV subtypes [19, 22, 25]. With the exception of disease progression between B and B-Br serotypes in Brazil, other studies did not find significant differences in disease progression among persons infected with different subtypes; however, these studies had relatively few subjects.

Other phenotypic differences of HIV-1 subtypes might lead to differences in pathogenicity. Virus loads differ by HIV-1 subtype [26–28], and subtypes might have different biologic characteristics, such as frequency of syncytium-inducing phenotype and patterns of coreceptor usage [29, 30], that may be capable of affecting pathogenesis.

In the developed world, where most studies on the natural history of HIV-1 disease have been done, subtype B is the major HIV-1 subtype. HIV-1 diversity is greatest in Africa, which offers the best sites for studies relating viral subtype to disease progression. However, there are few cohorts in Africa to provide the necessary clinical information. We have established well-characterized longi-

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tudinal cohorts that are suitable for studying disease progression and HIV-1 subtypes in Uganda [31, 32]. Two major subtypes (A and D) are approximately equally distributed in Uganda [4, 10, 12, 15]. Both subtypes are transmitted by heterosexual contact, and in this population antiretroviral drugs are rarely used.

Here we report the results of a study of HIV-1 disease progression in relation to HIV subtypes A and D, as measured by progression to death and by CD4 cell count during follow-up, in a population of >1000 HIV-positive Ugandan adults. For this study, we looked at the envelope subtype, including the V3 region, because of the importance of this region in the pathogenesis of HIV-1 disease and host immunity. The region is important in cell tropism, phenotype, neutralization, and cellular immunity [33–37].

Methods

Study population and follow-up. A cohort of HIV-1–infected adults was established in Entebbe, Uganda, between 1995 and 1998 in order to undertake a randomized controlled trial of a polyvalent pneumococcal vaccine [38]. Recruitment took place at 2 community clinics, the AIDS Support Organisation (TASO) and the Uganda Virus Research Institute (UVRI). The vaccine trial ended in June 1998, but patient enrollment in the cohort and follow-up continued.

The eligibility criteria for inclusion were as follows: attendance at TASO or UVRI, willingness to participate, and ability to give consent for the vaccine study. Persons who were bedridden because of HIV infection or another opportunistic infection were not recruited unless they recovered. The other important exclusion criterion was distance from the clinics: persons living >15 km from Entebbe were excluded.

At enrollment and every 6 months thereafter, a detailed medical history was taken, a clinical examination was done, and CD4 T cell subsets were measured. Participants had open access to the study clinics and were encouraged to attend when sick. Those too ill for outpatient care were hospitalized or visited at home by a study clinician. Failure to attend a routine clinic visit activated default tracing, and field workers would visit participants at home. Participants who were healthy were encouraged to attend the clinic. For cases of disappearance, refusal to take further part in the study, and departure from the study area, the default date was recorded as the date of last contact. Relatives, neighbors, and TASO counselors also routinely reported deaths if the participant was not under the immediate care of a trial physician at the time of death. Information obtained from clinical assessment at baseline and at each 6-month visit was used to assign participants to a clinical stage according to the World Health Organization (WHO) staging system [39].

Subtyping. At the time of study enrollment, 5–10 mL of blood was collected in EDTA vacutainers (Becton Dickinson) and transferred to the UVRI laboratory within 8 h of phlebotomy. The sample was divided into 2 aliquots, and plasma was separated from 1 aliquot; the other aliquot was transferred to a Sarsted tube and kept frozen at −20°C for DNA extraction. HIV-1 subtyping was done for all participants recruited during the trial. The samples were subtyped by use of an algorithm that employs peptide serology, followed by heteroduplex mobility assay (HMA) for the untypeable samples; those samples that were still untypeable were sequenced. For peptide serology, we used both V3 and gp41 peptides to assign the subtype [13]. In brief, consensus A–D V3 peptides were used in a peptide-limiting dilution ELISA, with peptides coated at concentrations of 1, 0.1, and 0.01 μg/mL. A serotype was the peptide that gave the highest optical density:cutoff ratio at the lowest peptide concentration. In addition, new gp41 peptides that discriminated between subtypes A and D were used at a concentration of 5 μg/mL. We designated samples as a particular subtype only if both assays gave consistent results. Quality assurance was also done on serotyped samples by sequencing or HMA.

HMA subtyping was done by standard procedures [40]. For samples that were untypeable by HMA, an aliquot of HMA second-round products was sent to the Centre for HIV Research, University of Edinburgh, where sequencing was done as described elsewhere [13]. Subtype data for 120 persons in this study were obtained from the pilot study previously reported [13]. We focused on persons infected with subtype A or D, who represented the majority of cases.

Statistical analysis. Characteristics at the enrollment visit were compared between persons infected with HIV-1 subtypes A and D. All the continuous characteristics were categorized in order to avoid any assumption about their distribution. Age 40 years was chosen to distinguish between the population characterized as "old" and that characterized as "young." Even though the older group was small because of the relative youth of this cohort, this cutoff seemed more meaningful than the median age (30 years). CD4 cell counts were divided into 3 groups: <200, 200–499, and ⩾500 cells/mm^3. These baseline characteristics were compared between subtypes A and D by χ^2 or Fisher's exact test.

A multivariate survival analysis was carried out to assess the prognostic value of HIV subtype for death (our primary end point) by using a Cox proportional hazards model. The use of categorized continuous characteristics (as described above) was chosen in order to increase the power of the statistical analysis and to avoid any assumptions about the log linear effect of any characteristics on the risk of death. A multivariate model was constructed to select which characteristics assessed at enrollment (except subtype) had the best prognostic value for death. The HIV subtype covariate was then added to the model in order to assess its prognostic value.

The effect of HIV subtype on the CD4 cell count during follow-up was investigated as a secondary end point. For this, a marginal general linear model was used [41]. To keep follow-up homogeneity between the 2 subtype groups, only subjects with ⩾2 CD4 cell counts measured at follow-up visits (in addition to the enrollment visit) were taken into account, and the follow-up was truncated after 6 visits (including enrollment). Because the primary end point (time to death) was highly correlated with length of follow-up, we needed to compare people with roughly the same length of follow-up in order to produce a meaningful complementary analysis of disease progression. Square-root transformation of the CD4 cell count was used to obtain an approximately normal distribution. Because CD4 cell counts recorded at multiple visits for the same patient cannot be assumed to be independent, a robust variance-covariance matrix was used to take this correlation into account. We assumed the correlation structure to be autoregressive (i.e., the closest visits exhibit a higher correlation between their associated CD4 cell count than do the remote visits).

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The effect of HIV subtype on average CD4 cell count during follow-up was adjusted for the CD4 cell count at enrollment in order to account for possible differences in disease stage at enrollment between the 2 subtype groups (see Appendix). Of note, unlike other common modeling techniques, this model does not make any assumptions about the linearity of the decline of CD4 cell counts for individuals. Here, we compare the average CD4 cell count during a given follow-up period for each subtype group rather than the rate of linear decline. We set the significance level at 5%. All analyses were performed with Stata software, version 6.0.

Results

Subtyping. Subtyping was attempted on 1259 cohort participants recruited until the end of 1998: 1058 (84%) were successfully subtyped. Of the subtyped participants, 1045 (99%) were infected with envelope subtype A (538) or D (507); the rest were infected with subtype C (11), B (1), or G (1). Subtypes were determined by a combination of V3 and gp41 peptide serology for 707 samples (68%), by HMA for 171 samples (16%), and by V3 sequencing for the remaining 167 samples (16%). In all, 115 serotyped samples had confirmatory DNA sequence data, and only 5 samples (4%) were wrongly identified. Of the 115 HMA-typed samples with confirmatory DNA sequencing data, only 1 sample (1%) was identified incorrectly [13].

Population comparison at enrollment. Table 1 shows the characteristics at enrollment of the subjects within the 2 subtype groups. Persons with subtype D were significantly younger than those infected with subtype A ($P = .02$, χ^2 test) and had significantly lower CD4 cell counts ($P = .001$, χ^2 test). These characteristics were also significantly different when considered as continuous variables (subtype A vs. subtype D: median age, 31 and 29 years, respectively, and interquartile range [IQR], 27–36 vs. 25–35 years, respectively [$P < .001$, Wilcoxon rank sum test]; median CD4 cell count, 266 and 162 cells/mm^3, respectively, and IQR, 118–460 vs. 46–426 cells/mm^3, respectively [$P < .001$, Wilcoxon rank sum test]). Nevertheless, distribution of clinical stage was not different between the subgroups.

Time to death. All 1045 participants with subtype A or D were included in this analysis. The median follow-up time was 1.5 years (IQR, 0.7–2.5 years). During this time, 460 deaths (44%) were recorded. In univariate analysis, subtype D was a significant prognostic factor for death (relative risk, 1.48; 95% confidence interval, 1.23–1.77; $P < .001$; figure 1). Other prognostic factors for death were low CD4 cell count and advanced WHO clinical stage. The effect of subtype was therefore adjusted for these factors, together with age, in order to account for the unbalanced age distribution at enrollment. After this adjustment, subtype D remained a significant prognostic factor for death ($P = .009$, Wald test; table 2).

CD4 cell counts during follow-up. In this study, 576 persons had $\geqslant 3$ CD4 cell counts done routinely at different visits (including at enrollment). The characteristics and the survival status of the participants excluded from this analysis are presented in table 3. The characteristics were similar for both subtypes. After truncation at 6 visits, there were 2131 CD4 cell counts to consider (mean, 3.7 CD4 cell counts per patient). Visits were equally spaced for both subtypes (median, 6.2 months; IQR, 5.9–7.0 months). The median follow-up for subtypes A and D were 2.09 and 2.05 years, respectively (IQRs, 1.6–2.5 and 1–2.5 years, respectively).

By use of the marginal general linear model and after adjustment for the CD4 cell count at enrollment, we found that subtype D was associated with a significantly lower average CD4 cell count during the follow-up period, compared with subtype A ($P = .001$, Wald test). Additional adjustment for WHO clinical stage or age at enrollment had no significant effect on CD4 cell count during follow-up. For example, a hypothetical person with an initial CD4 cell count of 200 cells/mm^3 would tend to have an average predicted CD4 cell count of 150 cells/mm^3 over the 2-year follow-up period if infected with subtype A and an average of only 120 cells/mm^3 if infected with subtype D.

Discussion

In this study, we found a significant difference in mortality between persons infected with HIV-1 subtype A and those infected with subtype D. In addition, persons infected with subtype

Table 1. Description and comparison of characteristics of subjects, by human immunodeficiency virus (HIV) type 1 envelope subtype at enrollment.

Characteristic	No. (%) of subjects: Subtype A (n = 538)	No. (%) of subjects: Subtype D (n = 507)	P
Age, years			
<40	456 (85)	454 (90)	.02
⩾40	82 (15)	53 (10)	
Sex			
Male	168 (31)	149 (29)	.51
Female	370 (69)	358 (71)	
CD4 cell count, cells/mm^3			
<200	198 (37)	272 (54)	
200–499	198 (37)	123 (24)	.0001
⩾500	115 (21)	100 (20)	
Missing value[a]	27 (5)	12 (2)	
World Health Organization clinical stage			
1	40 (7)	40 (8)	
2	204 (38)	167 (33)	.41
3	284 (53)	290 (57)	
4	10 (2)	10 (2)	
Year of enrollment			
1995	44 (8)	44 (9)	
1996	232 (43)	212 (42)	.97
1997	187 (35)	181 (35)	
1998	75 (14)	70 (14)	

[a] For these subjects, the CD4 T cell subset measurement failed.

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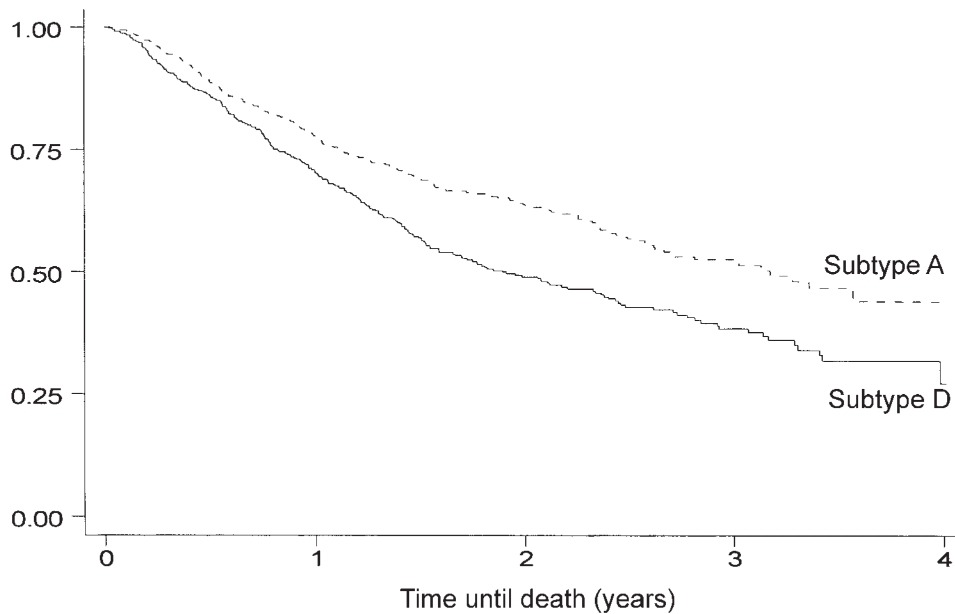

Figure 1. Kaplan-Meier nonparametric survival curves for human immunodeficiency virus type 1 envelope subtypes A and D in a Ugandan population, by time until death.

D had lower average CD4 cell counts during follow-up. This is the first report to demonstrate a significant difference in pathogenicity between HIV-1 subtypes A and D. We believe that this study is the largest of its kind: >1000 persons were followed in a population for which antiretroviral therapy is largely unavailable. There are, however, some limitations to our study, which must be considered when assessing these findings.

The study participants were initially recruited into a randomized controlled trial of the efficacy of a pneumococcal polysaccharide vaccine [38]. This study, therefore, was not designed to address whether HIV disease progression differs by subtype, and the population enrolled may not be fully representative of all HIV-1–infected people in the population. For example, 19.7% of newly registered TASO clients were not recruited, because of refusal to take part and/or severe illness.

Another limitation of the study was that 16% of samples could not be subtyped by serology, and we failed to amplify any DNA. This may have created bias if those not subtyped differed from our study population in terms of subtype or disease progression. A further limitation was that 68% of the samples were subtyped by peptide serology. This method cannot distinguish between subtypes A and C or between subtypes B and D. However, from our sequences [13] and from HMA data, very few persons were infected with subtype C or B. Other studies also have shown that subtypes B and C are rare in Uganda [3–16]. Thus, we believe that use of peptide serology did not substantially affect our interpretation of the results. In addition, we previously showed that an algorithm that uses 2 peptides (V3 and gp41), followed by HMA and DNA sequencing for untypeable samples, has an accuracy of 95% in Uganda, compared with HMA or DNA sequencing [13]. The inability of serotyping and HMA to determine whether persons were infected with recombinant or dual subtypes was another limitation of this study.

The date of seroconversion was not known for these patients. We estimated the prognostic value of envelope subtype on death, compensating for the undocumented seroconversion date, by

Table 2. Multivariate survival analysis for 1045 adults infected with human immunodeficiency virus (HIV) type 1.

Variable	No. (%) of deaths	Multivariate analysis RR (95% CI)	P[a]
Age, years			
<40	393 (43)	1	
⩾40	67 (50)	1.24 (0.95–1.61)	.12
CD4 cell count,[b] cells/mm^3			
<200	332 (65)	18.37 (11.02–30.64)	<.001
200–499	99 (31)	4.34 (2.55–7.37)	<.001
⩾500	16 (7)	1	
World Health Organization clinical stage			
1 or 2	135 (30)	1	
3	312 (54)	1.62 (1.31–2.00)	<.001
4	13 (65)	1.70 (0.94–3.10)	.08
HIV-1 subtype			
A	208 (39)	1	
D	252 (50)	1.29 (1.07–1.56)	.009

NOTE. CI, confidence interval; RR, relative risk.
[a] Based on the Wald test.
[b] Data were missing for 39 subjects, because the CD4 T cell subset measurement failed.

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Table 3. Description of subjects excluded from CD4 cell count analysis, by human immunodeficiency virus (HIV) type 1 envelope subtype, World Health Organization (WHO) clinical stage at enrollment, and vital status, during the first year of follow-up.

WHO clinical stage	No. of subjects with HIV-1 subtype A		No. of subjects with HIV-1 subtype D	
	Alive	Dead	Alive	Dead
1	6	1	5	1
2	39	20	35	28
3	55	90	68	106
4	3	3	4	5
Total	103	114	112	140

taking into account disease progression through adjustment for WHO stage and CD4 cell count at enrollment. Since time from infection cannot be controlled, such analyses are vulnerable to bias and confounding. However, we previously showed that WHO stage and CD4 cell count are comparable prognostic indicators and that WHO stage is a useful prognostic indicator [42] in Ugandan populations. It would be very difficult to achieve a comparable number of events by using a prospective study of recent seroconverters. For example, in a study of the natural history of HIV-1 in Uganda [31], we enrolled 149 seroconverters in 10 years and documented 31 deaths.

The results of the present study extend and are consistent with those we previously reported [24]. In the earlier study involving 117 participants with known dates of seroconversion, those infected with subtype D progressed faster than those infected with subtype A. Although this did not reach statistical significance for most indicators of progression, there were 3 times more deaths among those infected with subtype D, compared with subtype A. Alaeus et al. [22] also did not find significant differences in progression between persons infected with subtype A, B, C, or D in a racially heterogeneous population; however, there was a tendency for those with subtype D to progress faster than those with subtype A, and the rate of CD4 cell count decline in those with subtype D was twice that in those with subtype A. We therefore think that there are differences in the pathogenicity of these 2 subtypes (A and D), but large numbers are required to achieve statistical significance.

We chose death rather than AIDS as our primary end point because progression from an AIDS-defining event to death averages 9–10 months [43]; since the average intervisit interval was 6 months, we might have missed documenting this event. Death is less ambiguous than clinical AIDS as an end point, even when detailed clinical and laboratory investigations are available. We previously showed that, among HIV-1–positive adults, 80% of deaths are associated with AIDS [43].

We were not able to monitor virus loads in this study because of the cost. It would have been of interest to know whether there are differences in virus load by subtype. We recently reported that, in another cohort, persons infected with subtype D had significantly higher virus loads and a higher average annual increase in virus load than did persons with subtype A [28].

In the present study, those infected with subtype D were younger and had lower CD4 cell counts at enrollment than did those with subtype A. It is not clear which subtype was first present in Uganda; earlier studies reported both subtypes [1, 3, 4], and a study of the dynamics of HIV-1 in rural Uganda among incident cases has shown no major changes in subtype prevalence over a 10-year period (D. Yirrell, personal communication). In previous studies (data not shown) we found no relationship between subtype and age at seroconversion, suggesting that people are infected with subtypes A and D at the same age. The younger age of subtype D–infected persons therefore may reflect higher pathogenicity of subtype D, with the death of older people before the study started. We adjusted for age and CD4 cell count at enrollment to minimize these effects, but some residual confounding may have remained.

Subtype might be a confounding factor for disease progression because it is associated with more-important factors such as phenotype, virus load, or immune responses. However, there is a need to investigate viral set point at seroconversion for the different subtypes, since these set points may affect disease progression. If a person is infected with a particular subtype that is typified by "rapid high" phenotype or reduced cellular immune responses, it is possible that the person may have faster disease progression. We are currently investigating phenotype and cellular immune responses in such persons. Since the HIV-1 *gag* region is important in cellular immune responses and since ~30% of the viruses in our population are recombinant [17], we are now investigating the relationship between *gag* subtype and disease progression.

Another explanation for our observations is the possibility that these subtypes have ethnic clustering that could be associated with disease progression. This was not investigated.

If there are differences in subtype pathogenicity, this might have an influence on the dynamics of HIV-1 subtypes globally, whereby persons with less-virulent strains survive longer, and, as a result, these strains are transmitted more in the population. One study in Uganda, however, did not find significant differences in the proportions of subtypes A and D in selected sites over 3 years [15], and, in another cohort in Uganda with subtype prevalences similar to those in the current study, the proportions of HIV-1 subtypes A and D over a 10-year period appear to be stable (D. Yirrell, personal communication). This may indicate that subtype prevalence is also influenced by other factors, such as transmissibility efficiency, and that these factors need to be investigated. In addition, if the envelope region plays a major role in pathogenicity, this information may be important in vaccine research and therapeutic interventions. This would be an important target to either attenuate the virus or block virus spread in the host. Finally, subtype analysis should be considered when evaluating any future vaccine that alters disease progression.

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Appendix

Estimation of CD4 Count Decline, Using Generalized Estimating Equations

The marginal general linear model formulation for the estimation of the effect of human immunodeficiency virus (HIV) type 1 envelope subtype on CD4 cell count during follow-up can be written as follows: $E(Y_i) = \alpha + \beta X_i + \gamma Y_{i0}$ with $\text{corr}(Y_{ik}, Y_{il}) = \rho^{|l-k|}$, where i refers to patient i; Y_{ik} and Y_{il} are the square root transformation of the CD4 cell count at visits k and l, respectively ($k = 0$ for enrollment); γ is the effect of the initial CD4 cell count at enrollment (Y_{i0}) on CD4 cell counts recorded during the follow-up visits (to be estimated); α is the intercept (to be estimated); X_i is the subtype covariate for the patient (1 for subtype D and 0 for subtype A); β is the effect of subtype D, compared with subtype A, on CD4 cell count (to be estimated); and ρ is the correlation between the CD4 cell counts of consecutive visits (to be estimated).

By using our data set, the estimated model was $E(Y_i) = \hat{\alpha} + \hat{\beta} X_i + \hat{\gamma} Y_{i0}$, with $\hat{\beta} = -1.10$ (95% confidence interval [CI], -1.68 to -0.52; $P < .001$), $\hat{\alpha} = -0.61$ (95% CI, -1.54 to 0.33; $P = .20$), and $\hat{\gamma} = 0.94$ (95% CI, 0.89–0.98; $P < .001$). A high correlation was estimated between the CD4 cell counts of consecutive visits ($\hat{\rho} = 0.60$).

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