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Viral and Immune Risk Factors of HIV Rebound After Interruption of Antiretroviral Therapy

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Background. Identifying risk factors for human immunodeficiency virus (HIV) rebound after treatment interruption is crucial for designing effective remission strategies.

Methods. Peripheral blood mononuclear cells from participants in the Zurich HIV Primary Infection Cohort (ZPHI, n = 73) and ACTG study A5345 (n = 44) were analyzed before antiretroviral therapy (ART) interruption. We measured cell-associated HIV RNA, total HIV DNA, and proviral diversity (*env* gene). Immune phenotyping was conducted by flow cytometry. Cox proportional hazards (PH) models and penalized Cox PH models with an adaptive LASSO penalty identified risk factors for time to rebound (HIV RNA >1000 copies/mL).

Results. Late ART initiation was associated with higher rebound risk (shorter time to rebound) as compared to early ART. Higher pre-ART HIV RNA in plasma, total HIV DNA, and increased cellular HIV transcription at the time of ART interruption were associated with higher rebound risk. Higher proviral diversity was associated with higher rebound risk but only among male participants and those enrolled in the ZPHI cohort. Fewer $CD4^+$ T cells at ART interruption, higher proportions of effector and terminally differentiated T cells, and more activated and exhausted T cells were associated with higher rebound risk, primarily in early-treated participants. No significant immunological risk factors were found in participants treated during chronic HIV. In the combined cohort, total HIV DNA and terminally differentiated $CD8^+$ T cells appeared to be the most relevant risk factors for time to rebound, as indicated by variable selection in multivariable analysis.

Conclusions. These findings underscore the importance of early ART initiation and suggest that tailored interventions based on virologic, immunologic, and demographic factors may help achieve sustained viral suppression.

Clinical Trials Registration. NCT00537966 and NCT03001128.

Keywords. HIV; ART interruption; predictors.

Achieving a functional cure for human immunodeficiency virus (HIV) remains an elusive goal, and current methods to assess viral rebound upon antiretroviral therapy (ART) interruption are limited. Determining whether a person with HIV (PWH) has achieved remission depends solely on the observation of HIV RNA rebound when ART is stopped [1], as no reliable biomarkers have been identified as risk factors of viral rebound or indicators of the success of potential curative interventions. This knowledge gap hinders the efficient selection of participants for clinical trials and may expose individuals to ineffective therapies and unnecessary ART interruptions. There is an urgent need to identify virologic, immunologic, and other host biomarkers to predict the risk of HIV rebound after treatment interruption. Identifying these biomarkers could help prioritize interventions or participants in HIV clinical trials. Additionally, these biomarkers could guide the development of novel therapeutic agents aimed at achieving ART-free remission, a prospect that holds promise for improving the quality of life for PWH. Previous investigations have identified several factors that may influence the timing of HIV rebound, such as the early initiation of ART and the size of the total and transcriptionally active HIV reservoir [1–4]. However, a comprehensive understanding of the viral and host factors that influence HIV rebound dynamics remains an unmet challenge in the field.

Recently, the A5345 study team carried out a comprehensive investigation to assess virologic and immunologic risk factors

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of HIV rebound timing in PWH who had initiated ART during chronic (n = 33) or early (n = 11) stages after HIV acquisition [5]. In this study, the risk factors of HIV rebound timing exhibited differences between early and chronic-treated participants. For those who received early treatment, the most influential risk factor was the level of residual viremia, while in the case of chronic-treated individuals, the level of intact proviral DNA correlated strongly with rebound timing. Additionally, distinct sets of immune and inflammatory biomarkers were identified as risk factors of rapid (occurring in less than 4 weeks) versus slow (occurring after 4 weeks) rebound for both early and chronic-treated individuals.

Despite the valuable insights gained from this study, a limitation was noted concerning the relatively low number of participants who initiated ART during the early HIV stages. We sought to address this limitation by adding a large cohort of 73 PWH who commenced ART during the earliest stage of HIV as part of the Zurich HIV Primary Infection Cohort (ZPHI). Our goal is to corroborate and enhance the understanding of virologic and host factors in the context of HIV rebound dynamics after ART interruption. This approach will contribute to a more robust and comprehensive analysis of potential biomarkers, furthering our progress towards the goal of achieving an effective functional cure for HIV.

METHODS

Study Design and Sample Selection

Stored Peripheral Blood Mononuclear Cells (PBMC) were requested from 2 existing studies of ART interruption: the ZPHI (n = 73) and ACTG study A5345 (n = 44). Samples were selected immediately before ART interruption.

For ZPHI, individuals presenting after acute or recent HIV acquisition were enrolled between November 2002 and July 2007 [6]. The ZPHI study is an observational, open label, nonrandomized, single-center study (clinicaltrials.gov NCT00537966). Written informed consent was obtained from each participant. All participants with documented primary HIV infection were offered early ART initiation regardless of $CD4^+$ cell count or disease stage as part of this study protocol [7]. After 1 year of successful ART, participants could choose to stop treatment and join the observational study arm to examine the effects of analytical treatment interruption. Viral loads were monitored monthly until HIV RNA was detectable, then every 3 months until participants resumed ART according to clinical guidelines at the time.

A5345 is a prospective study of factors mediating the timing of HIV rebound after treatment interruption (ClinicalTrials.gov NCT03001128) [5, 8]. The study evaluated 2 cohorts of participants: individuals who initiated ART during chronic HIV (chronic treated, n = 33) or early HIV (early treated, n = 11). Chronic-treated participants initiated ART >6 months after acquiring HIV, while early treated participants initiated ART during Fiebig stages III–V of acute stage [9]. All participants were between 18 and 70 years of age, on suppressive ART for ≥2 years with $CD4^+$ count ≥500 cells/mm^3 and nadir CD4 count ≥200 cells/mm^3, and no history of AIDS-defining illness. Participants were followed for 48 weeks or until they met the ART restart criteria, whichever occurred first. During the first 8 weeks of the treatment interruption, viral loads were monitored twice weekly by the Roche COBAS assay (Quest) and $CD4^+$ T cell counts every 2 weeks at local Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories. Thereafter, viral loads were monitored weekly and $CD4^+$ T cell counts every 4 weeks. ART was restarted upon 2 successive viral loads ≥1000 copies/mL, 1 viral load ≥10 000 copies/mL, or based on another of the predefined criteria.

HIV Reservoir Quantifications

For each sample, cellular DNA and RNA were extracted from 5 million PBMC using AllPrep DNA/RNA Mini Kit (Qiagen) [10]. Total HIV DNA and 2-long terminal repeat (2-LTR) circles were quantified by droplet digital polymerase chain reaction (ddPCR) [11]. Copy numbers were calculated as the mean of replicate PCR measurements and normalized to 1 million cells as determined by ribonuclease P/MRP subunit p30 [12]. Unspliced HIV RNA (HIV RNA_{Gag}) and multiply spliced HIV RNA (HIV RNA_{TatRev}) were quantified in duplicate and normalized to total RNA input as determined by A260/A280 absorptivity ratio using a NanoDrop 2000 spectrophotometer (Thermo Scientific) [13].

Proviral Sequencing

To amplify FL Env, DNA was extracted from PBMCs. Primers used for the first round were 5′FENVouter (forward) TTAGGCATCTCCTATGGCAGGAA and 3′RENVouter (reverse) TCTTAAAGGTACCTGAGGTCTGACTGG. First-round PCRs were performed using the Advantage 2 PCR Kit from Takara Bio USA, Inc. (cat# 639206, supplied by Takara Bio), utilizing the 10X SA Buffer. Cycling conditions were 95°C for 1 min, 35 cycles of 95°C for 15 s, 57°C for 30 s, and 68°C for 3 min with a final extension at 68°C for 10 min. The second round of PCR was conducted using 5′FENVinner: GAG CAGAAGACAGTGGCAATGA (forward) and 3′RENVinner: CCACTTGCCACCCATBTTATAGCA (reverse). Cycling conditions were 95°C for 1 min, 30 cycles of 95°C for 15 s, 64°C for 30 s, and 68°C for 3 min with a final extension at 68°C for 10 min. PCR cleanups were done on the second-round reaction products using the QIAquick PCR Purification Kit from Qiagen (cat# 28106, supplied by Qiagen Inc.). DNA was quantified using the Qubit dsDNA HS Assay Kit from Invitrogen (cat# Q32854, supplied by Thermo Fisher Scientific). Quality and integrity were measured using Genomic DNA Screen Tape and reagents (Genomic DNA Screen Tape Cat# 5067-5365, Genomic DNA Reagents Cat# 5067-5366, supplied

by Agilent Technologies), in combination with the 2200 TapeStation System (Agilent Technologies). Subsequently, amplicons were prepared for deep sequencing using the Nextera XT DNA Library Preparation Kit from Illumina (FC-131-1096, supplied by Illumina, Inc.), with an indexing of 96 samples per run (Nextera XT Index Kit Set A, FC-131-2001, also from Illumina). Finally, we used a custom-designed pipeline to recover full-length Env HIV DNA sequences from the paired-end reads. The pipeline included a preliminary step of quality control, which involved trimming reads for PHRED quality ≥30 and removing Illumina adapters. Overlapping identically paired forward and reverse reads were merged and premapped to the HXB2 reference genome. The cleaned reads were remapped to the *de novo* assembled near the FL Env sequence. The minimum acceptable coverage was set to 10 000 reads. CliqueSNV [14] was used to identify viral haplotypes with a minimum frequency of 1% from these cleaned mapped reads. Shannon entropy [15] was computed for each sample.

Immune Phenotyping

For each sample, frozen cells were thawed in a 37°C water bath, washed, resuspended in phosphate-buffered saline (PBS; Corning), and counted using a BD Accuri C6 Plus (BD Biosciences). Live cells were stained using LIVE/DEAD aqua (Invitrogen) in PBS. Next, the cells were washed and stained for extracellular and intracellular markers [16]. Compensation and gating were performed using FlowJo (version 10). Using the gating strategy summarized in Supplementary Figure 1, we obtained proportions for $TGIT^+PD1^+$, $CD38^+HLADR^+$, $CD107a^+$, and $Ki67^+$ expressions on $CD4^+$ and $CD8^+$ T cells.

Statistical Analysis

Cox proportional hazards models were employed to assess the univariate association between the interval-censored time to rebound, defined as the first instance of HIV RNA >1000 copies/mL, and each of the virologic and host risk factors (Supplementary Table 1 for a complete list of analyzed variables). We assumed that the hazard ratios between different levels of covariates are constant over time. In this context, "hazard" refers to the likelihood of experiencing a viral rebound at any given time, if a rebound has not yet occurred up to that point. To explore potential effect modification, stratified analyses were performed by cohort (ZPHI vs A5345), timing of ART initiation (acute/early vs chronic), and sex at birth (female vs male), respectively. Analyses for the female-only subgroup were not conducted due to sample size limitations (n = 13). Because of the large number of immunological biomarkers under consideration, we performed the Benjamini-Hochberg procedure to control the false discovery rate (FDR) at the level of α. Given our small sample size, we set an $\alpha = .1$ to balance the risk of false positives and the chance of missing relevant biomarkers. Finally, a multivariable Cox proportional hazards model was constructed for the combined cohort. The initial model included all risk factors identified from the univariate analyses. Variable selection was performed using a penalized regression technique with an adaptive LASSO penalty [17]. The optimal tuning parameter was determined through a 10-fold cross-validation approach.

RESULTS

Cohort Description

The analysis population includes 73 ZPHI participants and 44 A5345 participants. The demographics of both cohorts are summarized in Table 1, while Supplementary Table 2 summarizes demographics by HIV staging at the time of ART initiation. As expected, we observed that the risk of viral rebound was 1.7 times higher for participants who started ART during the chronic HIV stage compared to those who started ART during acute/early HIV stage (hazard ratio = 1.703; 95% confidence interval, 1.035–2.804; P value = .036). None of the other evaluated clinical variables were associated with time to rebound in the combined cohort or in each cohort individually.

Virologic Variables Associated With Time to Viral Rebound

As a next step, we investigated associations between virologic variables (ie, HIV reservoir size [HIV DNA levels]; transcriptional activity [cellular HIV RNA levels, both multiply spliced and unspliced transcripts]; proviral molecular diversity [Shannon entropy]; and pre-ART plasma HIV RNA) and the time to viral rebound for all participants across both cohorts combined (Figure 1). Results are summarized in Table 2. Notably, in the combined cohort we observed that higher HIV DNA levels, increased cellular HIV transcription (HIV RNA_{Gag}) before ART interruption, and higher pre-ART plasma HIV RNA were significantly associated with a higher risk of rebound, therefore a shorter time to rebound ($P = .027$, $P = .015$, and $P = .016$, respectively). This relationship was not evident for HIV RNA_{TatRev}, 2-LTR circles, and proviral molecular diversity.

Based on the observations of our previous study and differences in putative mechanisms of viral controls [5], we stratified the analysis by timing of ART initiation (early vs chronic), by cohort (A5345 vs ZPHI), as well as by sex at birth (male vs female). While significance disappeared for some variables in stratified analysis likely due to the reduced sample size (with exception of pre-ART HIV RNA and HIV RNA_{Gag} in male participants), we observed a new association between higher proviral molecular diversity and higher risk of viral rebound in the ZPHI cohort and among male participants. Higher pre-ART plasma HIV RNA levels were consistently associated with higher risk of viral rebound in stratified analysis in ZPHI participants, participants who started ART during acute/early HIV, as well as among male participants.

Table 1. Demographic Characteristics of Each Cohort at the Time of ART Interruption

Variable	Combined	ACTG	ZPHI	*P* Value[a]
Total participants, No. (%)	117	44 (38)	73 (62)	
Age, y[b]	40 (19, 61)	43 (22, 61)	38 (19, 60)	.012
Age group, y, No. (%)				.055
<25	4 (4)	2 (5)	2 (3)	
25–34	34 (30)	8 (18)	26 (38)	
35–44	33 (29)	11 (25)	22 (32)	
45–55	29 (26)	15 (34)	14 (20)	
≥55	13 (11)	8 (18)	5 (7)	
Sex at birth, No. (%)				.764
Female	13 (11)	4 (9)	9 (12)	
Male	104 (89)	40 (91)	64 (88)	
Race/ethnicity, No. (%)				<.001
Others	25 (21)	19 (43)	6 (8)	
White non-Hispanic	92 (79)	25 (57)	67 (92)	
Staging at the time of ART start, No. (%)				<.001
Chronic	33 (28)	33 (75)	0 (0)	
Acute/early	84 (72)	11 (25)	73 (100)	
EDI to ART initiation, years[c]	0.44 (0, 11.4)	1.4 (0, 11.4)	0.15 (0.03, 0.97)	.765
ART duration, y	4.5 (0.4, 26.1)	9.5 (2.2, 26.1)	1.5 (0.4, 4.1)	<0.001
$CD4^+$ cell count/mm^3	779 (334, 1969)	851 (496, 1969)	735 (334, 1419)	.039
Pre-ART HIV RNA, log_{10}[d]	5.15 (2.35, 7.52)	4.72 (2.47, 7.26)	5.35 (2.35, 7.52)	.005
Total HIV DNA[e]	106 (0, 1014)	82 (0, 620)	122 (0, 1014)	.396
Cellular HIV RNA, unspliced[f]	7.6 (0, 266)	6.3 (0, 70)	8.4 (0, 266)	>.9
Cellular HIV RNA, TatRev[f]	4.8 (0, 244.5)	0.5 (0, 2.4)	7.7 (0, 244.5)	.002
Proviral diversity[g]	0.017 (0, 0.075)	0.023 (0, 0.075)	0.012 (0, 0.048)	.02

Abbreviations: ACTG, Advancing Clinical Therapeutics Globally for HIV/AIDS and Other Infections; ART, antiretroviral therapy; EDI, estimated date of infection; HIV, human immunodeficiency virus; ZPHI, Zurich HIV Primary Infection cohort.

Data are mean (minimum, maximum) except where indicated.

[a]Wilcoxon rank sum test for continuous variables; Fisher exact test for categorical variables.

[b]Age missing for 4 ZPHI participants.

[c]EDI missing for 23 participants (22 ACTG, 1 ZPHI).

[d]Pre-ART HIV RNA (log_{10} copies per ml) is missing for 10 ACTG participants.

[e]Total HIV DNA is normalized as copies/10^6 cells and missing for 7 participants (1 ACTG, 6 ZPHI).

[f]Cellular HIV RNA is normalized as copies/10^6 cells and missing for 8 participants (1 ACTG, 7 ZPHI).

[g]Proviral diversity missing for 12 ACTG participants and 33 ZPHI participants.

Immunological Variables Associated With Time to Viral Rebound

Subsequently, we evaluated associations between immune variables and time to viral rebound in the combined cohort. As expected, lower proportions of $CD4^+$ T cells at the time of ART interruption was associated with higher risk of viral rebound. Additional immunologic variables were identified as significant risk factors of time to rebound in the combined cohort after adjusting for FDR. Specifically, we found that a higher percentage of effector $CD4^+$ and terminally differentiated $CD8^+$ T cells, as well as various $CD4^+$ and $CD8^+$ T cell subsets expressing markers of activation ($CD38^+HLA\text{-}DR^+$) and exhaustion ($TIGIT^+PD1^+$ or $TIGIT^-PD1^+$) consistently correlated with a higher risk of viral rebound, and therefore shorter time to rebound (Figure 2 and Supplementary Table 3). Interestingly, there was no significant association between timing of viral rebound and T cells expressing markers of degranulation (CD107a) or cycling (Ki67).

In our stratified analysis (Supplementary Table 4), we noticed that most of the immunological biomarkers were significantly negatively associated with time to viral rebound only in the subset of participants who started ART during acute/early HIV. None of the immunological variables were significant in either cohort individually (ZPHI n = 70, A5345 n = 43), in participants who started ART during chronic HIV (n = 32), as well as when stratifying by sex at birth (male n = 100).

Multivariable Model

Finally, we incorporated all the significant risk factors detected by univariate analysis to construct a multivariable Cox proportional hazards model for the combined cohort. Given the high correlation among many of the tested variables (Supplementary Figures 3 and 4), we used variable selection to develop a robust and interpretable model. This selection process identified total HIV DNA and proportion of terminally differentiated $CD8^+$ T cells as the most relevant risk factors of viral rebound. The hazard ratio of both covariates were slightly larger in the multivariable model compared to the univariate associations (Supplementary Table 5). With the proportion of terminally

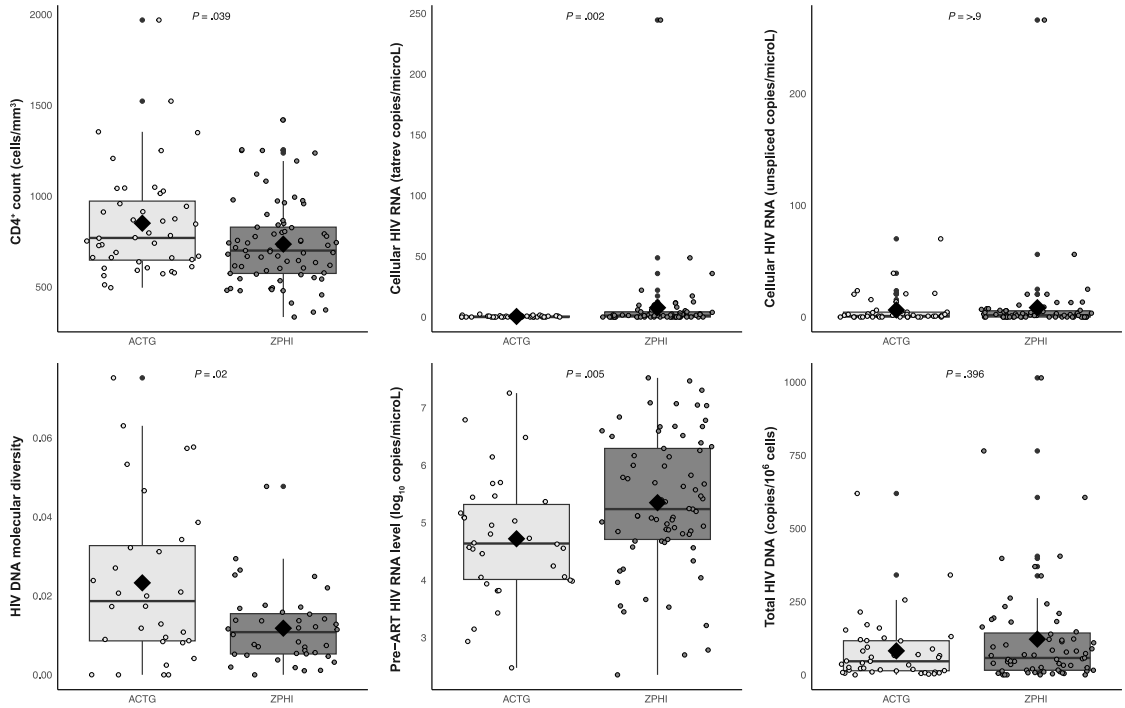

Figure 1. The first quartile (Q1, bottom line) and third quartile (Q3, top line) The height of the box represents the IQR, calculated as IQR = Q3 − Q1. Middle line is the median. Diamond shows average values. Abbreviations: ACTG, Advancing Clinical Therapeutics Globally for HIV/AIDS and Other Infections; ART, antiretroviral therapy; HIV, human immunodeficiency virus; ZPHI, Zurich HIV Primary Infection cohort.

differentiated $CD8^+$ T cells held constant, a 1-unit increase in total HIV DNA was associated with a 68% higher risk of viral rebound. Similarly, holding total HIV DNA constant, a 1-unit increase in the proportion of terminally differentiated $CD8^+$ T cells corresponded to a 3% increase in the risk of viral rebound.

DISCUSSION

We conducted a comprehensive assessment of virologic and immunologic risk factors of HIV rebound following treatment interruption, combining individuals from 2 cohorts: ZPHI (n = 73) and A5345 (n = 44). As anticipated, starting ART during the chronic stage of HIV was associated with a higher risk of viral rebound (therefore a shorter time to rebound) compared to those treated during the earlier stages. This is likely a consequence of initiating ART during the acute/early stage after HIV acquisition results in a smaller viral reservoir, better-preserved immune function, lower levels of immune activation, and fewer long-lived memory T cells harboring the virus [18–20].

Focusing on virologic variables, higher pre-ART plasma HIV RNA levels consistently correlated with a shorter time to rebound in the combined cohort, as well as in stratified analysis among ZPHI participants, those who started ART during acute/early HIV, and male participants. Higher levels of HIV DNA and increased cellular HIV transcription (HIV RNA_{Gag}) at the time of ART interruption were significantly linked to an increased risk of viral rebound in the combined cohort, which is consistent with previous reports [21, 22]. No significant association was found for HIV RNA_{TatRev}, 2-LTR circles, and proviral diversity in the combined cohort. Notably, stratified analysis revealed that higher proviral diversity was associated with an increased risk of viral rebound in the ZPHI cohort, as well as among male participants. This is consistent with a previous study showing that lower viral diversity before undergoing treatment interruption is associated lower replication capacity of the rebounding virus and spontaneous viral control [23]. Additionally, there are inherent biological differences between men and women that influence how HIV evolves and how the immune system responds to the virus [24]. Hormonal differences, genetic factors, and variations in immune system functioning could contribute to these discrepancies [25]. While limited by the low number of women in our study, these findings suggest potential sex differences in the impact of virologic factors on the timing of viral rebound. This underscores the importance of considering demographic variables in understanding HIV pathogenesis and treatment response, especially including enough women and other underrepresented participants to perform well-powered stratified analyses.

Table 2. Virologic Risk Factors of Time to Viral Rebound in Combined Dataset (ZPHI + ACTG) and Stratified by Cohort, HIV Staging at the Time of ART Initiation, and Sex at Birth

Strata	Sample Size	Hazard Ratio	(95% CI)	*P* Value
Total HIV DNA (gag)				
Combined	110	1.565	(1.059–2.311)	**.027**
By cohort				
ZPHI	67	1.645	(.77–3.513)	.193
ACTG	43	1.538	(.753–3.140)	.237
By HIV staging				
Early	78	1.541	(.875–2.715)	.129
Chronic	32	1.595	(.636–4.026)	.333
By sex at birth				
Male	98	1.463	(.868–2.467)	.149
Female[a]	12	…	…	…
HIV DNA molecular diversity				
Combined	72	1.153	(.951–1.397)	.154
By Cohort				
ZPHI	40	2.29	(1.017–5.154)	**.045**
ACTG	32	1.005	(.813–1.243)	.959
By HIV staging				
Early	45	1.379	(.742–2.563)	.313
Chronic	27	0.989	(.777–1.26)	.930
By sex at birth				
Male	64	1.271	(1.048–1.54)	**.015**
Female[a]	8	…	…	…
Cellular HIV RNA (unspliced, gag)				
Combined	109	1.032	(1.006–1.059)	**.015**
By cohort				
ZPHI	66	1.053	(.945–1.175)	.349
ACTG	43	1.018	(.972–1.067)	.449
By HIV staging				
Early	77	1.044	(.955–1.140)	.343
Chronic	32	1.021	(.972–1.071)	.409
By sex at birth				
Male	98	1.027	(1–1.054)	**.046**
Female[a]	11	…	…	…
Pre-ART plasma HIV RNA (log_{10} scaled)				
Combined	107	1.283	(1.047–1.572)	**.016**
By cohort				
ZPHI	73	1.398	(1.056–1.850)	**.019**
ACTG	34	1.294	(.895–1.870)	.170
By HIV staging				
Early	84	1.354	(1.055–1.738)	**.017**
Chronic	23	2.261	(.917–5.575)	.076
By sex at birth				
Male	96	1.325	(1.050–1.671)	**.018**
Female[a]	11	…	…	…

Analysis for 2-LTR and cellular HIV RNA encoding for *tat/rev* were not significant for any of the analyses conducted (data not shown). Significant *P* values are in bold.

Abbreviations: ACTG, Advancing Clinical Therapeutics Globally for HIV/AIDS and Other Infections; ART, antiretroviral therapy; CI, confidence interval; HIV, human immunodeficiency virus; LTR, long terminal repeat; ZPHI, Zurich HIV Primary Infection cohort.

[a]Analyses for these strata were not conducted due to limitations related to the small sample size.

When comparing our results with other studies on virologic risk factors, we observe some discrepancies. For instance, in previous A5345-only analysis, we found that intact (but not total) proviral DNA levels were the strongest predictor of HIV rebound in the chronic-treated group [5]. By contrast, in the SPARTAC trial [3], total HIV DNA was associated with time to rebound, whereas integrated HIV DNA was not. Similarly, the SALTO study group reported an inverse association between total HIV DNA levels and time to rebound [26], while another study found no association with HIV DNA but identified a link with cellular HIV RNA [1]. These differences may stem from several factors: variations in viral reservoir maturity depending on the timing of ART initiation, methodological differences in assay sensitivity and specificity, and individual host immune responses that influence reservoir dynamics.

While viral characteristics are crucial, they are not the sole factors influencing HIV rebound during treatment interruption and the immunologic environment is likely to play a crucial role. In our analysis, we found that lower $CD4^+$ T-cell proportion at the time of ART interruption were associated with increased risk of viral rebound in the combined cohort [8]. Several other immunologic factors were identified as negatively correlated with time to rebound after adjusting for FDR. Specifically, higher proportions of effector $CD4^+$ T cells and terminally differentiated $CD8^+$ T cells, as well as more activated and exhausted $CD4^+$ and $CD8^+$ T cells, were associated with increased risk of viral rebound in the subset of participants who started ART during acute/early HIV. No significant immunological risk factors were found in participants who started ART during chronic HIV, in either cohort individually, or when stratifying by sex at birth. Our findings are consistent with the previous A5345 study [5] as well as results from the SPARTAC trial showing that markers of T-cell exhaustion (but not activation) measured prior to ART initiation predicted time to the rebound in participants starting ART during acute/early HIV [27]. Interestingly, degranulation and cycling markers were not significantly associated with viral rebound in our study, possibly reflecting transient activation states rather than sustained functional capacity in our specific population.

Overall, these findings suggest that a less differentiated and less activated immune cell profile may protect against rapid viral rebound. Effector $CD4^+$ T cells and terminally differentiated $CD8^+$ T cells are more mature and specialized in their immune functions, often linked to higher levels of immune activation, pathogen exposure, and a diminished ability to control HIV [28, 29]. Similarly, chronically activated and exhausted T cells may contribute to reduced viral control, providing the virus with more opportunities to rebound when treatment is interrupted [30]. Exhausted T cells might also be associated with latently infected cells that are more prone to viral transcription [27]. In contrast, lower proportions of these differentiated and activated cells indicate a more quiescent immune environment, which could decrease the likelihood of viral reactivation and proliferation [31]. This underscores the importance of maintaining a balanced immune profile in managing HIV and preventing rebound [32].

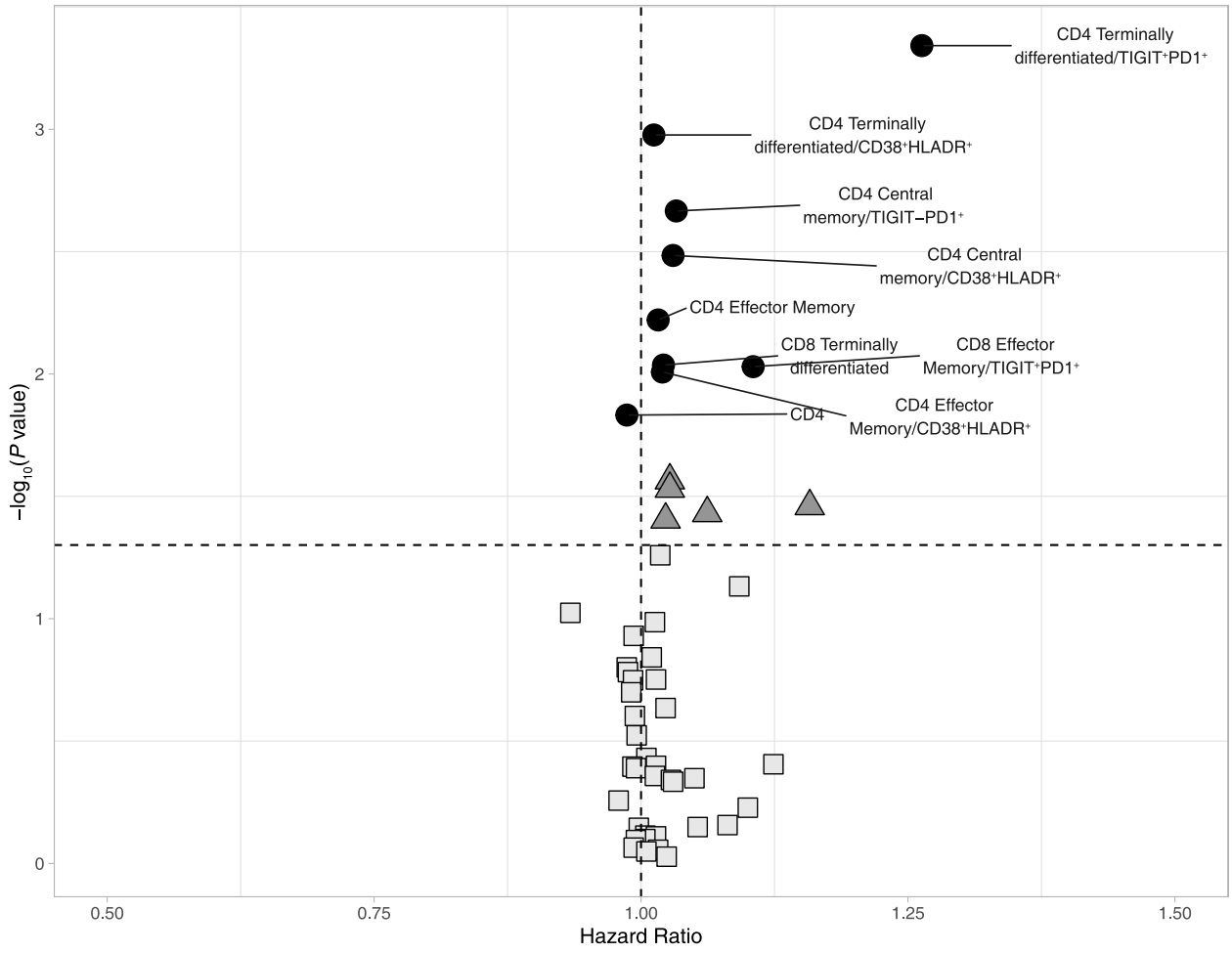

Figure 2. Immunological factors significantly associated with time to viral rebound after FDR adjustment in combined dataset (ZPHI + ACTG study A5345, n = 113). HR is shown on the X-axis: HR <1 indicates positive association, and HR > 1 indicates negative association with time to viral rebound. Y-axis shows *P* values after FRD adjustment. Gray squares are not significant, darker gray triangle are significant at $P < .05$ before FDR, and black dots are significant at $P < .05$ before and after FDR. Abbreviations: ACTG, Advancing Clinical Therapeutics Globally for HIV/AIDS and Other Infections; FDR, false discovery rate; HR, hazard ratio; ZPHI, Zurich HIV Primary Infection cohort.

These insights highlight the complexity of the immune system's role in controlling HIV and underscore the importance of targeting specific immune cell subsets in efforts to achieve sustained viral suppression or eradication. It is important to note that immune responses in early treated individuals are more homogenous and might be more uniformly effective at controlling the virus. This homogeneity could result in clearer correlations between immune factors and viral rebound and explain why these effects have been observed exclusively in the subset of participants who started ART during the acute and early stage of HIV.

Finally, when applying variable selection for multivariable analysis, we identified total HIV DNA and the proportion of terminally differentiated $CD8^+$ T cells as the most relevant risk factors for viral rebound in the combined cohort, highlighting the potential role of both viral reservoir size and immune cell differentiation status in influencing treatment outcomes.

This study has several limitations. First, we did not measure the intact HIV reservoir, and only immune markers from T cells were assessed. Additionally, the virologic and clinical analyses lacked FDR correction. While the study included a significant number of individuals who began ART during the acute/early HIV stages, the sample size remains small for stratified analyses, particularly for female and nonwhite subgroups. We also did not measure coinfecting viruses or evaluate non-blood reservoirs, which are critical for advancing cure research [33, 34]. Moreover, viral load monitoring after ART interruption was relatively sparse for participants enrolled in the ZPHI cohort, limiting our ability to precisely characterize rebound dynamics. Lastly, the ART restart criteria were stringent compared to more recent protocols of monitored treatment interruption.

In summary, our findings collectively highlight the complex interplay between virologic and immunologic factors in

determining HIV rebound dynamics especially in participants who started ART during chronic HIV, emphasizing the importance of early ART initiation. Furthermore, it will be important to consider demographic (eg, sex at birth) and immune profiles (eg, minimize T cell activation and exhaustion) to optimize treatment outcomes and inform strategies towards achieving sustained viral suppression or eradication.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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