Occurrence of Accelerated Epigenetic Aging and Methylation Disruptions in Human Immunodeficiency Virus Infection Before Antiretroviral Therapy

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Background: Whether accelerated aging develops over the course of chronic human immunodeficiency virus (HIV) infection or can be observed before significant immunosuppression on is unknown. We studied DNA methylation in blood to estimate cellular aging in persons living with HIV (PLWH) before the initiation of antiretroviral therapy (ART).

Methods: A total of 378 ART-naïve PLWH who had CD4 T-cell counts >500/μL and were enrolled in the Strategic Timing of Antiretroviral Therapy trial (Pulmonary Substudy) were compared with 34 HIV-negative controls. DNA methylation was performed using the Illumina MethylationEPIC BeadChip. Differentially methylated positions (DMPs) and differentially methylated regions (DMRs) in PLWH compared with controls were identified using a robust linear model. Methylation age was calculated using a previously described epigenetic clock.

Results: There were a total of 56 639 DMPs and 6103 DMRs at a false discovery rate of <0.1. The top 5 DMPs corresponded to genes *NLRC5*, *VRK2*, *B2M*, and *GPR6* and were highly enriched for cancer-related pathways. PLWH had significantly higher methylation age than HIV-negative controls ($P = .001$), with black race, low CD4 and high CD8 T-cell counts, and duration of HIV being risk factors for age acceleration.

Conclusions: PLWH before the initiation of ART and with preserved immune status show evidence of advanced methylation aging.

Keywords. HIV; aging; epigenetics; methylation.

With antiretroviral therapy (ART), persons living with human immunodeficiency virus (HIV) (PLWH) are living longer with fewer morbid effects related to AIDS-defining illnesses [1, 2]. Greater life expectancy has also meant that PLWH are also living long enough to experience common age-related illnesses, such as coronary artery disease, chronic obstructive pulmonary disease, and non-AIDS defining cancers [2, 3]. Whether the product of immunosuppression, unintended side effects from ART, or other still poorly defined factors, these age-related comorbid conditions may be occurring at earlier ages in PLWH

than in those without HIV [4]. Targeting or preventing periods of rapid cellular aging in PLWH may reduce negative outcomes associated with aging and HIV.

Given the complex and multifaceted process of aging, the estimation of biological or cellular age encompasses a wide range of measurements including telomere length and epigenetic markers, such as DNA methylation. A large difference between age as measured by these biomarkers and a person's chronological age may identify a rapid age acceleration process that could potentially portend the onset of age-related illnesses in PLWH. Leung et al [5] have previously reported on significant peripheral leukocyte telomere shortening detectable within the first 2 years after HIV seroconversion in a population of injection drug users, suggesting that cellular aging occurs fairly rapidly along the time course of HIV infection. DNA methylation (the dynamic addition or removal of a methyl group at cytosine-phosphate-guanine [CpG] sites), however, provides highly accurate measurements of biological age [5] and furthermore has the ability to identify epigenetically regulated genes associated

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with disease. For instance, methylation at CpG-rich island regions can influence gene expression.

A multitissue age predictor based on the weighted methylation status of 353 CpG residues (the Horvath clock) has recently been shown to correlate with disease status in HIV and numerous other conditions [6, 7]. Initial studies of DNA methylation in PLWH have shown that methylation age begins to deviate from the expected aging trajectory as the duration of HIV infection increases [5]. We hypothesized that PLWH, even before significant immunosuppression and the start of ART, demonstrate greater epigenetic aging compared to uninfected individuals. Furthermore, we hypothesize that distinct methylation patterns in PLWH when compared to uninfected individuals will identify epigenetically modified genes that can clarify the mechanisms of aging in PLWH.

METHODS

Study Cohorts

This cross-sectional study consisted of PLWH enrolled in both the genomics and pulmonary substudies of the previously described Strategic Timing of Antiretroviral Therapy (START; Clinicaltrials.gov NCT00867048 and NCT00867048) formed the HIV-infected cohort [8]. Briefly, this was a multicenter, international, randomized controlled trial that enrolled PLWH with CD4 T-cell counts >500/ μ L, subsequently randomizing them to either ART initiation immediately or deferred until the CD4 T-cell count declined to 350/ μ L or the onset of an AIDS-defining illness. The pulmonary substudy's design restricted enrollment to high-income countries to avoid confounding by biomass fuel exposure. The HIV-uninfected controls were taken from the Canadian Cohort of Obstructive Lung Disease (CanCOLD; Clinicaltrials.gov NCT00920348), a general population sample prospectively following Canadians for the presence and manifestations of airways diseases. The study was conducted approval by the University of British Columbia Ethics Board (approval no. H15-02166). Whole blood at study entry was collected from all participants and stored at -80°C .

DNA Methylation Profiling and Filtering Pipeline

DNA was extracted from whole-blood samples using the DNeasy Blood and Tissue Kit (Qiagen) and bisulfite converted using the EZ DNA Methylation Kit (Zymo). DNA methylation profiles were then obtained using the Illumina Infinium MethylationEPIC BeadChip microarray which interrogates 863 904 CpG sites [9]. This microarray covers 95% of RefSeq genes and 95% of CpG islands [10]. The β values (the ratio of the methylated probe intensity to the overall intensity, ranging from 0, indicating all unmethylated, to 1, indicating all methylated) are calculated for each CpG site and converted to M values (the $\log_2$ ratio of the intensity of the methylated probe versus unmethylated probe) for statistical analyses [11]. CpG sites were filtered for detection quality and probes with a detection P

value $>1 \times 10^{-10}$ were removed from downstream analysis. Non-CpG, XY-linked, and cross-hybridization probes and probes containing single-nucleotide polymorphisms were also removed. Background correction, normalization, and batch correction were performed using the normal-exponential out-of-band [12], beta-mixture quantile normalization [13], and ComBat (sva 3.30.1) [14] methods, respectively. Finally, Gaphunter (R package “minfi” 1.28.4) was used to remove outlier CpG probes [15].

Epigenetic Clock

Methylation age (DNAm) was calculated using the Horvath clock (<https://horvath.genetics.ucla.edu/html/dnamage/>) [16], a multitissue age predictor validated in numerous disease processes, including HIV [6, 7]. While DNAm is calibrated in units of years, the measure of age acceleration is based on the residual regressing DNAm on chronological age. The age acceleration residual refers to the regression residuals of the linear model, which regress DNAm on chronological age. Higher values of the age acceleration residual are therefore indicative of faster age acceleration.

The age acceleration residual is a global marker of methylation aging; 2 additional measures, however, can capture cell-intrinsic and cell-extrinsic methylation aging [17]. Intrinsic epigenetic age acceleration (IEAA), measuring methylation aging that is independent of blood cell composition, refers to the residuals of the linear model regressing DNAm on chronological age and peripheral blood immune cells (plasmablasts, naive and exhausted CD8 T cells, CD4 T cells, natural killer (NK) cells, monocytes, and granulocytes). Extrinsic epigenetic age acceleration (EEAA) is produced by using the methylation age calculated by methods described by Hannum et al [18], and increasing the weights assigned to 3 age-related immune cells (naive [CD45RA⁺CCR7⁺] and exhausted [CD28⁻CD45RA⁻] cytotoxic T cells, and plasmablasts), and then regressing this measure on chronological age and taking the residuals. As such, EEAA captures immunosenescence and the changes that occur in peripheral blood cell composition with aging.

Statistical Analysis

Two variables known to have a significant effect on methylation were inferred from the data: cell type proportion and ancestry. Cell type proportions of CD4 and CD8 T cells, NK cells, monocytes, neutrophils, eosinophils, and B cells were inferred using the deconvolution method of Houseman et al [19]. Ancestry was inferred using EPISTRUCTURE [20], a program using principal components computed from CpGs that are highly correlated with single-nucleotide polymorphisms to capture the genetic variation within a population. Additional covariates were chosen based on principal component analysis (PCA) identifying these variables as having significant ($P < .01$) association with methylation (Supplementary Figure 1). Of note,

sex (principal component [PC] 1 [$P = 2.1 \times 10^{-4}$] and PC4 [$P = .017$]) and current smoking status (PC4 [$P = 4.65 \times 10^{-3}$]) were significantly associated with methylation status.

To identify significantly differentially methylated CpG sites in PLWH compared with HIV-negative controls, a robust linear model was used, adjusting for these significant covariates, as follows:

$$\text{Methylation (M value)} \sim \text{HIV status} + \text{Sex} + \text{Age} + \text{Current smoking status} + \text{CD8 T cells} + \text{CD4 T cells} + \text{NK cells} + \text{B cells} + \text{Monocytes} + \text{Neutrophils} + \text{EPISTRUCTURE PC1-PC10}.$$

Eosinophils were excluded from the model because they did not have a significant association with methylation. Differentially methylated positions (DMPs) were identified if they had a false discovery rate (FDR) < 0.1 . Differentially methylated regions (DMRs), defined as ≥ 3 contiguous CpG probes, were identified using DMRcate (R, Bioconductor 1.18.0) [21]. To identify enriched biological pathways associated with HIV, DMPs were converted to their corresponding genes and entered into the Kyoto Encyclopedia of Genes and Genomes (KEGG; www.genome.jp/kegg) using the R package WebGestaltR (0.4.3), with statistical significance set at an FDR < 0.1 , comparing pathway-associated with non-pathway-associated genes.

To determine accelerated epigenetic aging in PLWH, the age acceleration residual, IEAA, and EEAA were compared between PLWH and HIV-negative controls using multivariable linear models. Sex, ethnicity, and smoking status were considered as potential covariates and were backward-selected using the Akaike information criterion. Chronological age was not considered as a potential covariate because the age acceleration residual, EEAA, and IEAA are the residuals of models regressing DNAm on chronological age and are thus independent of chronological age by the definition of regression residuals. Spearman correlation was calculated between the age acceleration residual and CD4 and CD8 T-cell counts, CD4/CD8 ratio, and HIV loads. A multivariable linear regression model using age acceleration residual as the response variable was used. Predictors to be included in the multivariable model, such as sex, baseline body mass index (BMI), smoking status, education level, HIV acquisition, race, baseline CD4 and CD8 T-cell counts, and time since HIV diagnosis, were again backward-selected using the Akaike information criterion. Statistical significance was set at $P < .05$. All statistical analyses were performed using R software (version 3.5.0).

Pyrosequencing Validation

We performed bisulfite pyrosequencing validation on selected DMPs (cg18805164, cg09858955, and cg07839457), which had both small P values and large absolute methylation β differences. Detailed pyrosequencing methods can be found in the [Supplementary Materials](#).

RESULTS

Study Demographics

Baseline characteristics of PLWH ($n = 378$) and HIV-negative controls ($n = 34$) are shown in [Table 1](#). PLWH were significantly younger and more likely to be male than the HIV-negative controls. They were also more likely to be current smokers, while HIV-negative controls were more likely to be former smokers. PLWH also had lower BMI values.

Association Between HIV Infection and Significant Changes in DNA Methylation

Overall, significant changes in global DNA methylation were observed between the 2 study groups. [Figure 1](#) shows the PCA plot for PLWH and HIV-negative controls, with significant separation of methylation patterns along PC1 ($P = 1.19 \times 10^{-11}$). After removing CpGs that were not annotated to any genes, in total, 56 639 DMPs and 6103 DMRs were found, with the breakdown by absolute β difference and position in an island region shown in [Supplementary Table 1](#). A full listing of DMPs and DMRs can be found in the [Supplementary Materials](#). As a sensitivity analysis, we also tried a more stringent significant FDR threshold of < 0.05 ; the majority of the DMPs and DMRs remained significant ([Supplementary Table 1](#)).

A volcano plot demonstrating the DMPs with the highest statistical significance and largest methylation β differences between the 2 groups is shown in [Figure 2](#). The only DMP demonstrating an absolute methylation β difference > 0.2 was mapped to gene *ARHGAP33*; the top 5 DMPs demonstrating an absolute methylation β difference > 0.1 were mapped to genes *NLR5*, *VRK2*, *B2M*, and *GPR6*. Within those DMPs demonstrating an absolute methylation β difference > 0.1 , the DMPs mapping to *NLR5*, *GPR6*, and *DUSP22* were found in island regions. The top 4 DMRs mapped to the genes *FOXG1/ RP11-966I7.1*, *MACROD2/SELIL2*, *DPP6*, and *CNOT2*. All 4 DMRs, except the DMR mapping to *CNOT2*, are in the island region ([Supplementary Figure 2](#)). Features of these DMPs and DMRs are provided in [Tables 2](#) and [3](#).

Pyrosequencing validation was performed on cg18805164 (corresponding to *ARHGAP33*), the only DMP with an absolute methylation β difference > 0.2 , as well as cg09858955 (corresponding to *VRK2*) and cg07839457 (corresponding to *NLR5*), which are in the top 3 DMPs that demonstrated an absolute methylation β difference > 0.1 . We did not perform pyrosequencing on cg16411857 (the top DMP with an absolute methylation β difference > 0.1) because it also maps to gene *NLR5* and the effect size is smaller than cg07839457. Validation of these CpGs is shown in [Supplementary Figure 3](#). All 3 CpGs were successfully validated. The top KEGG biological pathways associated with the DMPs are shown in [Figure 3](#). DMPs were significantly enriched for pathways related to cancer, including Hippo signaling pathway, pathways in cancer, phospholipase D

Table 1. Baseline Demographics of the Study Cohort

Variable	Study Participants, % ^a		PValue
	PLWH (n = 378)	HIV-Negative Controls (n = 34)	
Age, median (IQR), y	37 (31–45)	46 (45–48)	<.001
Female sex	8	35	<.001
Current smokers	46	18	.002
Former smokers	19	35	.025
Never smokers	35	47	.198
Smoking pack-years, median (IQR)	2 (0–10)	0 (0–16)	.694
BMI, median (IQR)	24.3 (22.3–27.5)	27.8 (24.7–30.3)	<.001
Race			
Black	18	0	<.001
Asian	1	6	
Hispanic	17	0	
White	62	94	
Other	2	0	
Time since HIV diagnosis, median (IQR), d	402 (162–1275)	...	...
CD4 T-cell count, median (IQR), cells/μL			
Baseline	641 (583–739)	...	...
Nadir	535 (460–640)	...	...
Baseline HIV load, median (IQR), copies/μL	17 805 (4450–56 453)	...	...
HIV risk factors, %			
Injection drug use	2	...	...
Same sex contact	72	...	...
Opposite sex contact	21	...	...
Other	5	...	...

Abbreviations: BMI, body mass index; HIV, human immunodeficiency virus; IQR, interquartile range; PLWH, persons living with HIV.

^aData represent % of study participants unless otherwise specified.

^bP values calculated with Mann-Whitney U or Fisher exact tests.

^cBMI calculated as weight in kilograms divided by height in meters squared.

Figure 1. Principal component analysis demonstrates significant overall differences in global methylation patterns between persons living with human immunodeficiency virus (HIV) (PLWH) (blue) and HIV-negative controls (red). Separation of the 2 patterns is significant along the principal component (PC) 1 axis ($P = 1.19 \times 10^{-11}$).

Figure 2. Volcano plot showing statistically significant differentially methylated positions (DMPs), with human immunodeficiency virus (HIV)–negative controls serving as the reference group. DMPs in red are hypermethylated in persons living with HIV compared with HIV-negative controls; those in blue are hypomethylated. Horizontal dotted line indicates a false discovery rate threshold of 0.1; vertical dashed lines, an absolute β difference of 10%.

Table 2. Features of the 15 Most Significantly Affected Differentially Methylated Positions^a

CpG	Gene	Chromosome	Relationship to Island ^b	Methylation β Difference ^c	PValue	FDR
cg16411857	NLRC5	16	Island	-0.11572	1.06×10^{-20}	1.96×10^{-16}
cg09858955	VRK2	2	OpenSea	-0.14734	2.04×10^{-14}	6.21×10^{-11}
cg07839457	NLRC5	16	N_Shore	-0.18981	3.79×10^{-14}	1.08×10^{-10}
cg12828896	B2M	15	S_Shore	-0.17806	4.64×10^{-13}	9.29×10^{-10}
cg11372636	GPR6	6	Island	0.107029	7.88×10^{-12}	1.00×10^{-8}
cg00218406	HCP5	6	OpenSea	-0.12763	7.73×10^{-11}	6.46×10^{-8}
cg03425812	B2M	15	S_Shore	-0.16671	1.53×10^{-10}	1.13×10^{-7}
cg27537252	B2M	15	S_Shelf	-0.12704	3.48×10^{-6}	3.09×10^{-4}
cg11671940	RP11-351M16.3	10	OpenSea	0.151881	1.83×10^{-5}	1.09×10^{-3}
cg14827090	WDR25	14	OpenSea	-0.11712	5.25×10^{-5}	2.40×10^{-3}
cg00959259	PARP9,DTX3L	3	N_Shore	-0.10508	1.45×10^{-4}	5.10×10^{-3}
cg18805164	ARHGAP33	19	N_Shore	-0.21586	1.72×10^{-4}	5.80×10^{-3}
cg11235426	DUSP22	6	Island	0.156257	3.24×10^{-3}	.0468
cg07332563	DUSP22	6	N_Shore	0.145178	3.38×10^{-3}	.0482
cg17876578	DUSP22	6	N_Shore	0.131111	4.87×10^{-3}	.0616

Abbreviations: CpG, cytosine-phosphate-guanine; N, north (upstream, 5'end); S, south (downstream, 3'stream).

^aIncluding differentially methylated positions with absolute β values >0.1.

^b"Relationship to Island" described the density of CpG sites. CpG islands are regions with a high frequency of CpG sites; shores, <2-kb flanking CpG islands; and shelves, <2-kb islands flanking outward from a CpG shore; remaining genomic regions are considered as open sea area.

^cHuman immunodeficiency virus uninfected controls served as the standard for the methylation β value.

Table 3. Features of the 4 Most Significantly Affected Differentially Methylated Regions^a

Genes	Chromosome	Position, Mb		CpGs, No.	Methylation β Difference ^a		Minimum FDR	Stouffer PValue ^b
		Starting	Ending		Maximum	Mean		
FOXG1 RP11-966I7.1	14	29.2342	29.2375	29	0.035	0.016	1.24×10^{-80}	2.69×10^{-45}
MACROD2 SEL1L2	20	13.9746	13.9777	22	0.056	0.023	2.51×10^{-86}	2.64×10^{-43}
DPP6	7	153.5829	153.5847	14	0.088	0.034	6.06×10^{-65}	9.02×10^{-43}
CNOT2	12	70.7209	70.7213	5	-0.055	-0.036	1.60×10^{-54}	2.37×10^{-39}

Abbreviations: CpG, cytosine-phosphate-guanine; FDR, false discovery rate.

^aHuman immunodeficiency virus uninfected controls served as the standard for the methylation β value.

^bCombined FDR of all the CpG sites within a region using the Stouffer method.

Figure 3. Top 10 the Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways meeting a false discovery rate (FDR) threshold of <0.1. Circle sizes correspond to the number of genes overlapped between each pathway and the differentially methylated position genes. Abbreviations: ARVC, arrhythmogenic right ventricular cardiomyopathy; ECM, extracellular matrix.

signaling, proteoglycans related to cancer, and extracellular matrix receptor interaction.

Association Between HIV Infection and Accelerated Epigenetic Aging

DNAm was highly correlated with chronological age in the study cohort ($P < 2.2 \times 10^{-16}$; Spearman $\rho = 0.871$) as shown in [Supplementary Figure 4](#). For models comparing age acceleration residual, EEAA, and IEAA between HIV-negative controls and PLWH, ethnicity was the only covariate included in the final model after model selection. Compared with HIV-negative controls, PLWH demonstrated significantly higher age acceleration residual ($P = .016$) and higher EEAA ($P = .044$) ([Figure 4A](#) and [4B](#)), suggesting both global epigenetic aging and cell-extrinsic aging related to immune cell proportion changes occur early in HIV infection. In contrast, IEAA was not significantly different between the 2 groups ($P = .642$) ([Figure 4C](#)). To further confirm our findings, we performed the same analysis in whites (32 HIV-negative controls vs 237 PLWH); the results remained the same: PLWH demonstrated higher age acceleration residual ($P = .020$) and higher EEAA ($P = .022$), but there was no difference in IEAA between the 2 groups ($P = .584$).

The age acceleration residual was weakly but significantly negatively correlated with baseline CD4 T-cell count ($P = .024$; Spearman $\rho = -0.116$), nadir CD4 T-cell count ($P = .016$; Spearman $\rho = -0.123$), and CD4/CD8 ratio ($P = 4.52 \times 10^{-5}$; Spearman $\rho = -0.208$). The age acceleration residual was also weakly but significantly positively correlated with baseline CD8 T-cell count ($P = 8.26 \times 10^{-4}$; Spearman $\rho = 0.172$) and with time since HIV diagnosis ($P = 4.94 \times 10^{-6}$; Spearman $\rho = 0.232$). There was no significant relationship between the age acceleration residual and HIV load ($P = .212$). The correlations are summarized in [Supplementary Table 2](#).

After a backward model selection from all the demographic variables such as sex, baseline BMI, smoking status, education level, HIV acquisition, race, baseline CD4 and CD8 T-cell counts, and time since HIV diagnosis, we performed a

multivariable linear regression with age acceleration residual as the response variable and sex, race, baseline CD4 and CD8 T-cell counts, and time since HIV diagnosis as the predictors. Sex was not significantly associated with age acceleration residual, while black race ($P < .001$), low baseline CD4 T-cell count ($P = .004$), high baseline CD8 T-cell count ($P < .001$), and more time since HIV diagnosis ($P < .001$) remained significant risk factors for age acceleration ([Table 4](#)). Similarly, as a sensitivity analysis, we performed the same model in whites only and the risk factors remained the same (baseline CD4 T-cell count, $P = .049$; baseline CD8 T-cell count, $P = .016$; time since HIV diagnosis, $P < .001$), which further confirmed our findings.

DISCUSSION

Our epigenome-wide methylation analysis is one of the largest of its kind in PLWH and one of the first to our knowledge to use the broader MethylationEPIC BeadChip microarray for adult PLWH. The study revealed that even PLWH with relatively preserved immune status, before ART initiation, can display significant methylation changes and greater methylation age acceleration compared with HIV-negative controls. This would suggest that prolonged periods of immunosuppression, the onset of opportunistic infections, or long-term exposure to ART are not necessarily required for epigenetic changes to occur. While clinically important outcomes related to advanced methylation age in PLWH are yet to be determined, previous studies in the general population have demonstrated that this measure is associated with increased death rates [17, 22], including deaths related to cancer and cardiovascular disease [22].

We detected profound differences in methylation patterns between PLWH and HIV-negative controls, with a total of 56 639 DMPs and 6103 DMRs distinguishing the 2 groups at an FDR threshold of <0.1 . This is a sizeable increase over the differences detected in previous epigenome-wide analyses comparing PLWH with HIV-negative individuals, using the Illumina 450k

Figure 4. A, B, Age acceleration residual (A) and extrinsic epigenetic age acceleration (EEAA) (B) are significantly higher in persons living with PLWH human immunodeficiency virus (HIV) compared with HIV-negative controls ($P = .016$ and $P = .044$, respectively). C, There was no significant difference in intrinsic epigenetic age acceleration (IEAA) between the 2 groups ($P = .642$).

Table 4. Factors Associated With Age Acceleration

Factor ^a	Estimate (SE)	PValue
Race (white [reference] vs black)	2.409 (0.586)	<.001
Baseline CD4 T-cell count (per 100/ μ L)	−0.374 (0.130)	.004
Baseline CD8 T-cell count (per 100/ μ L)	0.122 (0.036)	<.001
Time from HIV diagnosis (per year)	0.271 (0.068)	<.001

Abbreviations: HIV, human immunodeficiency virus; SE, standard error.

^aNonsignificant terms (sex and race [white vs other races]) were not reported.

platform [23, 24]. Our increased sample size and the use of the broader MethylationEPIC BeadChip microarray (which nearly doubles the number of measured CpG sites) could account for these differences in results. Importantly, though, we were able to replicate, with both microarray and pyrosequencing techniques, the findings of Zhang et al [24] that *NLR5* is an epigenetically modified, hypomethylated gene in HIV infection. *NLR5*, found on chromosome 16, is a transactivator of major histocompatibility complex class I genes; is involved in cellular immune response, inflammation, and host interactions with infectious pathogens; and has also been associated with frailty, injection drug use, and hepatitis C in PLWH [25]. Although the exact role that *NLR5* plays in HIV needs to be further clarified, the consistent identification of this gene in methylation studies suggests that HIV may modify its epigenetic regulation.

In addition, we discovered new genes whose methylation may be altered by HIV infection. Among these was *VRK2*, encoding for a serine/threonine kinase, which was significantly hypomethylated among PLWH. Although little is known as to whether *VRK2* plays a role in early HIV infection, it has known associations with apoptosis [26] and tumor suppression. Down-regulation of *VRK2* expression, for instance, is protective against tumor invasion [27]. Altogether, the most differentially methylated genes were highly enriched for pathways related to cancer. The links between HIV and cancer are well known, not just for AIDS-defining cancers, such as Kaposi sarcoma and non-Hodgkin lymphoma, but also for non-AIDS-defining cancers that afflict the lung, anus, and liver [28]. Our study raises the possibility that the initial insults leading to this increased oncogenic risk may come in the early phases of HIV infection and may be mediated through epigenetic disruptions.

In our study cohort, PLWH with low CD4 and high CD8 T-cell counts were at elevated risk of developing epigenetic age acceleration, confirming Horvath and Levine's previous finding in a cohort of 109 male PLWH [6]. The age acceleration signal was observed when using a global measure such as the age acceleration residual and when using the EEAA measure that specifically reflects the changing composition of peripheral blood cells, a hallmark of HIV infection. The relationship between immunosuppression and accelerated epigenetic aging may be partially explained by the theory of "inflammaging" [29], used to describe how chronic inflammation and inflammatory

cytokines may help drive the process of cellular senescence. Greater depletion of CD4 T cells with HIV infection contributes to the breakdown of the gut mucosal lining, driving microbial translocation and a persistent inflammatory response. This inflammatory response may in fact alter the epigenetic aging process. Recent evidence suggests that certain genetic polymorphisms in *IL-6* and *IL-10* are associated with advanced epigenetic age in PLWH [30]. In particular, PLWH who are *IL-6* −174C allele carriers have a >3-fold higher chance of developing an HIV-associated non-AIDS condition, such as cerebrovascular disease, chronic obstructive pulmonary disease, cancer, and diabetes.

Black PLWH in this cohort were also at greater risk for age acceleration in our study. The associations between epigenetic age and race have been somewhat conflicting. While the Horvath clock has previously demonstrated that African Americans have lower EEAA than Hispanics and whites [31], other analyses using different measures of epigenetic aging have reported the opposite association [32, 33]. Whether advanced epigenetic age might account for racial disparities in HIV outcomes is unknown; black PLWH are less likely to achieve viral suppression [34, 35] and in certain cohorts display higher HIV-related mortality rates than non-Hispanic whites [36, 37]. However, only in certain age-related comorbid conditions, such as chronic kidney disease [38] and cardiovascular disease [39], has there been a demonstrably higher prevalence among black PLWH. Given the uneven distribution of race and ethnicity in our cohort, we recommend interpreting our findings with caution and recommend further validation.

Although the current study provides one of the largest epigenome-wide assessments of PLWH, several limitations should be noted. First, our study cohort comprised PLWH with detectable viral loads and preserved CD4 T-cell counts, who had not yet started ART. As such, our results cannot be generalized to the many PLWH who have achieved viral suppression during long-term ART. It is entirely possible that DMPs and DMRs identified in this analysis may no longer be differentially methylated after chronic ART use. Longitudinal analyses of methylation in PLWH would help to clarify whether these changes can be reversed with treatment. Our cohort could have also included slow progressors and elite controllers, whose aging processes may differ from those of other PLWH; future analyses specifically targeting these groups would give greater insight into the process of aging in HIV.

Second, it is unclear in this study whether methylation disruptions result in downstream gene expression and ultimately protein changes. As such, how the genes identified in this analysis might link to cell injury and aging cannot be answered. Third, there were significant demographic differences between our PLWH and HIV-negative cohorts. While our statistical modeling attempted to account for these differences, future validation studies should occur in more equally matched controls.

Nonetheless, we still detected an age acceleration difference between the 2 cohorts despite the fact that our HIV-negative cohort was considerably older.

The results of our study suggest that the aging process can potentially start early in the course of HIV infection and that substantial methylation changes can occur even in the absence of opportunistic infections and AIDS-defining illnesses. Whether the immediate initiation of ART can help mitigate these changes and temper age acceleration should be the focus of future work, as should the exploration of methylation age as a biomarker in the care of PLWH.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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