# Differences in epigenetic age by HIV status among patients with a non-AIDS defining cancer

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**Objective:** People with HIV (PWH) are living longer and experiencing higher numbers of non-AIDS-defining cancers (NADC). Epigenetic aging biomarkers have been linked to cancer risk, and cancer is now a leading cause of death in PWH, but these biomarkers have not been investigated in PWH and cancer.

**Design:** In order to compare epigenetic age by HIV status, HIV-uninfected participants were matched to PWH by reported age, tumor site, tumor sequence number, and cancer treatment status.

**Methods:** DNA from blood was assayed using Illumina MethylationEPIC BeadChip, and we estimated immune cell composition and aging from three epigenetic clocks: Horvath, GrimAge, and epiTOC2. Age acceleration by clock was computed as the residual from the expected value, calculated using linear regression, for each study participant. Comparisons across HIV status used the Wilcoxon rank sum test. Hazard ratios and 95% confidence intervals for the association between age acceleration and survival in PWH were estimated with Cox regression.

**Results:** Among 65 NADC participants with HIV and 64 without, biological age from epiTOC2 ($P < 0.0001$) and GrimAge ($P = 0.017$) was significantly higher in PWH. Biological age acceleration was significantly higher in PWH using epiTOC2 ($P < 0.01$) and GrimAge ($P < 0.0001$), with the difference in GrimAge remaining statistically significant after adjustment for immune cell composition. Among PWH, GrimAge acceleration was significantly associated with increased risk of death (hazard ratio 1.11; 95% confidence interval (CI) 1.04–1.18).

**Conclusion:** We observed a higher epigenetic age in PWH with a NADC diagnosis compared with their HIV-uninfected counterparts, as well as a significant association between this accelerated biological aging and survival for patients diagnosed with a NADC.



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## Introduction

Due to widespread use of antiretroviral therapy (ART), people with HIV (PWH) are living longer. By 2030, the proportion of PWH over the age of 65 will more than double [1,2]. Cancers related to aging, including many non-AIDS-defining cancers (NADCs) such as prostate cancer, are not traditionally linked to severe immunosuppression or an oncogenic virus, but are now increasing within this population. It is projected that prostate and lung cancers will be the most common cancer types among PWH by 2030 [1].

Age as measured by chronological time, or years since birth, does not represent the complete spectrum of the aging process that occurs at a cellular level [3]. Several molecular markers of cellular aging have been identified, including the changing level of DNA methylation (DNAm) on CpG sites over time [4–6]. Leukocyte DNAm profiles change with aging, cellular replication, lifestyle, and exposure to carcinogens. Patterns of DNAm can be translated using epigenetic clocks that may estimate an age more reflective of the biologic aging process compared with a person's reported age [7,8]. Several clocks exist, each using a unique set of CpG sites and methylation patterns [9,10]. For example, Horvath's clock uses DNAm patterns that are highly correlated with a person's chronological age (i.e. estimate their reported age in years) and was developed and validated by identifying DNAm patterns using 8000 publicly available microarray samples and 30 tissue and cell types from children and adults [3]. GrimAge was built using methylation patterns that correlate with distinct clinical features related to mortality in the Framingham Heart Study [11]. epiTOC2 was built using methylation patterns that can be used to estimate the total number and rate of stem cell divisions [5,12].

Prior research has demonstrated that biological age in PWH, determined using epigenetic clocks, is accelerated beyond chronological or reported age. Studies have found that both ART-naive PWH and PWH receiving effective ART therapy have significantly higher epigenetic age than people without HIV. Epigenetic age is accelerated in PWH with depressed immunity (i.e. $CD4^+$ cell count below 200 cells/μl) [13], and in a small study of 15 PWH compared with 15 HIV-uninfected controls, age acceleration from four clocks [Horvath, Hannum, PhenoAge, and GrimAge] remained higher in PWH even after ART was initiated [14]. To date studies have not yet demonstrated the association of HIV with genome-wide methylation patterns and potentially accelerated aging among PWH in the context of cancer. We know that individuals who have developed a tumor have experienced some degree of advanced aging, but we hypothesized that even among cancer patients, those with HIV would have the highest levels of epigenetic aging.

As the burden of NADCs continues to increase among PWH, identification of clinically relevant aging biomarkers associated with cancer outcomes is a critical step towards understanding the cancer outcome disparity in PWH [15] and to inform cancer prevention and screening strategies. Using epigenetic changes could provide the ideal tool to achieve this goal, although HIV-specific factors like degree of immune competence should be taken into consideration. Therefore, we aimed to assess in this study HIV-related differences in epigenetic age that accounted for differences in patient immune cell composition among NADC patients with and without HIV.

## Methods

This cross-sectional study evaluated patients older than 18 years of age receiving care for cancer at Moffitt Cancer Center in Tampa, Florida, USA. PWH were evaluated for inclusion if they had an electronic medical record (EMR)-verified HIV diagnosis. Inclusion criteria for all participants were diagnosis with a solid organ NADC (all sites; further description can be found in Table 1) between 2004 and 2021, availability of a DNA-amenable specimen (whole blood, buffy coat, or PBMC) or stored genomic DNA extracted from blood, and specimen collection prior to cancer treatment or within 1 year of starting cancer treatment. HIV-uninfected participants were selected by matching to PWH on tumor site, reported age at cancer diagnosis within 5 years, tumor sequence number (primary cancer diagnosis, nonprimary diagnosis), and treatment status. Treatment status matching was based on whether the specimen was collected before or after the start of systemic cancer treatment (chemotherapy, radiation, immunotherapy, hormone therapy). Consent was provided by all participants prior to specimen collection. Ethical approval from Advarra IRB was provided prior to study initiation.

DNA was extracted from specimens using the Autopure LS automated DNA extractor (QIAGEN, Hilden, Germany) and quantified using Qubit fluorometric quantification (Invitrogen, Waltham, Massachusetts, USA). Five hundred nanograms of extracted and quantitated DNA was bisulfite converted using Zymo EZ DNA Methylation Kits (Zymo Research, Irvine, California, USA), and the converted DNA was hybridized to the Illumina Infinium MethylationEPIC BeadChip (Illumina, Inc., San Diego, California, USA), which provides the methylation status of more than 850 000 CpG sites across the human genome. The processed arrays were then scanned on an Illumina iScan scanner and QC was performed using the Illumina GenomeStudio software.

Methylation data were normalized using Noob [16]. $\beta$ values with a corresponding detection $P$ value greater than 0.05 were set to missing. Chip-wide control probes, frequency of missing values, a histogram of $\beta$ values, and

**Table 1. Characteristics of the study population.**

| | Without HIV ($n = 64$) [*N* (%)] | PWH ($n = 65$) [*N* (%)] |
|---|---|---|
| Age at cancer diagnosis | | |
| Median (IQR) | 55.5 (50.5–60.0) | 55 (51–60) |
| ≤50 | 16 (25.0) | 14 (21.5) |
| >50 | 48 (75.0) | 51 (78.5) |
| Vital status | | |
| Alive | 56 (87.5) | 41 (63.1) |
| Deceased | 8 (12.5) | 24 (36.9) |
| Race | | |
| Black | 2 (3.1) | 18 (27.7) |
| White | 58 (90.6) | 42 (64.6) |
| Other[a] | 4 (6.3) | 5 (7.7) |
| Sex | | |
| Female | 31 (48.4) | 15 (23.1) |
| Male | 33 (51.6) | 50 (76.9) |
| Primary site | | |
| Breast | 2 (3.1) | 3 (4.6) |
| GI[b] | 31 (48.4) | 33 (50.8) |
| GU[c] | 14 (21.9) | 12 (18.5) |
| GYN[d] | 2 (3.1) | 2 (3.1) |
| Head and neck[e] | 4 (6.3) | 4 (6.2) |
| Lung | 8 (12.5) | 8 (12.3) |
| Skin[f] | 3 (4.7) | 3 (4.6) |
| Tumor sequence number | | |
| 0 | 44 (68.8) | 48 (73.8) |
| 1 | 4 (6.3) | 2 (3.1) |
| 2 | 16 (25.0) | 15 (23.1) |
| Specimen collection relative to cancer treatment | | |
| Surgery only | 14 (21.9%) | 17 (26.2%) |
| Prior to any systemic therapy | 28 (43.8%) | 25 (38.5%) |
| ≤72 days of systemic therapy[g] | 9 (14.1%) | 6 (9.4%) |
| >72 days of systemic therapy[g] | 9 (14.1%) | 14 (21.9%) |
| No treatment | 4 (6.3%) | 3 (4.6%) |
| Specimen type | | |
| Whole blood | 52 (81.3%) | 51 (78.5%) |
| Buffy coat | 2 (3.1%) | 4 (6.2%) |
| PBMC | 0 | 1 (15.4%) |
| Genomic DNA | 10 (15.6%) | 9 (13.8%) |
| $CD4^+$ cell count at cancer diagnosis (cells/μl) | | |
| Mean (standard deviation) | | 392.8 (±376) |
| Median (range) | | 231 (16–1411) |
| *Missing* | | *38* |
| ART class at cancer diagnosis[h] | | |
| NRTI | | 52 (80.0%) |
| NNRTI | | 14 (21.5%) |
| Protease inhibitor | | 19 (29.2%) |
| Integrase inhibitor | | 20 (30.8%) |
| Entry/fusion inhibitor | | 1 (1.5%) |
| Year of HIV diagnosis | | |
| Median (range) | | 1994 (1984–2002) |
| *Missing* | | *47* |

ART, antiretroviral therapy; IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.
[a]American Indian, Asian Indian, Japanese, Pacific Islander, other.
[b]GI includes esophagus, stomach, colon, appendix, anorectal, pancreas.
[c]GU includes penis, prostate, kidney, bladder.
[d]GYN includes vulva, ovary.
[e]Head and neck includes tongue, cheek, larynx.
[f]Skin includes melanoma.
[g]Days relative to systemic therapy was categorized by the median number of days since treatment initiation within the patients who initiated systemic therapy.
[h]Proportions for ART class are not mutually exclusive.

principal component analysis (PCA) plots were used to visualize data quality and detect outlier samples to confirm no batch effects were observed. CpG sites differentially methylated by HIV status were identified using a false discovery rate (*q* value) less than 0.05 (statistical significance of difference by HIV status) and mean difference (beta) of 0.15 (magnitude of difference by HIV status). Details on differentially methylated regions are provided in Supplemental Material, http://links.lww.com/QAD/C933.

Epigenetic age using three clocks (Horvath, GrimAge, and epiTOC2) was estimated using output from the Illumina methylation array and translated through the EstimAge website (http://estimage.iac.rm.cnr.it). Horvath's clock is an epigenetic clock that was designed by identifying sets of CpG sites that are predictive of chronological or reported age in years across multiple tissues and cell lines [7,9]. GrimAge is an epigenetic clock that was built in the Framingham Heart Study and is based on DNAm predictors of seven circulating plasma proteins, smoking history, chronological age, and sex. This clock, validated in five independent cohorts, is highly predictive of mortality [11]. Both Horvath and GrimAge epigenetic clocks produce epigenetic age estimates in years. In contrast, epiTOC2 is a mitotic clock, which provides an estimate of the total number of stem cell divisions (TNSC), which can be translated to a rate of cell division per years of life lived (irS) for easier comparison to clocks that output in years. EpiTOC2 was built using Hannum's samples (whole blood from 656 adults) to approximate the number of stem cell divisions and was found to be correlated with cancer, which included tissue from 15 solid organ cancer types [12].

Data from the methylation array were also deconvoluted to determine the immune cell composition of each patient sample. TheFlowSorted.BloodExtended.EPIC R package [17] provided a reference library of 56 methylation profiles for 12 cell types: basophils, memory B cells, naive B cells, $CD4^+$ T-memory cells, $CD4^+$ T-naive cells, $CD8^+$ T-memory cells, $CD8^+$ T-naive cells, eosinophils, monocytes, neutrophils, natural killer (NK) cells, and T-regulatory cells [18]. In conjunction with the estimateCellCounts2 function provided by the R/Bioconductor package FlowSorted.Blood.EPIC [19] and using the CP/QP reference-based method described by Houseman *et al.* [20], the relative proportions of the 12 cell types were estimated.

### Statistical analysis

Differences in epigenetic age (clock outputs) and immune cell composition (proportion by cell type) were compared by HIV status using the Wilcoxon rank sum test. The correlation between reported age and epigenetic age was evaluated using the Pearson correlation coefficient. Age acceleration was computed as the difference (residual) between observed epigenetic age for an individual and the expected epigenetic age (mean output in the population at a given reported age) by regressing epigenetic age from each clock against reported age using normal linear regression and calculating the residuals [21]. The age acceleration residuals by HIV status were computed using both an unadjusted linear regression model and a linear regression model adjusted for immune cell subsets that were observed to differ significantly (Wilcoxon rank sum $P$ value $<0.05$) by HIV status. Vital status was assessed for PWH through 2022 and the association of unadjusted epigenetic age acceleration and age acceleration adjusted for immune cell types with survival following cancer diagnosis was determined using Cox proportional hazards model. All statistical analyses were completed in R.

## Results

Sixty-five PWH with a NADC (~50% gastrointestinal tumors) were included in this study. They were matched by age, tumor site, sequence number, and treatment status to 64 HIV-uninfected NADC patients whose DNA was successfully extracted and assayed. Median age and interquartile range were similar between groups [PWH: 55 (51–60); without HIV: 55.5 (50.5–60.0)]. Evaluation of study population characteristics (Table 1) revealed that a higher proportion of PWH were male individuals (76.9 vs. 51.6%), black (27.7 vs. 3.1%), and recorded as deceased in the EMR (36.9 vs. 12.5%) compared with HIV-uninfected participants. An equal proportion of participants with and without HIV were over the age of 50 at the time of cancer diagnosis (78.5 vs. 75%), were diagnosed with a first primary cancer (73.8 vs. 68.8%) and had the study specimen collections prior to any systemic therapy (40 vs. 47%). Analysis of genome-wide methylation patterns found 1804 CpG sites differentially methylated ($q$-value $<0.05$ and beta-difference $>0.15$) by HIV status (Supplemental Figure 1, http://links.lww.com/QAD/C933).

Epigenetic age from Horvath's clock was highly correlated with reported age in years among NADC participants with ($R = 0.85$) and without HIV ($R = 0.94$). The correlation with reported age in years was lower for GrimAge (PWH: 0.66; without HIV: 0.74) and epiToc2 (PWH: −0.24; without HIV: −0.26) (Fig. 1), indicative of these clocks measuring pieces of information beyond chronological age.

When examining epigenetic age from the three clocks, chronological age (Horvath's clock) did not significantly differ between PWH and those without HIV ($P = 0.16$). In contrast, significantly higher biological age among PWH was observed using GrimAge ($P = 0.017$) and epiTOC2 ($P < 0.0001$) (Fig. 2).

The mean age from the GrimAge clock was 67.7 vs. 63.1 years for PWH and NADC patients without HIV, respectively (difference of ~5 years). Using epiTOC2, the mean epigenetic age reported as rate of cell divisions per year of life was 69.8 vs. 41.4 for PWH compared with those without HIV, respectively. Epigenetic age differences persisted among PWH regardless of treatment status (Supplemental Figure 2, http://links.lww.com/QAD/C933) or tumor site (Supplemental Figure 3, http://links.lww.com/QAD/C933).

The proportion of immune cells in NADC patient samples differed by HIV status. Specifically, memory B

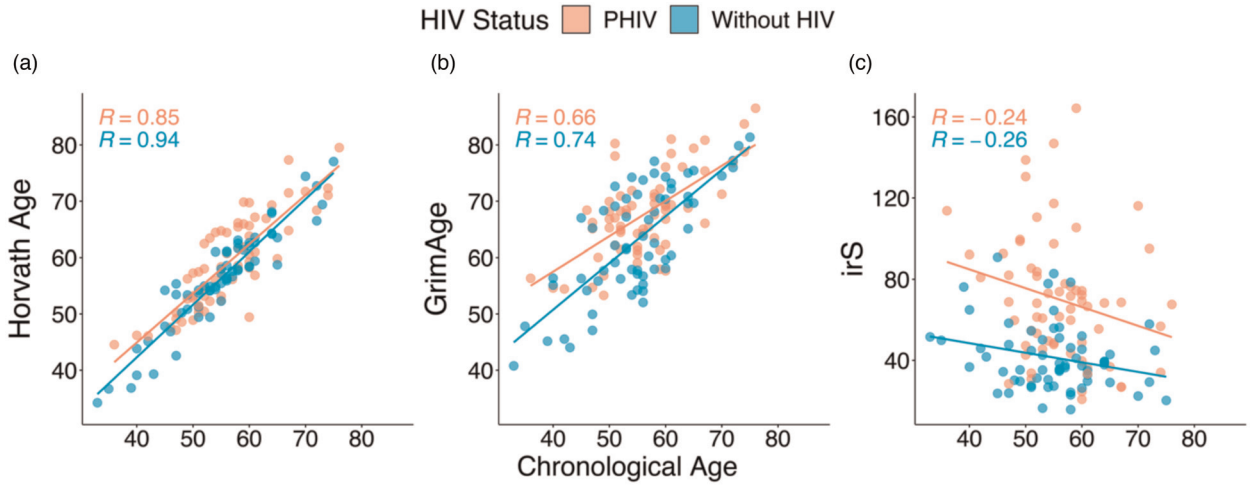


**Fig. 1. The correlation of estimated epigenetic age with reported age (years since birth) was examined.** (a) Horvath, a chronological clock, and (b) GrimAge, a biological clock, estimate an age in years, while (c) epiTOC2, estimated a rate of stem cell divisions per year of life lived.

cells and memory $CD8^+$ T cells were present at significantly higher proportions in PWH, whereas memory and naive $CD4^+$ T cells and neutrophils were significantly lower in PWH ($P < 0.05$) (Fig. 3).

Age acceleration for each clock was modeled both before and after adjustment for immune cell populations that significantly differed by HIV status. Age acceleration was significantly higher for NADC participants with HIV compared with their HIV-uninfected counterparts when assessed using either GrimAge (mean age acceleration for PWH = 1.78 years and without HIV = −1.81 years; $P = 0.0028$) or epiTOC2 (mean age acceleration for PWH = 792 TNSC and without HIV = −804 TNSC;

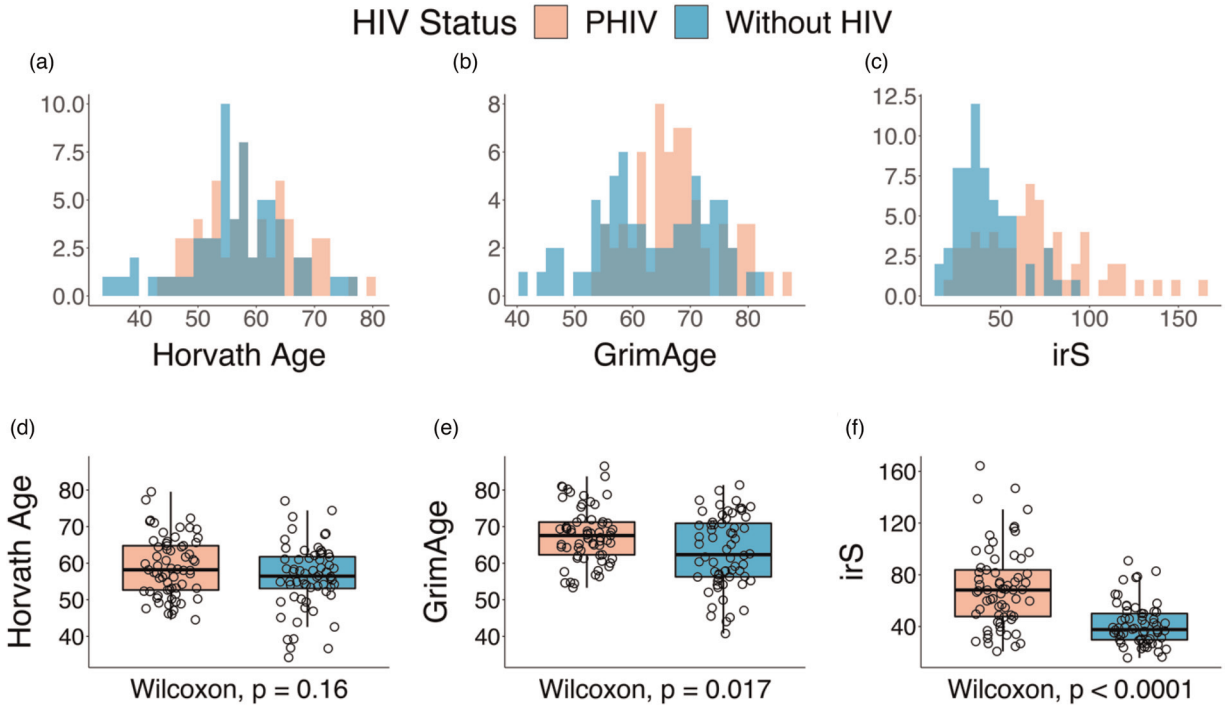


**Fig. 2. Differences in epigenetic age are described by non-AIDS-defining cancer patient HIV status using three epigenetic clocks.** Horvath and GrimAge estimate an age in years, while epiTOC2 is represented in irS and estimates a rate of stem cell divisions per year of life lived. Upper figures (a–c) describe the frequency distribution of epigenetic age in NADC patients with and without HIV. Lower figures (d–f) illustrate the average epigenetic age by HIV status. *P* values were calculated using Wilcoxon rank sum tests. NADC, non-AIDS-defining cancers.

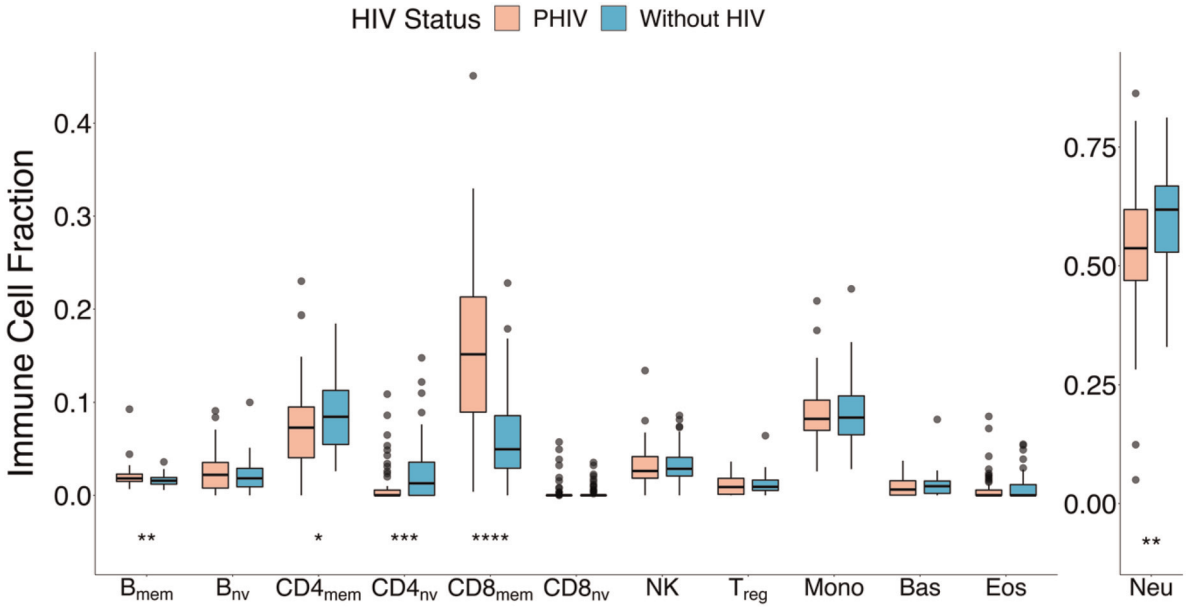


**Fig. 3. Immune cell deconvolution methods were used to determine the distribution of immune cells by non-AIDS-defining cancer patient HIV status.** Cell types investigated included (left to right) memory B cells, naive B cells, $CD4^+$ T-memory cells, $CD4^+$ T-naive cells, $CD8^+$ T-memory cells, $CD8^+$ T-naive cells, natural killer cells, T-regulatory cells, monocytes, basophils, eosinophils, and neutrophils. *P* values were generated using Wilcoxon Rank sum test to compare each cell type distribution by HIV status and considered significantly different if *P* less than 0.05. In the figure, the following indicates the level of statistical significance: *($P < 0.05$); **($P < 0.01$), ***($P < 0.001$), ****($P < 0.0001$)

$P < 0.0001$) in unadjusted models (Fig. 4). After adjustment for observed differences in immune cell distributions by patient HIV status, age acceleration remained statistically significantly different by HIV status for GrimAge (mean age acceleration for PWH = 1.74 years and without HIV = −1.62 years; $P = 0.0006$) (Fig. 4). Age acceleration from epiTOC2, a clock derived from total cell divisions, was no longer different by HIV status when adjusted for differing immune cell proportions. These results were maintained when also adjusted by race and sex, two notable differences across the study groups (data not shown). To mirror a metric used in the clinical management of PWH, we also adjusted GrimAge models for the $CD4^+/CD8^+$ T-cell ratio and observed persistent, HIV-associated differences in biological age acceleration ($P = 0.0062$) (Supplemental Figure 4, http://links.lww.com/QAD/C933).

Due to the observed acceleration by HIV status for GrimAge, as well as the clock's association with patient prognosis, age acceleration from GrimAge was examined for its association with survival. Among PWH, each year of GrimAge acceleration was significantly associated with an ∼11% increased risk of mortality after a NADC diagnosis [hazard ratio: 1.11; 95% confidence interval (CI) 1.04–1.18; $P = 0.003$]. The association remained significant when GrimAge acceleration was adjusted for observed differences in immune cell proportions (1.09; 95% CI 1.01–1.19; $P = 0.031$).

## Discussion

Using three epigenetic clocks, we investigated differences in epigenetic aging by HIV status among participants diagnosed with a solid organ NADC. PWH and a NADC had significantly higher levels of mortality-associated GrimAge and higher levels of epiTOC2-measured cell divisions per year of life compared with HIV-uninfected NADC participants matched on age, tumor site and sequence number, and timing of cancer treatment. Significant acceleration in GrimAge among PWH persisted after adjustment for noted differences in immune cell distribution. Importantly, higher biological age acceleration was associated with mortality risk after a cancer diagnosis among PWH.

The epigenetic age as computed using GrimAge was 4 years higher, on average, in NADC participants with vs. without HIV. A parallel comparison using epiTOC2 revealed a difference in stem cell divisions per year of life lived of 30 between NADC participants with vs. without HIV. These differences were observed despite matching based on reported age as part of the study design. Remarkably, prior studies in younger participants without cancer have reported epigenetic age to be accelerated approximately 5 years in PWH compared with the general population [22,23]. The clocks in this study were chosen a priori because of the relevant outcomes they were known to be correlated with, and the

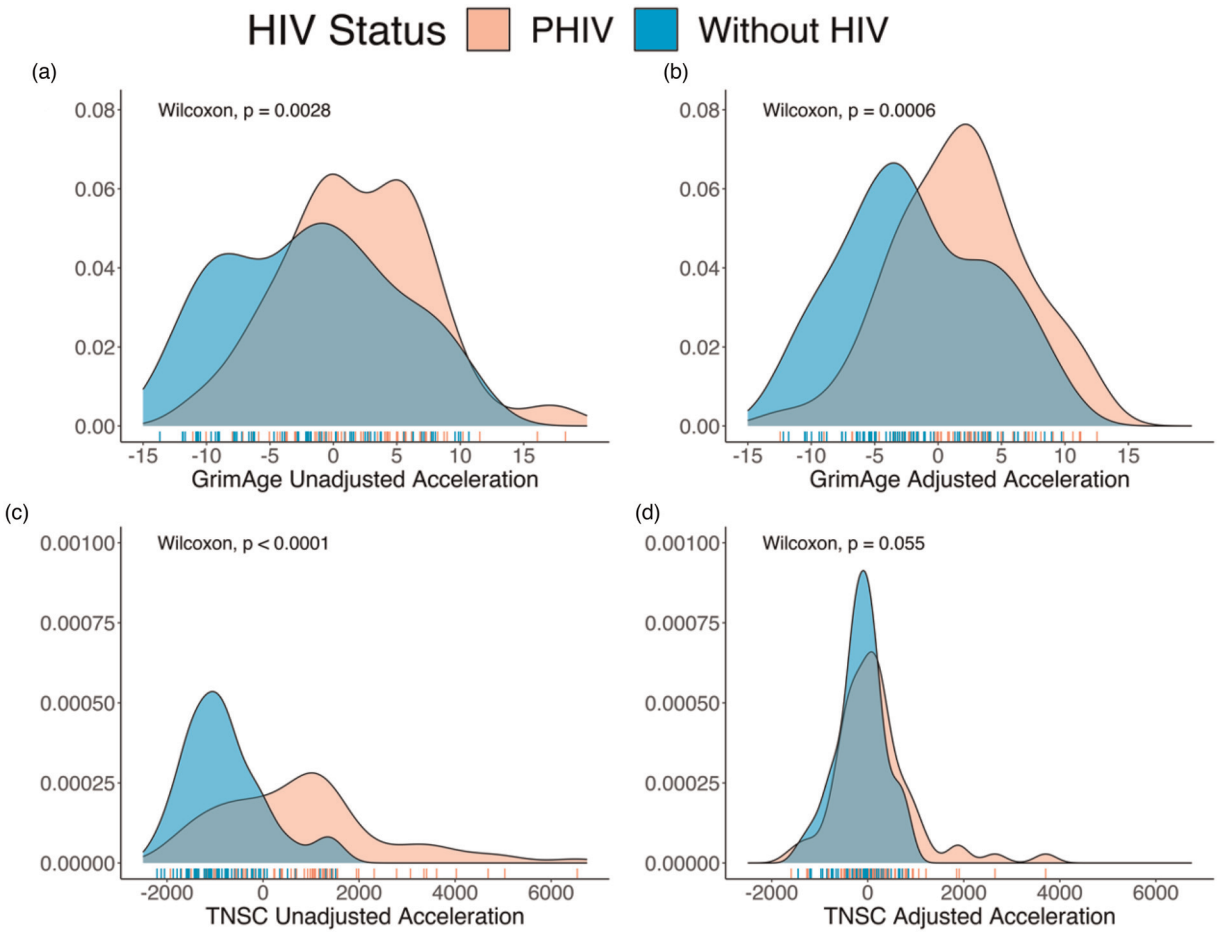


**Fig. 4. Age acceleration using biological clocks by HIV status.** (a) Unadjusted using GrimAge; (b) GrimAge adjusted for differences in immune cell distribution by HIV status; (c) unadjusted using epiTOC2; (d) epiTOC2 adjusted for differences in immune cell distribution by HIV status. Selected cell types in (b) and (d) included those that were significantly ($P < 0.05$) different by NADC patient HIV status: memory B cells, memory $CD8^+$ T cells, memory and naive $CD4^+$ T cells, and neutrophils. Both were also additionally adjusted for race and sex only (GrimAge $P = 0.003$; epiTOC2 $P < 0.0001$) and race, sex, and the described immune cell types (GrimAge $P = 0.002$; epiTOC2 $P = 0.145$). NADC, non-AIDS-defining cancers.

fact that these outcomes were independent. For example, the chosen clocks covered a chronological clock that correlates well with reported age in years, a clock linked to a clinical outcome (mortality), and a clock linked to a lab-based metric (cell division rate). Our data (Fig. 1) show how the biological clocks (GrimAge and epiTOC2) represent a measure independent of reported age.

In a study of 24 PWH and 24 without HIV (age 20–56), data from DNA methylation levels across more than 450k CpG sites in blood concluded that HIV accelerates age-related methylation by 14 years [24]. Among 378 ART-naive PWH with well controlled immune function ($CD4^+$ T-cell counts >500 cells/μl), epigenetic age from Horvath's clock was higher compared with 34 HIV-negative controls ($P = 0.016$) [25]. However, our study is the first to extend the investigations of advanced biological aging among PWH to the context of cancer, now the leading cause of non-HIV-related death in PWH [15].

Biological age acceleration remained higher among PWH after adjusting for differences in immune cell composition when using GrimAge. PWH characteristically have lower $CD4^+$ cell counts [26], higher $CD8^+$ cell counts [27], and overall immune dysfunction [28,29]. As such, we noted the possibility that methylation-derived metrics of biological aging might differ by HIV status because of known expected differences in immune cell composition (e.g. $CD4^+$ T cells). Accounting for such differences is critical to interpretation given that many epigenetic clocks are built using DNA methylation patterns for immune cell lineages that naturally change over time as a part of the aging process [20]. It is therefore notable that in our study, the differences in GrimAge persisted by HIV status even after accounting for different immune cell distribution. This demonstrates that PWH and a NADC diagnosis have patterns of genome-wide methylation indicative of aging and poor prognosis that persist even after accounting for their expected differences in immune cells. Further, age acceleration from

epiTOC2, a clock, which reports age in number of cell divisions, was attenuated when adjusted for immune cell proportions. Although immune cells do not encompass the entirety of the cell populations epiTOC2 is reporting in its calculation of cell division, the fact that PWH experience a high amount of immune cell turnover is likely a key explanation for why adjustment for cell turnover attenuated HIV-related differences in this particular clock.

The development of the GrimAge clock is based on the correlation of DNAm patterns with smoking pack-years and an assortment of plasma proteins (adrenomedullin, C-reactive protein, plasminogen activation inhibitor, and growth differentiation factor 15) previously associated with mortality and morbidity. This clock was developed using data from participants of the Framingham Heart Study and validated in five independent cohorts [11]. Our study found that GrimAge was not only higher in PWH but that acceleration of GrimAGE in PWH (i.e. an average increase of ~2 years in a patient's epigenetic age compared with what they report) from this clock was significantly associated with poor overall survival in PWH. This is of particular importance as NADC-specific mortality is higher in PWH compared with HIV-uninfected participants [15], with nearly 20% of all deaths among PWH now being attributed to an NADC [30], a trend only expected to grow with the continued aging of the HIV population. It is important to note that smoking rates are higher in PWH compared with those without HIV, and the GrimAge clock was developed in the Framingham cohort using smoking-related differences in methylation patterns to help build the clock. However, studies have shown age acceleration using GrimAge in PWH even after adjusting for smoking history [31].

This study utilized a novel biomarker for aging in a population that is experiencing age-related comorbidities disproportionately. It was the first to do so in the context of cancer and HIV. Participants were matched by age and cancer characteristics to account for effects these factors may exhibit in methylation-based assessment of epigenetic age and cancer outcome. Matching in this fashion increased our confidence that the results observed are associated with HIV-associated biological age differences rather than known differences in patient demographics by HIV status. Finally, all participants were recruited and followed at Moffitt Cancer Center, reducing any potential recruitment or collection bias and eliminating any confounding associated with location.

However, there are study limitations that should be mentioned. First, directly-measured HIV metrics such as nadir $CD4^+$ cell count, viral load suppression, and ART history were not included because data was abstracted from the EMR at a tertiary care cancer center, with limited complete history of infectious disease care. However, using methylation data to deconvolute immune cell composition provided a way to account for immune competence at the time of biological sampling, and it was noted that all participants were currently using ART per their electronic medical record. Second, the analysis was not cancer site-specific. NADCs, while generally associated with aging, still have unique risk factors by anatomic site that we could not examine separately in this study. We tried to address this by limiting to solid organ tumors and by matching on cancer site and sequence number during participant selection to ensure that epigenetic aging comparisons were made in a group balanced by cancer site. The cross-sectional nature of this study limits an understanding of how DNA methylation markers may change over time. Longitudinal studies with long-term follow-up and regular intervals of methylation assessment are needed to address this limitation. Although this study revealed several key findings in the field of epigenetic aging and HIV, future studies are needed. First, a better understanding of how ART type, duration, and adherence may mitigate the changes that lead to age acceleration in PWH diagnosed with cancer is crucial as the majority of PWH are now using ART, likely for decades prior to cancer diagnosis. Studies are also needed to investigate methylation-based epigenetic aging in precancerous populations, which may help to understand biological aging during the early stage of carcinogenesis that could aid early cancer detection. Finally, a larger study sample is needed to also investigate relevant comorbidities and co-infections that are often present in PWH.

In conclusion, this study found that epigenetic age and acceleration of epigenetic age beyond reported age in years were both significantly higher in NADC participants with HIV compared with those without HIV, despite matching on reported age. These differences persisted after accounting for expected differences in immune cell composition, leading us to conclude that the advanced aging observed in PWH with an NADC is likely attributable to impacts of HIV beyond $CD4^+$ T-cell depletion. Importantly, this age acceleration was linked to poor NADC patient prognosis for PWH. Further studies examining DNA methylation-based biological aging in PWH and cancer is warranted as the cancer burden continues to grow in this underserved patient population.

## Acknowledgements

Data availability statement: the data generated in this study are not publicly available because of inclusion of information that could compromise patient privacy but may be available upon reasonable request from the corresponding author. This research was enabled, in part, by the use of the FlowSorted.BloodExtended.EPIC R package developed at Dartmouth College, which software is subject to the licensing terms made available

by Dartmouth Technology Transfer and which software is provided "as is" with no warranties whatsoever.

Author contributions: B.L.D. and A.E.C. were responsible for study conceptualization, data acquisition, interpretation of data. R.M.P. provided data analysis and interpretation. J.K.K., A.M.S., A.B.P., M.T., M.E., A.R.G., N.G., and A.B. provided further interpretation of the data based on their subject-matter expertise. B.L.D. drafted and revised the work after contribution and comment from all co-authors. All authors have reviewed and approved this work.

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## Conflicts of interest

There are no conflicts of interest.

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