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Do people living with HIV experience greater age advancement than their HIV-negative counterparts?

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Objectives: Despite successful antiretroviral therapy, people living with HIV (PLWH) may show signs of premature/accentuated aging. We compared established biomarkers of aging in PLWH, appropriately chosen HIV-negative individuals, and blood donors, and explored factors associated with biological age advancement.

Design: Cross-sectional analysis of 134 PLWH on suppressive antiretroviral therapy, 79 lifestyle-comparable HIV-negative controls aged 45 years or older from the Co-morBidity in Relation to AIDS (COBRA) cohort, and 35 age-matched blood donors.

Methods: Biological age was estimated using a validated algorithm based on 10 biomarkers. Associations between 'age advancement' (biological minus chronological age) and HIV status/parameters, lifestyle, cytomegalovirus (CMV), hepatitis B (HBV) and hepatitis C virus (HCV) infections were investigated using linear regression.

Results: The average (95% CI) age advancement was greater in both HIV-positive [13.2 (11.6–14.9) years] and HIV-negative [5.5 (3.8–7.2) years] COBRA participants compared with blood donors [−7.0 (−4.1 to −9.9) years, both P's < 0.001)], but also in HIV-positive compared with HIV-negative participants ($P < 0.001$). Chronic HBV, higher anti-CMV IgG titer and $CD8^+$ T-cell count were each associated with increased age advancement, independently of HIV-status/group. Among HIV-positive participants, age advancement was increased by 3.5 (0.1–6.8) years among those with nadir $CD4^+$ T-cell count less than 200 cells/μl and by 0.1 (0.06–0.2) years for each additional month of exposure to saquinavir.

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Conclusion: Both treated PLWH and lifestyle-comparable HIV-negative individuals show signs of age advancement compared with blood donors, to which persistent CMV, HBV co-infection and $CD8^+$ T-cell activation may have contributed. Age advancement remained greatest in PLWH and was related to prior immunodeficiency and cumulative saquinavir exposure.

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Introduction

Despite the success of combination antiretroviral therapy, people living with HIV (PLWH) have an increased burden of noncommunicable age-associated comorbidities compared with HIV-negative individuals [1,2]. The causes of this increased burden of comorbidities remain unclear but may involve an accelerated or accentuated aging process [3,4], resulting from a complex mix of HIV infection, antiretroviral treatment, chronic viral co-infections and lifestyle/behavioral factors.

Aging can be defined as the time-dependent decline of functional capacity and stress resistance associated with increased risk of disability, morbidity and mortality [5]. There is clear evidence that the rate of aging differs significantly between individuals, because of genetic heterogeneity and environmental factors [6]. Therefore, chronological age may not represent the best way of measuring aging and may not accurately reflect an individual's position in his/her total lifespan [7]. This has led to a search for reliable biomarkers of aging, defined as biological parameters that capture the age-related changes in body function or composition. These biomarkers could serve to measure 'biological' age, a hypothetical value denoting the extent of age-related changes in function and composition of a human body, and predict the onset of age-related diseases and/or expectant residual lifetime more accurately than chronological age [8].

Many candidate biomarkers of aging have been proposed in the scientific literature and have been used to investigate the association between HIV and aging [9]. Among these, there are markers of chronic systemic immune activation (soluble $CD14^+$ and $CD163^+$ [10,11]), inflammation (C-reactive protein and interleukin-6 [12]), coagulation (D-dimer [13]), leukocyte telomere length [14], somatic mitochondrial DNA mutations [15], expression levels of the cell cycle regulator CDKN2A [14], DNA methylation levels [16], ophthalmological parameters [17] and age-related brain atrophy [18].

Combinations of biomarkers may more reliably measure these age-related changes (or what is called also 'biological age'), giving more accurate estimates of residual lifetime than that obtained from any single biomarker in isolation. The MARK-AGE project has proposed a method to combine powerful biomarkers of human aging (most of which have been shown to be good markers of age when taken in isolation [19,20]) to predict biological age of individuals, and therefore, assess the aging process [21,22].

The current study aimed to compare established biomarkers of aging and their combination (as proposed by the MARK-AGE study) in order to evaluate the biological age of PLWH, demographically and lifestyle-matched HIV-negative individuals, and blood donors with similar chronological age. Furthermore, we investigated the associations between any observed age advancement and lifestyle risk factors, chronic viral co-infections including hepatitis B virus (HBV), hepatitis C virus (HCV) and cytomegalovirus (CMV), HIV-related parameters and (cumulative) past or current exposure to antiretroviral drugs.

Methods

Study participants

HIV-positive ($n = 134$) and HIV-negative individuals with similar sociodemographic and lifestyle factors ($n = 79$) were prospectively enrolled in the COmorBidity in Relation to AIDS (COBRA) cohort from HIV outpatient clinics and sexual health centres, respectively, located in Amsterdam (Netherlands) and London (UK) [23]. Inclusion criteria were age 45 years or older ($\geq$50 years in London), laboratory-confirmed presence or absence of HIV-1 infection, and the HIV-positive people were required to have plasma HIV-RNA less than 50 copies/ml for at least 12 months on antiretroviral therapy. Exclusion criteria were: current major depression [as indicated by a Patient Health Questionnaire-9 (PHQ-9) score $\geq$15 at screening], confounding neurological diseases, previous severe head injury (with loss of consciousness for $\geq$30 min), a history of cerebral infections (including AIDS-defining diseases involving the central nervous system), current intravenous drug use (in the past 6 months, based on self-report), daily use of recreational drugs (with the exception of cannabis), excess alcohol intake (>48 units per week), severe

psychiatric disease or contraindication to MRI scan or lumbar puncture examination. Recruitment took place between December 2011 and October 2014. The study was approved by the institutional review board of the Academic Medical Center (AMC) in Amsterdam (reference number NL 30802.018.09) and a UK Research Ethics Committee (REC) (reference number 13/LO/0584 Stanmore, London). All participants gave written informed consent.

In addition, 35 blood donors were selected from the Dutch national blood bank in Amsterdam, the Netherlands (https://www.sanquin.nl/en/). Blood donors were age-matched with the HIV-positive and HIV-negative COBRA participants and, as a requirement for blood donation in the Netherlands, they had screened negative for HIV, HBV, HCV, syphilis, and human T-lymphotropic virus 1 and 2 (HTLV) infections. Candidate blood donors in the Netherlands are also eventually excluded from blood donation based on a questionnaire with regard to health (general and sexual), medication use, sexual risk behavior and travel.

Biomarkers of aging

A 10-item panel of biomarkers of aging identified by the MARK-AGE project [21,22] were measured and are listed in Supplementary Table 1, http://links.lww.com/QAD/B389. Briefly, these biomarkers have been selected as best predictors of chronological age among nearly 400 candidate biomarkers in a population of approximately 3300 individuals aged between 30 and 74 years (mean age was 56 years) recruited from eight European countries. Lycopene, and α-tocopherol in plasma were simultaneously determined by high performance liquid chromatography with UV and fluorescence detection as previously described [24]. Alpha-2-macroglobulin in plasma was measured on an autoanalyzer (DxC 800; Beckman-Coulter, Woerden, The Netherlands) by an immunoturbimetric method using reagents from Dialab, Wiener Neudorf, Austria as described by Jansen *et al.* [25]. Dehydroepiandrosterone sulfate, ferritin (women only), and prostate-specific antigen (men only) in plasma were analyzed using an immuno-analyzer (Access-2; Beckman–Coulter) [25]. ELOVL2 and FHL2 DNA methylation in purified PBMC were analyzed using the Agena Bioscience's EpiTYPER DNA methylation analysis technology [26]. The N-glycans present on the proteins in plasma were released, labelled, and analyzed by DSA-FACE technology, as described previously [20].

Age advancement

The biological age of each individual was derived separately for men and women as a linear combination of these biomarkers using the method and the weights described in Bürkle *et al.* [22]. Age advancement for each individual was then defined as the difference between biological and chronological age. Positive age advancement is, therefore, indicative of more age-related changes in body function and composition than what would be expected in the average population, given the observed chronological age; negative age advancement would suggest less age-related changes.

Statistical analysis

Participants' characteristics and distribution of the 10 biomarkers used to derive biological age are reported as raw and relative frequencies or median and interquartile range (IQR) and differences across groups were evaluated using chi-square or Wilcoxon signed-rank tests, as appropriate. Age advancement in each group is reported as mean and 95% confidence interval (CI) and assessed for significance using the one-sample *t*-test. Differences between groups were assessed using linear regression with HIV status/study group as categorical independent variable.

Associations were considered between age advancement and the following factors:

(1) Sociodemographic factors (age, sex, ethnicity, sexuality, years of education)
(2) Self-reported smoking, alcohol consumption and current recreational drug use
(3) CMV (CMV serostatus, CMV total IgG and CMV high-avidity IgG titers), chronic HBV (defined as detectable hepatitis B surface antigen and/or HBV DNA) and HCV (defined as detectable HCV RNA)
(4) HIV disease parameters such as $CD4^+$ and $CD8^+$ T-cell counts, $CD4^+$: $CD8^+$ T-cell ratio, nadir $CD4^+$ T-cell count, nadir $CD4^+$ T-cell count <200 cells/μl, years since HIV diagnosis, prior AIDS diagnosis (defined as prior category C event as per the Centers for Disease Control and Prevention's classification system for HIV infection)
(5) Exposure to antiretroviral drugs considered as both prior exposure (yes or no) and months of cumulative exposure.

The associations of age advancement with each factor, unadjusted and adjusted for HIV status/study group, were assessed through a series of linear regression models (with each factor considered one at the time in separate models). Factors that remained significantly associated with age advancement after adjustment for HIV status and group ($P < 0.1$), were then included in a multivariable regression model to identify factors independently associated with age advancement. All statistical analyses were performed using SAS v9.4 (Cary, North Carolina, USA).

Results

Cohort characteristics

The 79 HIV-negative COBRA participants were comparable with the 134 HIV-positive participants in terms of age, sex, years of education, smoking and recreational drug use. HIV-positive participants were

Table 1. Characteristics of the COBRA participants and blood donors (P^1: HIV-positive vs. HIV-negative COBRA participants; P^2: HIV-positive COBRA participants vs. blood donors: both were obtained from chi-square or Wilcoxon signed-rank tests, as appropriate).

	COBRA participants				
n (%) or median (IQR)	HIV-positive ($n=134$)	HIV-negative ($n=79$)	P^1	Blood donors ($n=35$)	P^2
Age (years)	55 (51–62)	57 (52, 64)	0.24	59 (52, 65)	0.37
Sex: female	9 (6.7%)	6 (7.6%)	0.79	17 (48.6%)	<0.001
Male	125 (93.3%)	73 (92.4%)		18 (51.4%)	
Ethnicity: black-African	16 (12.0%)	2 (2.6%)	0.03	n/a	
White	117 (88.0%)	76 (97.4%)		n/a	
Sexuality: MSM	104 (77.6%)	59 (74.7%)	0.45	n/a	
Bisexual	10 (7.5%)	4 (5.1%)		n/a	
Heterosexual	18 (13.4%)	16 (20.2%)		n/a	
Years of education	14 (13, 16)	16 (14, 17)	0.23	n/a	
Smoking status: current smoker	40 (29.9%)	20 (25.3%)	0.24	n/a	
Ex-smoker	58 (43.2%)	29 (36.7%)		n/a	
Never smoked	36 (26.9%)	30 (38.0%)		n/a	
Alcohol consumption: current drinker	104 (77.6%)	71 (89.9%)	0.04	n/a	
Previous drinker	18 (13.4%)	3 (3.8%)		n/a	
Never drunk	12 (9.0%)	4 (5.1%)		n/a	
Recreational drugs use in past 6 months	44 (32.8%)	18 (22.8%)	0.16	n/a	
Chronic HBV infection	7 (5.3%)	0 (0.0%)	0.05	0 (0.0%)	0.34
Chronic HCV infection	5 (3.7%)	0 (0.0%)	0.16	0 (0.0%)	0.58
CMV infection	129 (97.7%)	63 (79.8%)	<0.001	8 (22.9%)	<0.001
Total anti-CMV IgG (AU)	51.2 (29.0–107.0)	21.1 (11.8–53.8)	<0.001	11.3 (10.2–16.8)	0.003
High avidity anti-CMV IgG (AU)	29.4 (14.9–61.1)	12.6 (7.2–25.3)	<0.001	10.7 (10.0–13.2)	0.02
$CD4^+$ T-cell count (cells/μl)	618 (472–806)	900 (692–1174)	<0.001	n/a	
$CD8^+$ T-cell count (cells/μl)	770 (611– 947)	422 (313–614)	<0.001	n/a	
$CD4^+$: $CD8^+$ T-cell count ratio	0.84 (0.60–1.12)	2.01 (1.44–2.64)	<0.001	n/a	
Nadir $CD4^+$ T-cell count (cells/μl)	180 (90–250)	n/a		n/a	
Years since HIV diagnosis	15.0 (9.1–20.0)	n/a		n/a	
Duration of antiretroviral therapy (years)	12.5 (7.4–16.9)	n/a		n/a	
Nadir $CD4^+$ T-cell count less than 200 cells/μl	83 (61.9%)	n/a		n/a	
Prior AIDS	42 (31.3%)	n/a		n/a	

ARV, antiretroviral therapy; COBRA, Co-morBidity in Relation to AIDS; CMV, cytomegalovirus; HBV, hepatitis B virus; HCV, hepatitis C virus.

more likely to be of black-African origin ($P=0.03$) and were less likely to report current alcohol consumption ($P=0.04$) than the HIV-negative participants. Compared with both groups of COBRA participants, the blood donor group included a greater proportion of women ($P<0.001$). CMV, chronic HBV and HCV co-infections were all more frequent in HIV-positive COBRA participants compared with both the HIV-negative participants and blood donors, with CMV also being more frequent in HIV-negative participants than in blood donors. All HIV-positive COBRA participants had plasma HIV RNA less than 50 copies/ml and were on antiretroviral therapy at study visit, they had a median (IQR) $CD4^+$ T-cell count of 618 (472–806) cells/μl, and 31% had a prior clinical AIDS diagnosis (Table 1). The number and proportion of people who had ever been exposed to each antiretroviral drug and the median (IQR) duration of exposure are reported in Supplementary Table 2, http://links.lww.com/QAD/B389.

Biomarkers of aging

The distribution of each of the 10 biomarkers of aging used to derive biological age is summarized in Supplementary Table 3, http://links.lww.com/QAD/B389. HIV-positive COBRA participants had a greater cumulative proportion of cytosine methylation at four out of five gene positions compared with HIV-negative COBRA participants (all Ps ≤ 0.01). DHEAS and A2M (in male participants only) concentrations were also significantly different in the two COBRA groups ($P=0.03$ and $P=0.004$, respectively). In all the five gene positions, the cumulative proportion of cytosine methylation in blood donors was significantly lower than that seen in both COBRA groups. A2M concentration (in males only) in blood donors was lower compared with both COBRA groups (both Ps <0.001), whereas N-glycan peak 6 was higher compared with COBRA HIV-positive ($P=0.04$) but not with HIV-negative participants ($P=0.18$).

Age advancement in HIV-positive and HIV-negative COBRA participants and blood donors

Biological age was significantly greater than chronological age by a mean of 13.2 (95% CI 11.6–14.9) years in HIV-positive COBRA participants and by 5.5 (3.8–7.2) years in HIV-negative participants ($P<0.001$ for each). In contrast, biological age was a mean of 7.0 (4.1–9.9) years lower than chronological age in blood donors ($P<0.001$, Fig. 1a). Whilst age advancement was greater in both COBRA groups compared with blood donors ($P<0.001$ for each), the HIV-positive COBRA

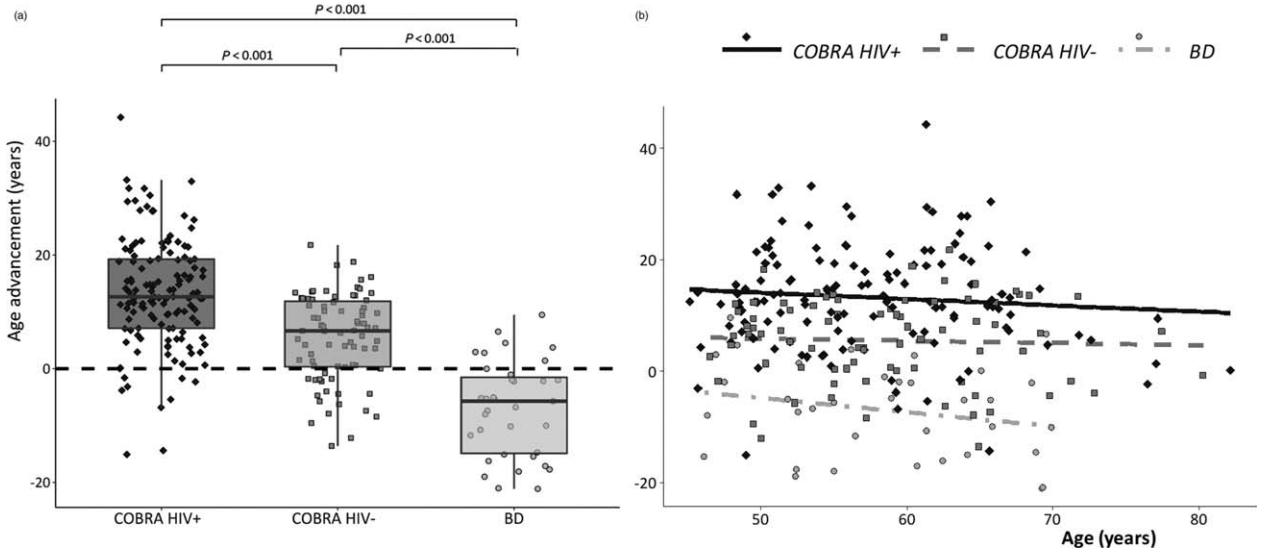

Fig. 1. (a) Age advancement (biological minus chronological age) in HIV-positive and HIV-negative COBRA participants and blood donors (*P*s from linear regression); (b) Correlation between age advancement and chronological age in HIV-positive and HIV-negative COBRA participants and blood donors (no interaction between chronological age and HIV-status/group, $P = 0.66$). BD, blood donors; COBRA, Co-morBidity in Relation to AIDS.

participants also demonstrated greater age advancement than the HIV-negative participants ($P < 0.001$). Age advancement was also negatively correlated with chronological age (Pearson's $r = -0.17$, $P = 0.08$) with no significant interaction with HIV-status/group ($P = 0.66$, Fig. 1b).

Factors associated with age advancement

Across the entire study population, no significant associations were found between age advancement and ethnicity, sexual orientation or lifestyle factors (Table 2). Overall, men had greater age advancement than women even after adjustment for HIV-status/group. However, among COBRA participants only, the difference (95% CI) between men and women was only 0.09 (−5.04 to 5.22) years ($P = 0.97$) and the difference between COBRA groups and blood donors remained significant even after adjusting for sex (both *P*s <0.001). Viral co-infections such as CMV and chronic HBV, as well as $CD4^+$ and $CD8^+$ T-cell count and their ratio appeared to be associated with increased age advancement. However, only chronic HBV, total and high avidity anti-CMV IgG antibody titer and $CD8^+$ T-cell count showed significant associations, which were also independent of HIV-status/group.

In multivariable analysis including significant factors after adjusting for HIV-status/group ($P < 0.1$), the average increase in age advancement (95% CI) remained significant for chronic HBV [10.05 (3.45−16.64) years, $P = 0.003$], total anti-CMV IgG antibody titer [1.83 (0.51− 3.15) years per one log (AU) increase, $P = 0.007$] and $CD8^+$ T cell [0.44 (0.02−0.85) years per 100 cells/μl increase, $P = 0.04$]. Even after adjusting for chronic HBV, total anti-CMV IgG and $CD8^+$ T-cell, HIV-positive COBRA participants exhibited a greater age advancement compared with both the HIV-negative participants [mean increase (95% CI) 4.5 (1.6−7.5), $P = 0.003$] and blood donors [mean increase (95% CI): 19.0 (12.6−25.4), $P < 0.001$], with the HIV-negative COBRA participants also exhibiting greater age advancement than blood donors [mean increase (95% CI) 13.5 (7.1−20.0, $P < 0.001$].

Associations with HIV parameters and exposure to antiretroviral drugs

Among the HIV-positive participants in COBRA, in univariable regression analyses, positive correlations were found between age advancement and time since HIV diagnosis, duration of antiretroviral therapy and nadir $CD4^+$ T-cell count <200 cells/μl (Table 3). Cumulative exposure and/or prior exposure to some antiretroviral drugs was also associated with an increased age advancement (Supplementary Table 4, http://links.lww.com/QAD/B389); in particular each additional year of exposure to saquinavir was associated with a 1.39 (95% CI 0.71−2.07) years increase in age advancement ($P < 0.001$), but also prior exposure to stavudine or any d-drug were associated with greater age advancement ($P = 0.02$ and $P = 0.03$, respectively). Due to the strong correlations between these factors, we ran a multivariable regression analysis with all HIV-parameters and exposures to antiretroviral drugs that were significantly associated with age advancement in univariable analysis ($P < 0.1$), also accounting for chronic HBV, total anti-CMV IgG antibody titer and $CD8^+$ T-cell count. In this multivariable analysis, only cumulative exposure to saquinavir (average increase of 1.17 (0.49, 1.85) years per year of

Table 2. Mean (95% confidence interval) increase (if positive) or decrease (if negative) in age advancement among HIV-positive and HIV-negative COBRA participants and blood donors (combined), unadjusted, and adjusted for HIV status/group.

Variable	Mean (95% CI) increase/decrease (years)			
	Unadjusted	*P*	Adjusted for HIV status/group	*P*
Age (per 5 years)	−0.17 (−0.35 to 0.02)	0.08	−0.11 (−0.26 to 0.04)	0.15
Gender: male vs. female	4.19 (0.01–8.36)	0.05	4.55 (0.92–8.18)	0.01
Ethnicity: black-African vs. white	3.40 (−1.28 to 8.09)	0.15	1.18 (−3.21 to 5.56)	0.60
Sexuality: MSM vs. non-MSM	0.92 (−1.86 to 3.69)	0.51	−0.01 (−3.23 to 3.20)	0.99
Smoking: current smoker vs. never smoked	2.46 (−0.95 to 5.88)	0.16	1.47 (−1.69 to 4.64)	0.36
Ex-smoker vs. never smoked	0.71 (−2.41 to 3.83)	0.65	−0.28 (−3.18 to 2.62)	0.85
Alcohol: current drinker vs. never drunk	−1.80 (−6.81 to 3.21)	0.48	−0.58 (−5.24 to 4.08)	0.81
Previous drinker vs. never drunk	0.60 (−5.57 to 7.16)	0.81	−0.03 (−5.93 to 8.87)	0.99
Recreational drugs use	−0.88 (−3.77 to 2.01)	0.55	−1.80 (−4.48 to 0.87)	0.18
Chronic HBV infection	14.47 (6.12– 22.83)	<0.001	9.11 (2.41–15.82)	0.008
Chronic HCV infection	6.50 (−3.50–16.50)	0.20	1.09 (−6.89 to 9.08)	0.79
CMV infection	11.46 (8.18–14.73)	<0.001	0.54 (−3.11 to 4.19)	0.77
Total anti-CMV IgG [per 1 log (AU)]	3.49 (2.15–4.85)	<0.001	2.05 (0.73–3.36)	0.002
High avidity anti-CMV IgG (per 1 AU)	0.06 (0.03–0.09)	<0.001	0.04 (0.01–0.07)	0.01
$CD4^+$ T-cell count (per 100 cells/μl)	−0.85 (−1.30 to −0.41)	<0.001	−0.31 (−0.79 to 0.17)	0.21
$CD8^+$ T-cell count (per 100 cells/μl)	0.91 (0.53–1.28)	<0.001	0.49 (0.07–0.90)	0.02
$CD4^+$: $CD8^+$ T-cell count ratio (per 1-unit)	−2.87 (−4.08 to −1.66)	<0.001	−1.11 (−2.59 to 0.38)	0.14

Blood donors were included only in analyses concerning age, sex, chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, cytomegalovirus (CMV) infection and anti-CMV IgG. Estimates were obtained with separate linear regression models (one for each factor considered).

exposure, $P < 0.001$) and nadir $CD4^+$ T-cell count less than 200 cells/μl (average increase of 3.00 (−0.22 to 6.22) years, $P = 0.07$), as well as chronic HBV (7.35 (0.42–14.29) years, $P = 0.04$) and total anti-CMV IgG antibody titer (1.86 (0.22–3.51) years per one log increase, $P = 0.03$), retained their significant associations.

Discussion

Both successfully treated PLWH and people without HIV with similar lifestyles show signs of age advancement compared with healthy blood donors who, conversely, appear younger than their chronological age. This may be explained by the strict requirements around blood donation in the Netherlands. This hypothesis is supported, for example, by the observation that the prevalence of CMV infection was higher in HIV-negative participants (79.8%), and lower in blood donors (22.9%), than in the general Dutch population (50% prevalence at 50 years of age) [27].

Whilst both groups of COBRA participants exhibited age advancement, this was significantly greater in HIV-positive than in HIV-negative participants. Whilst this difference in age advancement did not appear to be explained by differences in participant characteristics, chronic viral co-infections such as CMV and HBV, prior immunosuppression and exposure to some antiretroviral drugs may have contributed to this age advancement. CMV and HBV co-infections may cause premature aging of the immune system in PLWH by their chronic antigenic stimulation, inducing systemic immune activation [28]. In particular, CMV reactivation and concurrent immune responses to control infection are associated with aging and increased morbidity and mortality in both the general population and PLWH [29,30]. In the same group of people reported here, we previously found that increased anti-CMV IgG levels are associated with a higher proportion of terminally differentiated $CD4^+$ and $CD8^+$ T cells as well as $CD4^+$ T-cell activation [31], which may, in turn, have contributed to the observed greater age advancement.

Table 3. Mean (95% confidence interval) increase (if positive) or decrease (if negative) in age advancement among HIV-positive COBRA participants associated with each HIV-related factor.

Variable	Mean (95% CI) increase/decrease (in years)	*P*
Years since HIV diagnosis (per year)	0.28 (−0.01–0.56)	0.05
Duration of antiretroviral therapy (per year)	0.25 (0.01–0.49)	0.05
Nadir $CD4^+$ T-cell count (per 100 cells/μl)	−0.73 (−1.87–0.41)	0.21
Nadir $CD4^+$ T-cell count less than 200 cells/μl	3.48 (0.14–6.83)	0.04
Prior AIDS	1.70 (−1.85–5.24)	0.35

Estimates were obtained with separate linear regression models (one for each factor considered). CI, confidence interval; COBRA, Co-morbidity in Relation to AIDS.

Interestingly, current or past smoking did not appear to affect age advancement. Whilst more direct and accurate measures of smoking (including duration and frequency of smoking) would be more appropriate to evaluate the effect on the aging process, we cannot exclude that our findings reflect a lack of association with markers used in this study, especially in cohorts with relatively low smoking frequency (COBRA participants who self-reported current smoking, reported a mean of 12 cigarettes smoked per day). Other factors that have been shown to be associated with different aspects of the aging process of PLWH in previous studies (i.e. HCV co-infection [32] and $CD4^+$: $CD8^+$ T-cell count ratio [33]) were not linked to greater age advancement. Whilst this is likely to be because of a combination of low statistical power (only five participants were infected with chronic HCV) and collinearity with other lifestyle factors (e.g. recreational drugs for HCV and $CD8^+$ T-cell count for $CD4^+$: $CD8^+$ ratio), further studies are needed to elucidate potential contributors to the age advancement seen in PLWH.

Among antiretroviral drugs, we found a significant association between age advancement and duration of past exposure to saquinavir, which was independent of concomitant exposure to other drugs generally considered to have the greatest mitochondrial toxicities and potential effects on aging such as didanosine, stavudine, zalcitabine and zidovudine (data not shown). Whereas saquinavir has been shown to directly induce vascular endothelial toxicity *in vitro* [34], other HIV protease inhibitors were shown to be able to induce vascular smooth muscle cell senescence by downregulating ZMPSTE24, leading to prelamin A accumulation and potential premature vascular aging, changes, which were also observed in peripheral blood mononuclear cells from HIV-infected patients who were treated with the same protease inhibitors [35]. The reason why we only observed an association between age advancement and exposure to saquinavir, but not other HIV protease inhibitors, remains unclear, and suggests this observation should be interpreted with caution. Importantly, having experienced more pronounced immunodeficiency in HIV-positive participants was also linked to an increased age advancement suggesting that a greater effect on aging is likely to occur as the infection remains untreated for longer.

Our findings are consistent with previous reports of brain aging in the COBRA study [18] and with other studies of cellular and molecular markers of biological aging. Indeed, studies of telomere length and CDKN2A [14,36], $CD8^+$ T-cell senescence [37], and DNA methylation profiles ('epigenetic clock') [16,38] showed similar indications of age advancement in PLWH. Other studies reported evidence of accentuated aging only in PLWH with low nadir $CD4^+$ T-cell counts compared with HIV-negative individuals [39,40]. Of note, however, these previous studies often included untreated PLWH (some with detectable HIV RNA) and HIV-negative controls that differed with regards to some lifestyle behaviors, such as smoking and alcohol consumption. In our study, effectively treated PLWH with high $CD4^+$ T-cell counts and highly comparable HIV-negative controls were purposely recruited in order to reduce the influence of sociodemographic and lifestyle confounding factors. The importance of an appropriately chosen control group of HIV-negative individuals is also highlighted by the finding that HIV-negative COBRA participants showed greater age advancement compared with blood donors. Furthermore, these studies only focus on one single tissue or body system, and are therefore unable to reflect the intrinsic multicausal and multisystem nature of the aging process [41]. Chronic HIV may differently affect the rate of aging at the levels of cells, tissues or body systems within the same organism and this complexity is more likely reflected by a method used in our study, which integrates multiple sources of molecular, cellular and physiologic data.

Age advancement reflects the difference in body function and composition of an individual relative to that of a similarly-aged 'healthy' individual. As such, it is not surprising that we found a weak negative correlation with chronological age that, if anything, may reflect survivorship bias. Moreover, as we found no interaction between the correlation of age advancement with chronological age and HIV-status/group, our results are more suggestive of accentuated rather than accelerated aging in the context of treated HIV disease. However, longitudinal follow-up is required to more appropriately address this issue. Accentuated aging occurs when there is an increased burden of aging-related damage but the year-on-year damage remains static over time whereas accelerated aging occurs when the decline arises earlier than expected and implies a progressive increase in the rate of decline [42].

Unmeasured confounders (e.g. living settings (urban vs. rural), diet, physical activity, sleep habits and direct, rather than self-reported, information on smoking and substance/alcohol use) may also have contributed to the increased age advancement we observed in both PLWH and lifestyle-matched HIV-negative individuals and also to the apparent increase due to HIV. Our study has further limitations. Given the cross-sectional design, it can only assess associations and longitudinal studies would be necessary to evaluate the potential causal role of HIV and the interplay with antiretroviral drugs and viral co-infections. The sample size was believed to be sufficient to allow meaningful analysis; however, the study may not be powered enough to evaluate associations with cumulative exposure to antiretroviral drugs as they were evaluated only on participants with prior exposure to a drug. Also our cohort predominantly constitutes white, northern European MSM over the age of 45 years. Results may,

therefore, not be generalizable to younger populations or populations within a different HIV epidemic setting. Also, individuals with major depression were excluded from the study; as psychological stress has been linked with molecular changes that can affect the aging process [43], this could have resulted in an underestimation of age advancement in both COBRA groups. Finally, the age advancement observed in PLWH, COBRA HIV-negative individuals and blood donors is relative to the population used by the MARK-AGE project to develop the algorithm to estimate biological age. Individuals with self-reported HIV, HBV (except seropositivity by vaccination), HCV or cancer were excluded but the representativeness of the sample to European countries involved in the MARK-AGE study still needs to be assessed. Whilst the algorithm has not been yet validated in external population (including cohort of PLWH), it showed promising results in individuals affected by Down's syndrome who, as expected, showed greater age advancement compared with the general population (manuscript in preparation). Moreover, sensitivity analysis in our cohort suggest a positive correlation between age advancement and the number of age-associated comorbidities (Pearson's $r = 0.18$, $P = 0.007$) and time needed to walk 15 feet (Pearson's $r = 0.13$, $P = 0.04$) as well as a negative correlation with hand grip (Pearson's $r = -0.12$, $P = 0.05$), a validated measure of frailty.

In conclusion, our results suggest that PLWH with undetectable plasma HIV RNA may experience accentuated aging compared with HIV-negative individuals with similar lifestyles, as estimated using a set of validated biomarkers of aging. This age advancement appears to be related to viral co-infections such as CMV and chronic HBV, but also to historic severe immunosuppression and possibly exposure to particular antiretroviral drugs. Future longitudinal studies are required to further help clarifying the effect of HIV and its treatment on the natural aging process and the functional and clinical consequences in the millions of PLWH worldwide.

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Conflicts of interest

There are no conflicts of interest.

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