Epigenetic age acceleration changes 2 years after antiretroviral therapy initiation in adults with HIV: a substudy of the NEAT001/ANRS143 randomised trial

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Summary

Background DNA methylation-based estimators of biological age are reliable biomarkers of the ageing process. We aimed to investigate a range of epigenetic ageing biomarkers in a substudy of the NEAT001/ANRS143 clinical trial, which compared ritonavir-boosted darunavir with either raltegravir or tenofovir disoproxil fumarate and emtricitabine in antiretroviral therapy (ART)-naive adults.

Methods We analysed frozen whole blood samples from 168 ART-naive participants with HIV from the NEAT001/ANRS143 trial, before ART initiation and after 2 years of ART (84 participants on ritonavir-boosted darunavir with raltegravir and 84 participants on ritonavir-boosted darunavir with tenofovir disoproxil fumarate and emtricitabine). We also included 44 participants without HIV with a similar age and sex distribution. We analysed DNA methylation. Epigenetic age estimators (Horvath's clock, Hannum's clock, GrimAge, and PhenoAge) and estimated leucocyte compositions were generated using Horvath's New Online Methylation Age Calculator and Houseman's method. We calculated epigenetic age acceleration measures for each estimator of epigenetic age. The NEAT001/ANRS143 trial is registered with ClinicalTrials.gov, NCT01066962.

Findings Compared with the HIV-uninfected group, ART-naive participants with HIV showed higher epigenetic age acceleration (EAA) according to all EAA estimators (mean 2·5 years, 95% CI 1·89–3·22 for Horvath-EAA; 1·4 years, 0·74–1·99 for Hannum-EAA; 2·8 years, 1·97–3·68 for GrimAge-EAA; and 7·3 years, 6·40–8·13 for PhenoAge-EAA), with all differences being statistically significant except for Hannum-EAA (Horvath-EAA p=0·0008; Hannum-EAA p=0·059; GrimAge-EAA p=0·0021; and PhenoAge-EAA p<0·0001). Epigenetic ageing was more pronounced in participants who had CD4 counts less than 200 cells per µL (significant for PhenoAge and Hannum's clock, p=0·0015 and p=0·034, respectively) or viral loads over 100 000 copies per mL at baseline (significant for PhenoAge, p=0·017). After 2 years of ART, epigenetic age acceleration was reduced, although PhenoAge and GrimAge remained significantly higher in participants with HIV compared with participants without HIV (mean difference 3·69 years, 95% CI 1·77–5·61; p=0·0002 and 2·2 years, 0·47–3·99; p=0·013, respectively). There were no significant differences in the ART effect on epigenetic ageing between treatment regimens. At baseline, participants with HIV showed dysregulation of DNA methylation-based estimated leucocyte subsets towards more differentiated T-cell phenotypes and proinflammatory leucocytes, which was also partly restored with ART.

Interpretation ART initiation partly reversed epigenetic ageing associated with untreated HIV infection. Further studies are needed to understand the long-term dynamics and clinical relevance of epigenetic ageing biomarkers in people with HIV.

Funding NEAT-ID Foundation.

Introduction

Antiretroviral therapy (ART) has transformed HIV infection into a chronic and manageable disease in which life expectancy among people living with HIV approaches that of the general population.[1] However, this prolonged survival in people living with HIV has been linked to an increased burden of age-related comorbidities, such as cardiovascular disease, cancer, cognitive impairment, osteoporosis, and frailty.[2,3] Although the cause of this accelerated or accentuated ageing is not completely understood, it might be related to residual immunosenescence and inflammation that persists despite successful ART.[4,5]

Until roughly the past 5 years, the study of ageing and its consequences lacked precise and reproducible biomarkers. Although blood telomere length is negatively associated with chronological age, its ability to predict life expectancy is restricted.[6] Among the most reliable

Lancet HIV 2021; 8: e197–205

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See Online for appendix

Research in context

Evidence before this study

Data on the effect of HIV and antiretroviral therapy (ART) on epigenetic biomarkers of ageing are scarce. We searched PubMed for reports published in English, with no restrictions on publication date, using combinations of the following keywords: "HIV infection", "antiretroviral therapy", "premature aging", "epigenetic clocks", "epigenetic aging", and "epigenetic age acceleration". We also searched for relevant publications from international HIV congresses (2017–20) using the same search criteria. Our search yielded six publications. Five of these reports found an association between HIV infection and epigenetic age acceleration (EAA), both in untreated and treated HIV infection. Nevertheless, none of these studies evaluated the effect of ART on epigenetic ageing in treatment-naive adults with HIV in the context of a clinical trial. We found only one longitudinal study that reported a positive effect of ART initiation on epigenetic ageing in people with HIV. However, this study included a small number of participants (n=19) and was not fully powered to establish how epigenetic ageing dynamics change immediately after introducing ART.

Added value of this study

To our knowledge, this is the first study to assess changes in biomarkers of epigenetic ageing after ART initiation in a population of participants with HIV enrolled in a clinical trial (NEAT001/ANRS143). Our results support evidence that untreated HIV infection is associated with EAA, which is more pronounced in participants with severe immunodeficiency. Our study also suggests that ART partly reverses epigenetic ageing only 2 years after initiation. We also compared, for the first time to our knowledge, the effect of different ART regimens on epigenetic ageing dynamics. We found no significant difference in epigenetic ageing reversal between participants receiving darunavir and ritonavir plus raltegravir or darunavir and ritonavir plus tenofovir disoproxil fumarate and emtricitabine regimens.

Implications of all the available evidence

To our knowledge, our study is one of the first examples of how biomarkers of epigenetic ageing can capture the initial beneficial effect of a therapeutic intervention that significantly prolongs lifespan. The partial reversal of HIV-induced EAA supports an additional beneficial effect of ART. EAA predicts a higher risk of emergence of comorbidities and mortality in the general population, but its clinical relevance in people living with HIV remains to be appropriately defined. More evidence is needed to elucidate whether biomarkers of epigenetic ageing could help to identify people with chronic HIV who are more likely to suffer premature age-related comorbidities.

biomarkers of age are so-called epigenetic clocks, mathematical algorithms that predict epigenetic age as a surrogate of biological age based on the DNA methylation levels of different sets of CpG dinucleotide sites in the genome that are known to change with ageing.[7] The first to be developed were Horvath's multi-tissue epigenetic clock, based on 353 CpG sites,[8] and Hannum's epigenetic clock for blood samples, based on 71 CpG sites.[9] These epigenetic clocks are attractive biomarkers of ageing because they have a strong correlation with chronological age and because epigenetic age acceleration (EAA), defined as an epigenetic age greater than that predicted based on chronological age, predicts the occurrence of age-related comorbidities and mortality.[7,10] In the past two years, two new DNA methylation-based estimators of ageing have been developed: PhenoAge and GrimAge. PhenoAge predicts a surrogate measure of phenotypic age based on 513 CpG sites, and is considered an accurate predictor of mortality, healthspan (ie, the period of time in which a person is in good health), cardiovascular disease, and other morbidities.[11] The GrimAge estimator, which is calculated based on chronological age, sex, and DNA methylation-based surrogates for seven plasma proteins and smoking pack-years, also predicts time to death and comorbidities.[12] There are almost no data concerning the evolution of these biomarkers after successful treatment of diseases that shorten the lifespan. An example of such a disease is HIV, which has a median survival from age 25 of 19·9 years when untreated, compared with 51·1 years for people without HIV.[13]

Previous studies have reported that HIV is associated with accelerated epigenetic ageing in adults and perinatally HIV-infected children on ART.[14–16] Furthermore, in a cohort of 31 injection drug users, this accelerated ageing started soon after HIV seroconversion.[17] However, there is a paucity of data concerning the effect of ART and specific antiretroviral regimens on the evolution of epigenetic ageing. We aimed to investigate, for the first time to our knowledge, a range of epigenetic ageing biomarkers in a substudy of the NEAT001/ANRS143 clinical trial, which compared ritonavir-boosted darunavir combined with either raltegravir or tenofovir disoproxil fumarate and emtricitabine in ART-naive adults.[18] We hypothesised that ART would have a beneficial effect on epigenetic ageing.

Methods

Study design and participants

We included participants from the NEAT001/ANRS143 clinical trial. Briefly, NEAT001/ANRS143 was a randomised, open-label, non-inferiority trial at 78 clinical sites in 15 European countries (appendix pp 6–7) between August, 2010, and October, 2013. The trial showed non-inferiority over 96 weeks of ritonavir-boosted darunavir combined with raltegravir versus ritonavir-boosted darunavir combined with tenofovir disoproxil fumarate and emtricitabine in ART-naive adults aged ≥18 years

with HIV.[18] For the current study, we selected blood samples from participants in the NEAT001/ANRS143 blood telomere length substudy, which randomly selected 201 participants from the parent trial.[19] 186 (93%) of 201 selected participants had remaining available samples both at baseline and 96 weeks after ART initiation, but 18 were excluded because their samples did not meet the quality criteria for methylation analysis. Thus, we analysed samples from 168 NEAT001/ANRS143 participants (84 from the ritonavir-boosted darunavir with tenofovir disoproxil fumarate and emtricitabine group and 84 from the ritonavir-boosted darunavir with raltegravir group). Additionally, we analysed blood samples of 44 individuals without HIV from an anonymised collection of samples from healthy volunteers only once, for whom only age and sex data were available. We used frequency matching to obtain an HIV-uninfected group with a similar age and sex distribution.

All participants provided written informed consent, and the ethics committee and competent authority for all participating centres approved the study.

Procedures

We purified DNA from frozen whole blood samples using the MagPurix Blood DNA Extraction Kit 200 (Zinexts Life Science; New Taipei, Taiwan) and sodium bisulfite converted using the EZ DNA Methylation Kit (Zymo Research; Irvine, CA, USA). A genome-wide methylation analysis was done using the Infinium MethylationEPIC BeadChip Kit (Illumina; San Diego, CA, USA) following the manufacturer's instructions. To avoid technical variance, DNA methylation data from HIV-infected and HIV-uninfected participants were generated at the same time as follows: participants were randomly distributed across plates and chips, and the two timepoints per HIV-infected participant were analysed in the same chip. Experiments were done in the same core facility (Genomics Laboratory, Instituto de Genética Médica y Molecular, Hospital Universitario La Paz-IdiPAZ, Madrid, Spain) and by the same technician (PB). Raw array data were processed through the minfi R package (version 1.32.0) and background corrected with the noob normalisation method before obtaining the DNA methylation levels (β-values).[20,21] We kept samples that satisfied the quality criteria established in the guidelines from the minfi package for analysis.[20]

Epigenetic age estimators and estimated leucocyte compositions (total CD4 and CD8 T cells, naive CD4 and CD8 T cells, exhausted CD8 T cells, B cells, plasmablasts, monocytes, natural killer cells, and granulocytes) were generated from methylation profiles using Horvath's online DNA Methylation Age Calculator and Houseman's method.[22] We analysed four different epigenetic age estimators in our samples as follows: Horvath's epigenetic clock,[8] Hannum's epigenetic clock,[9] PhenoAge,[11] and GrimAge.[12] For each estimator, we calculated EAA measures, denoted as Horvath-EAA, Hannum-EAA, PhenoAge-EAA, and GrimAge-EAA. These measures corresponded to the residuals (difference between the real epigenetic age and the predicted value) resulting from the linear regression of each epigenetic age estimator and chronological age. All regression models used the samples from participants without HIV as a reference group, so a positive value of an EAA measure means that epigenetic age is higher than that predicted from the regression model for an HIV-uninfected individual of the same chronological age.

Statistical analysis

Participant characteristics were reported as absolute and relative frequencies and medians (IQR) for categorical and continuous variables, respectively. We analysed differences between treatment groups using χ^2 test or Wilcoxon rank-sum test as appropriate. Correlations were assessed using Spearman's correlation.

We used linear mixed-effects models (ie, random intercepts) to assess the longitudinal changes in EAA measures and estimated leucocyte composition in participants with HIV. We included the interaction term between study visit and group in the mixed models correspondingly to evaluate differences among the groups of participants with HIV (according to CD4 counts, viral load, and treatment allocation). Despite the marginal benefit of including random slopes in the model, the data presented an adequate fit including only random intercepts. We calculated non-longitudinal comparisons using linear regression analysis. Models were also created to adjust for covariates (age, sex, ethnicity, method of HIV infection, time since HIV diagnosis, statin treatment, body-mass index [BMI], and tobacco and alcohol use) when comparing participants with HIV according to their viral load and CD4 counts at baseline, and their antiretroviral regimen. We also adjusted by baseline data when comparing longitudinal changes in EAA measures among these groups. No adjustment was made for multiple comparisons given the exploratory design of the study. To determine whether EAA in untreated HIV was affected by leucocyte composition, we did multivariable linear regression, modelling EAA measures as a function of HIV status, adjusting for chronological age, sex, and estimated leucocyte composition. These models excluded the estimated levels of granulocytes to avoid multicollinearity.

Statistical analyses were done using Stata (version 16.0). The NEAT001/ANRS143 trial is registered with ClinicalTrials.gov, NCT01066962.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

For **Horvath's online DNA Methylation Age calculator** see https://dnamage.genetics.ucla.edu/new

	All participants (n=168)	Ritonavir-boosted darunavir plus raltegravir group (n=84)	Ritonavir-boosted darunavir plus tenofovir disoproxil fumarate and emtricitabine group (n=84)
Age, years*	38·1 (30·5–46·5)	39·5 (30·7–46·3)	37·4 (29·9–46·5)
Sex	..	..	..
Female	20 (12%)	11 (13%)	9 (11%)
Male	148 (88%)	73 (87%)	75 (89%)
Ethnicity	..	..	..
White	138 (82%)	70 (83%)	68 (81%)
Black	22 (13%)	11 (13%)	11 (13%)
Asian	2 (1%)	2 (2%)	0
Other	6 (4%)	1 (1%)	5 (6%)
Tobacco use*	..	..	..
Currently smoking	59 (35%)	26 (31%)	33 (39%)
Stopped smoking	18 (11%)	8 (10%)	10 (12%)
Never smoked	91 (54%)	50 (60%)	41 (49%)
Alcohol use*	..	..	..
Current drinker†	13 (8%)	3 (4%)	10 (12%)
Ex-drinker‡	1 (1%)	1 (1%)	0
Non-drinker or moderate drinker§	154 (92%)	80 (95%)	74 (88%)
Body-mass index, kg/m²*	23·8 (22–26·3)	24·2 (22·0–27·2)	23·1 (21·8–25·7)
Statin treatment*	13 (8%)	8 (10%)	5 (6%)
Time since HIV diagnosis, years	1·2 (0·4–2·5)	1·2 (0·4–2·5)	1·2 (0·3–2·5)
Method of HIV infection	..	..	..
Same-sex sexual activity	118 (70%)	56 (67%)	62 (74%)
Heterosexual sexual activity	41 (24%)	22 (26%)	19 (23%)
Injection drug use	1 (1%)	0	1 (1%)
Other	8 (5%)	6 (7%)	2 (2%)
CD4 count, cells per µL			
Baseline	346·0 (254·3–412·2)	347·8 (268·5–419·0)	344·3 (242·8–403·6)
Week 96	557·3 (430·0–688·0)	561·0 (453·0–695·0)	544·0 (424·0–687·0)
CD8 count, cells per µL			
Baseline	865·0 (641·6–1159·0)	879·0 (650·0–1256·0)	840·0 (624·0–1079·0)
Week 96	768·0 (529·0–971·0)	807·0 (545·0–1002·0)	728·5 (504·0–960·0)
CD4 to CD8 ratio			
Baseline	0·37 (0·25–0·51)	0·37 (0·24–0·49)	0·38 (0·26–0·52)
Week 96	0·74 (0·55–1·06)	0·75 (0·58–1·06)	0·73 (0·52–1·07)
Nadir CD4 count, cells per µL	305·5 (235·8–360·5)	312·5 (244·0–368·5)	296·3 (222·8–353·0)
Log HIV viral load, copies per mL at baseline	4·7 (4·3–5·1)	4·6 (4·1–5·2)	4·7 (4·4–5·1)
HIV RNA ≤50 copies per mL at week 96	159 (95%)	82 (98%)	77 (92%)

Data are median (IQR) or n (%). *Baseline characteristics. †More than four alcoholic drinks for men and three for women in a single day, and more than14 drinks for men and seven for women per week. ‡Meet criteria for non-drinker or moderate drinker but have met criteria for current drinker in the past. §Up to four alcoholic drinks for men and three for women in a single day, and a maximun of 14 drinks for men and seven for women per week.

Table: **Characteristics of participants with HIV**

Results

In participants with HIV, baseline characteristics were similar between treatment groups, with the exception of the nadir CD4 count (table). At week 96, the immunological and virological responses did not differ between the treatment groups. 159 (95%) of 168 participants had virological suppression and the median CD4 to CD8 ratio increased after ART initiation (table). Participants with HIV and HIV-uninfected individuals were well balanced for age (median 38·1 years, IQR 30·5–46·5 *vs* 39 years, 29·5–47·8; p=0·86) and sex (20 [12%] of 168 women participants *vs* seven [16%] of 44 women participants; p=0·48).

In participants with HIV (at baseline and week 96) and participants without HIV, we found strong correlation between chronological age and the four epigenetic clocks tested (in participants with HIV at baseline: Horvath's clock ρ=0·90; p<0·0001, Hannum's clock ρ=0·92; p<0·0001, PhenoAge ρ=0·87; p<0·0001, GrimAge ρ=0·82; p<0·0001; in participants with HIV at follow-up: Horvath's clock ρ=0·90; p<0·0001, Hannum's clock ρ=0·91; p<0.0001, PhenoAge ρ=0·86; p<0·0001, GrimAge ρ=0·82; p<0·0001; in participants without HIV: Horvath's clock ρ=0·89; p<0·0001, Hannum's clock ρ=0·87; p<0·0001, PhenoAge ρ=0·83; p<0·0001, GrimAge ρ=0·90; p<0·0001). The Horvath's and GrimAge epigenetic clocks tended to overestimate epigenetic age over chronological age, whereas the Hannum's and PhenoAge clocks tended to underestimate it (figure 1).

Compared with the HIV-uninfected group, ART-naive participants with HIV showed higher EAA according to all the EAA estimators (mean 2·5 years, 95% CI 1·89 to 3·22; p=0·0008 for Horvath-EAA; 1·4 years, 0·74 to 1·99; p=0·059 for Hannum-EAA; 2·8 years, 1·97 to 3·68; p=0·0021 for GrimAge-EAA; and 7·3 years, 6·40 to 8·13; p<0·0001 for PhenoAge-EAA; figure 2). After 96 weeks of ART, levels of these EAA measures significantly decreased (mean differences at week 96 compared with baseline –1·1 years, 95% CI –1·51 to –0·66 for Horvath-EAA, –1·6 years, –2·08 to –1·21 for Hannum-EAA, –0·6 years, –1·14 to –0·05 for GrimAge-EAA, and –3·6 years, –4·27 to –2·88 for PhenoAge-EAA), revealing a positive effect of ART on epigenetic ageing (figure 2). However, PhenoAge-EAA and GrimAge-EAA remained significantly higher in participants with HIV compared with participants without HIV after 2 years of ART (figure 2). In participants with HIV, we found no differences in EAA between those receiving raltegravir or tenofovir disoproxil fumarate and emtricitabine at any timepoint. Additionally, longitudinal changes in EAA measures after ART initiation did not differ between the treatment groups (appendix p 1).

Since our findings suggested a negative effect of HIV infection on epigenetic ageing, we assessed whether more advanced HIV infection was associated with higher EAA. Compared with participants with baseline CD4 counts above 200 cells per µL (n=140), those with CD4 counts less than 200 cells per µL (n=28) had higher levels of all the EAA measures tested, with significant

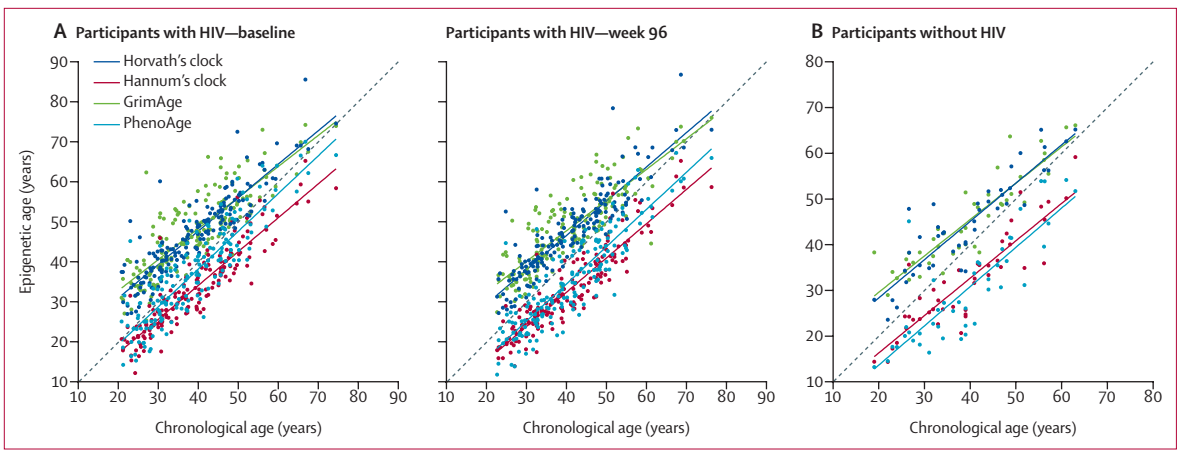

Figure 1: **Correlations between estimators of epigenetic age and chronological age in whole blood samples from 168 participants with HIV**
At baseline and 2 years after antiretroviral therapy initiation (week 96; A) and in 44 participants without HIV (B). Chronological age versus epigenetic age according to Horvath's clock, Hannum's clock, GrimAge, and PhenoAge. Grey dashed lines indicate y=x.

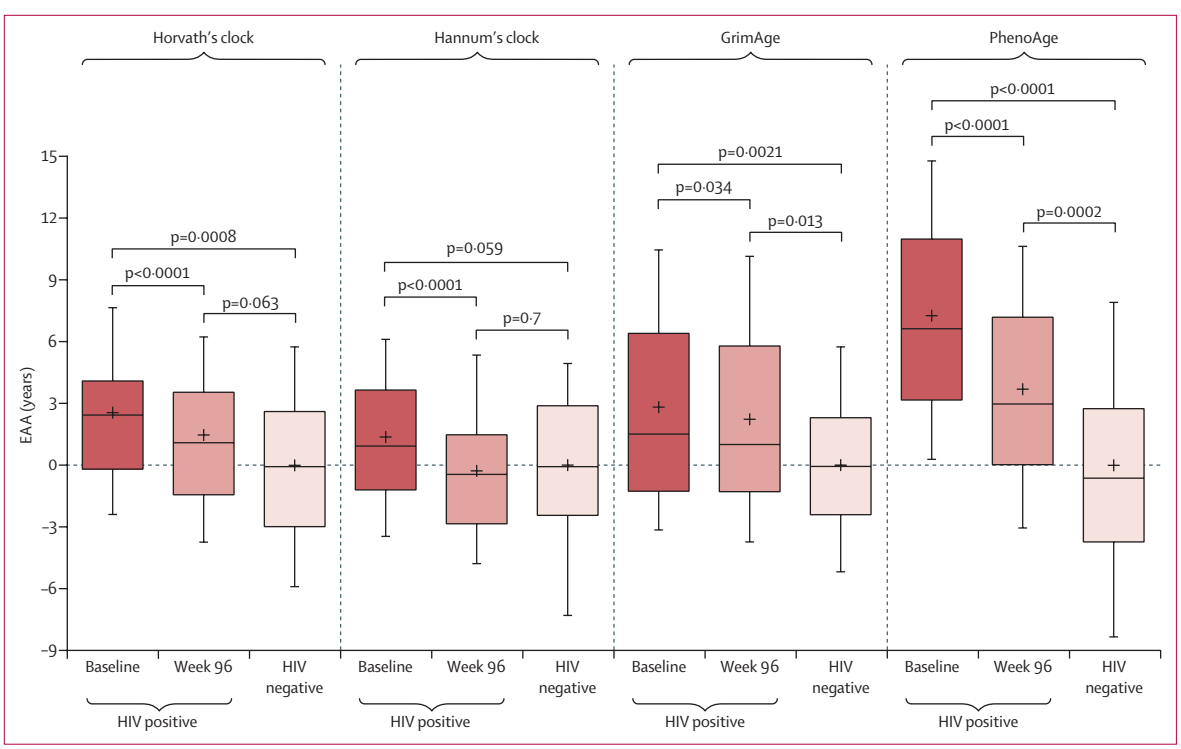

Figure 2: **Epigenetic age acceleration in ART-naive participants with HIV before and 2 years after ART initiation**
EAA according to Horvath's clock, Hannum's clock, GrimAge, and PhenoAge in 168 ART-naive participants with HIV at baseline and 2 years after ART initiation (W96), and in 44 HIV-uninfected participants. Boxes show medians and IQRs; whiskers correspond to 10th and 90th percentiles. The + symbol indicates the mean. ART=antiretroviral therapy. BL=baseline. EAA=epigenetic age acceleration.

differences for PhenoAge-EAA (mean difference 3·6 years, 95% CI 1·31–5·83; p=0·0015) and Hannum-EAA (1·8 years, 0·13–3·42; p=0·034; figure 3A). After adjusting for viral load and other covariates (age, sex, ethnicity, method of HIV infection, time since HIV diagnosis, statin treatment, BMI, and tobacco and alcohol use), only the difference in PhenoAge-EAA was maintained (p=0·034) and difference in GrimAge-EAA became significant (mean difference 2·5 years, 95% CI 0·54–4·53; p=0·013). All EAA measures were increased in participants in whom viral load was greater than 100 000 copies per mL at baseline (n=51), with significantly higher levels only for PhenoAge-EAA (mean difference 2·2 years, 95% CI 0·40–4·08; p=0·017; figure 3B). After adjusting for CD4 counts and other covariates, the difference in PhenoAge-EAA lost its

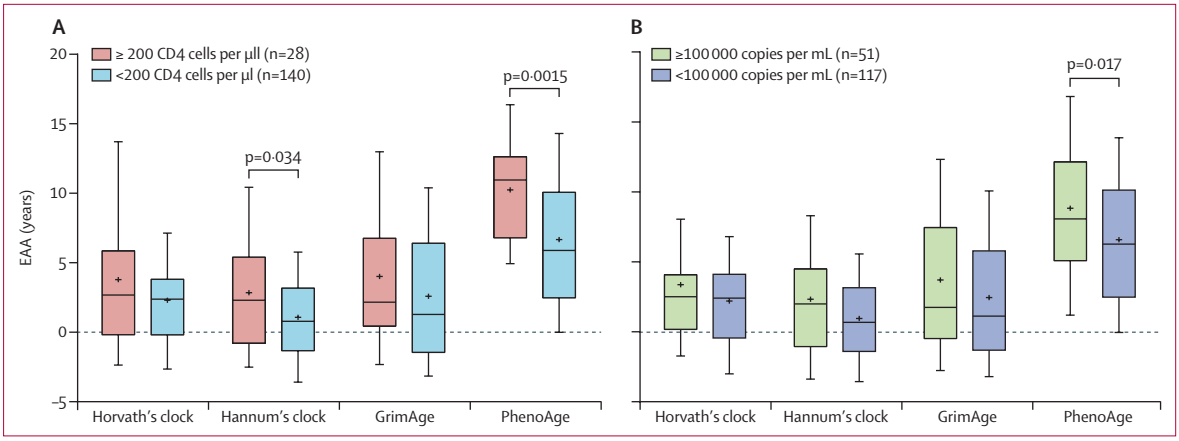

Figure 3: **EAA according to baseline CD4 counts (A) and baseline viral load (B) in participants with untreated HIV infection**
EAA according to Horvath's clock, Hannum's clock, GrimAge, and PhenoAge in ART-naive participants with HIV. Boxes show medians and IQRs; whiskers correspond to 10th and 90th percentiles. The + symbol indicates the mean. ART=antiretroviral therapy. EAA=epigenetic age acceleration.

significance (p=0·095) and the difference in GrimAge-EAA became significant (mean difference 1·7 years, 95% CI 0·11–3·30; p=0·036).

After 2 years of ART, a model adjusted by baseline EAA measures and other covariates showed that participants with CD4 counts less than 200 cells per µL at baseline underwent a more pronounced decline in EAA than did participants with CD4 counts above 200 cells per µL (GrimAge-EAA 1·7 years more, 95% CI 0·69 to 2·72; p=0·0010, PhenoAge-EAA 2·2 years more, 0·86 to 3·53; p=0·0012, and Hannum-EAA 1·2 years more, 0·30 to 2·00; p=0·0079). We observed no differences in EAA declines among participants according to their viral loads at baseline. We found that reductions in Hannum-EAA and PhenoAge-EAA measures were weakly correlated with an increase in CD4 counts (ρ=–0·24, p=0·0017 and ρ=–0·23, p=0·0022, respectively).

We estimated some leucocyte subset levels based on methylation profiles in participants with HIV and participants without HIV to investigate whether the higher EAA observed in untreated HIV was related to a different blood cell distribution. We assayed the accuracy of this estimation by a correlation model between the estimated levels of CD4 and CD8 T cells and the real counts of these lymphocytes determined by flow cytometry (data only available for participants with HIV), obtaining moderate correlations for both cell types (ρ=0·64, p<0·0001 for CD4 T cells and ρ=0·67, p<0·0001 for CD8 T cells).

In ART-naive participants with HIV, the estimated levels of total CD4 T cells, naive CD4 T cells, naive CD8 T cells, B cells, and granulocytes significantly increased after ART initiation, whereas the estimated total CD8 T cells, exhausted CD8 T cells, natural killer cells, and monocytes decreased significantly (figure 4). However, in participants with HIV, estimated levels of most leucocyte subsets remained higher after 96 weeks of treatment compared with participants without HIV, who had higher levels of total CD4 and naive CD4 and CD8 T cells, and lower levels of total CD8 and exhausted CD8 T cells and natural killer cells (figure 4). Estimated levels of plasmablasts did not differ among groups (data not shown). We also found no difference in estimated leucocyte composition between treatment groups at any timepoint (appendix p 2).

Multivariable linear regression models showed that untreated HIV, compared with HIV-uninfected status, was associated with a greater Horvath-EAA, GrimAge-EAA, and PhenoAge-EAA after adjusting for sex and chronological age (appendix p 3). Because of the differences in leucocyte subsets among groups, we also constructed models to adjust for the estimated levels of total CD4 and CD8 T cells, B cells, monocytes, and natural killer cells to control for confounding effects, and found that only GrimAge-EAA remained associated with untreated HIV infection (appendix p 3).

Discussion

In this study, we found that ART-naive adults with HIV had accelerated epigenetic ageing, which was more pronounced in those with CD4 counts less than 200 cells per µL or viral loads over 100 000 copies per mL. We observed a partial reversal of epigenetic ageing after only 2 years of ART, with no difference between participants on ritonavir-boosted darunavir with raltegravir or ritonavir-boosted darunavir with tenofovir disoproxil fumarate and emtricitabine. However, it is possible that other antiretrovirals could have a more pronounced effect on reversing EAA. The epigenetic ageing reversal was more marked in participants with more severe baseline CD4 immunodeficiency, and was accompanied by a shift of leucocyte subsets towards undifferentiated T-cell phenotypes and reduced proinflammatory leucocytes. These findings support that ART initiation partly reverses HIV-induced EAA, and is one of the first examples, to our knowledge, of how epigenetic clocks can capture the initial beneficial effect of

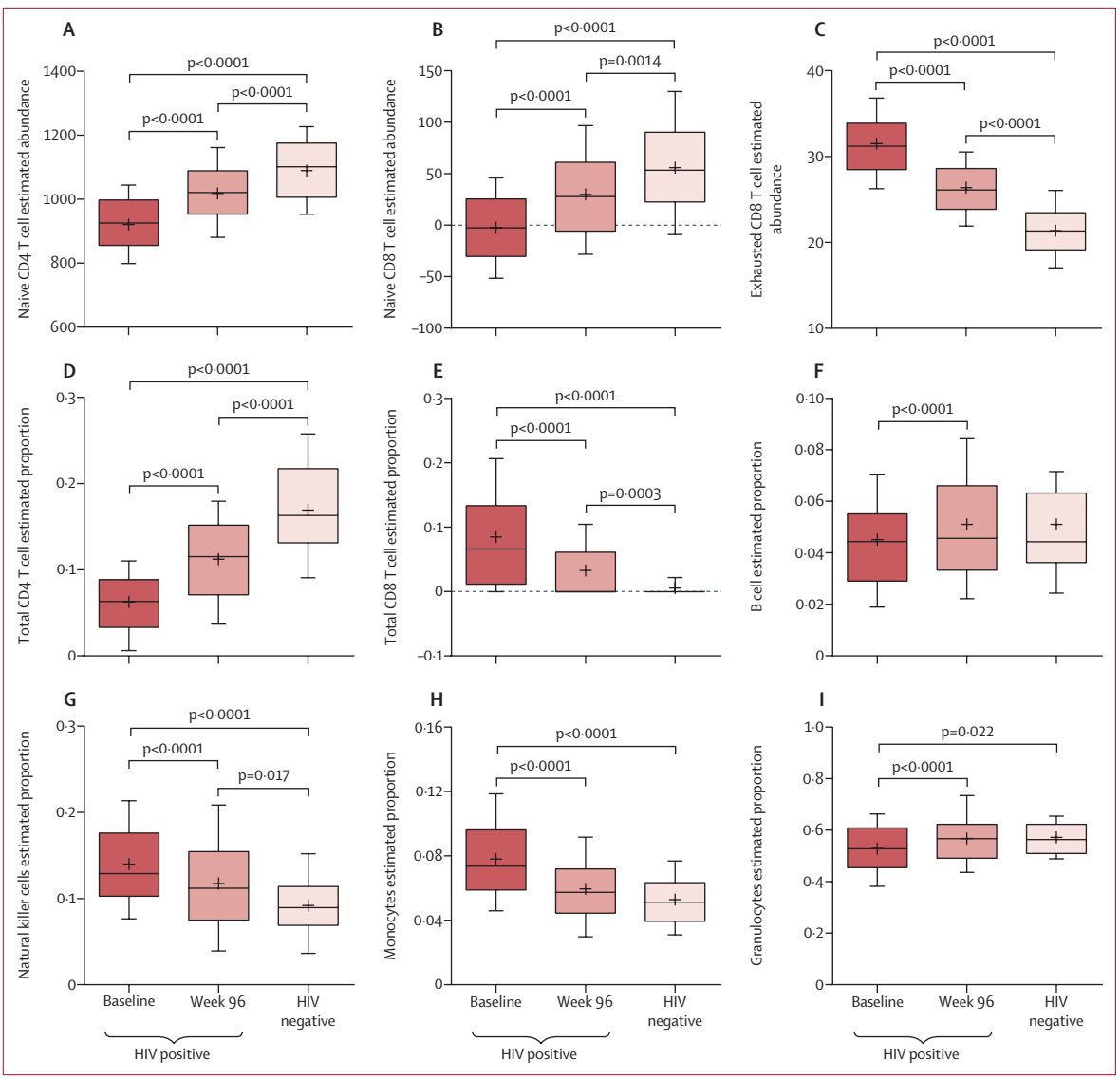

Figure 4: **DNA methylation-based estimated leucocyte composition in participants with HIV and participants without HIV**
Estimated leucocyte composition in participants with HIV at baseline and 2 years after ART initiation (W96), and in participants without HIV. (A) Naive CD4 T cells. (B) Naive CD8 T cells. (C) Exhausted CD8 T cells. (D) Total CD4 T cells. (E) Total CD8 T cells. (F) B cells. (G) Natural killer cells. (H) Monocytes. (I) Granulocytes. The y-axis corresponds to estimated abundance measures in parts A–C and estimated proportions in parts D–I, not to real cell counts. Boxes show medians and IQRs; whiskers correspond to 10th and 90th percentiles. The + symbol indicates the mean. ART=antiretroviral therapy.

a therapeutic intervention that significantly improves survival. In support of these data, we previously found a positive effect of ART initiation on blood telomere length, another biomarker of ageing, in data from the same clinical trial.[19]

In our study, we evaluated, for the first time to our knowledge in participants with HIV, the PhenoAge and GrimAge measures, two novel biomarkers of epigenetic ageing that have a greater ability to predict all-cause mortality and morbidities in the general population compared with traditional epigenetic clocks.[11,12] Although we found that EAA decreased after ART initiation according to the four selected indicators, PhenoAge-EAA and GrimAge-EAA were the only indicators that remained significantly elevated in participants with HIV after 2 years of follow-up. We could speculate that PhenoAge-EAA and GrimAge-EAA remained elevated because of the increased risk of morbidities, such as cardiovascular disease or malignancies, associated even with adequately treated HIV, and also because PhenoAge is associated with various markers of immunosenescence.[7]

Our longitudinal data are consistent with a previous study that found an improvement in epigenetic ageing 7–11 years after ART introduction based on Horvath's epigenetic clock in a group of 19 ART-naive US veterans with HIV from the Veterans Aging Cohort Study.[23]

Other cross-sectional studies have shown that HIV infection is associated with increased EAA. A study that compared age-related methylation patterns between ART-naive participants with HIV and a population of age-matched participants without HIV reported that untreated HIV infection induced an age advancement of 14 years.[24] Regarding chronic HIV infection on ART, two previous studies found that people living with HIV had an EAA around 5 years, according to Horvath's epigenetic clock.[14,15] In this study, we found a smaller effect on ART-naive participants with HIV before starting ART and 2 years after ART initiation (2·5 and 1·5 years, respectively), and a similar observation of restricted EAA among people with HIV on ART has been reported.[25] Although these data suggest that long-term chronic infection might have a negative effect on epigenetic ageing, we think these differences could also be explained by different sociodemographic, immunological, and virological characteristics of the study populations.

All previous studies we mentioned suggest an association between HIV infection and EAA, but the underlying causes of this process remain unclear. Since this study was done with frozen whole blood samples, we estimated the composition of the main leucocyte subsets to investigate their relationship with EAA. As expected, ART-naive participants with HIV had substantial immune dysregulation, with reduced levels of naive CD4 and CD8 T cells and increased levels of exhausted CD8 T cells, monocytes, and natural killer cells. We observed that the association between HIV status and higher levels of Horvath-EAA, Hannum-EAA, and PhenoAge-EAA disappeared after adjusting for estimated leucocyte composition in a multivariable model, and a similar finding has been previously reported.[23] As different leucocyte subsets have different methylation patterns,[26] this could indicate that these associations are driven by changes in cell composition. However, given that HIV infection is the cause of the immune dysregulation, the infection itself might be confounding the association between the estimated leucocyte composition and epigenetic ageing. Additionally, this hypothesis would not explain the accelerated ageing effects also reported in brain tissue samples from people with HIV.[14] Thus, further studies in specific blood cell subsets are needed to better understand what leads to epigenetic ageing in people with HIV.

The main limitation of our study is the selection of the HIV-uninfected group. The sample size was small (n=44), and chronological age and sex were the only available variables. Thus, comparisons between people with HIV and people without HIV could not be adjusted for potential confounders, such as socioeconomic status, lifestyle (eg, obesity, alcohol consumption, or smoking), and prevalence of cytomegalovirus co-infection, which have been shown to be associated with epigenetic ageing.[27] A previous study revealed that people living with HIV on ART and people without HIV with similar lifestyles had signs of age advancement compared with healthy blood donors.[28] Future studies are needed to know the real impact of HIV infection on epigenetic ageing, including control groups with similar lifestyle behaviours to people living with HIV (eg, alcohol, drug, and tobacco consumption or diet). However, our study shows that controlling HIV replication has a positive effect on ageing. Another limitation is the fact that we could not determine real counts of all leucocyte subsets by flow cytometry. Nevertheless, our data and other previously reported data support that estimated leucocyte composition based on DNA-methylation patterns has good strong correlation with corresponding flow cytometry measurements.[29]

The clinical relevance of the EAA observed in people living with HIV is not completely understood. Sánchez-Conde and colleagues[30] found that an older HIV-infected frail population had accelerated epigenetic ageing of up to 10 years compared with non-frail people with HIV who were matched for age and nadir CD4 count.[30] Another study that analysed post-mortem brain tissue samples from a group of people with HIV found that individuals who had been diagnosed with HIV-associated neurocognitive disorder within a year of death had higher EAA compared with those who were neurocognitively healthy.[31] Thus, although more studies are necessary to assess the relationship between epigenetic ageing and other pathologies, epigenetic ageing biomarkers could be useful tools to identify which individuals living with HIV have an elevated risk of age-related comorbidities, and therefore might benefit from more aggressive preventive interventions.

In conclusion, our results showed the ability of ART to partly reverse the EAA and leucocyte dysregulation associated with untreated HIV infection. Further research is needed to evaluate the long-term effect of ART in epigenetic ageing dynamics and to reveal the clinical relevance of epigenetic ageing biomarkers in people living with HIV.

Contributors

JRA, BR, and FR designed and oversaw the study. AE-C, JR-C, PB, BA, BR, and JRA reviewed and interpreted the data analyses. AE-C, JR-C, BA, BR, and JRA accessed and verified the data. AE-C, JR-C, BR, and JRA wrote the first draft of this report. All authors acquired the data, reviewed and amended the draft report, and approved the final version. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Declaration of interests

AE-C reports grants from Instituto de Salud Carlos III, during the conduct of the study. JR-C reports personal fees from Gilead, during the conduct of the study, and personal fees from ViiV and development of educational lectures for Gilead, outside the submitted work. RDM reports personal fees and non-financial support from ViiV and Gilead and grants from Fondo de Investigaciones Sanitarias, outside the submitted work. JIB reports grants and personal fees from Gilead, personal fees from ViiV, and personal fees and non-financial support from MSD, outside the submitted work. RM reports personal fees from Gilead, ViiV, Merck, and Janssen, outside the submitted work. JC reports grants from Instituto de Salud Carlos III, during the

conduct of the study, and other from MSD (funding for assistance to the XXIII Spanish Society of Clinical Microbiology and Infectious Diseases congress), outside the submitted work. EF reports grants from ViiV Healthcare, Gilead, and Janssen-Cilag NV, outside the submitted work. AA reports grants, personal fees, and non-financial support from Gilead and ViiV, and grants and personal fees from Janssen and Merck, outside the submitted work. AP reports grants and personal fees from ViiV and Gilead, outside the submitted work. CW reports grants from the European Commission and Inserm-ANRS, non-financial support from Gilead, and grants and non-financial support from Janssen and Merck, during the conduct of the study. FR reports personal fees from Gilead, Janssen, MSD, Theratechnologies, and ViiV, outside the submitted work. BR reports non-financial support from ViiV, outside the submitted work. JRA reports grants from NEAT-ID, during the conduct of the study, grants and personal fees from ViiV, and personal fees from MSD, Gilead, Janssen, Aelix, Serono, and Teva, outside the submitted work. All other authors declare no competing interests.

Data sharing

De-identified individual participant data that underlie the results reported in this Article will be made available upon request to the corresponding author.

Acknowledgments

This study was funded by the NEAT-ID Foundation through a grant from Gilead Sciences. NEAT is a project funded through the Instituto Superiore di Sanita–Rome by the EU under the Sixth Framework Programme (project number LSHPCT-2006-37570). The NEAT001/ANRS143 trial was also supported by Gilead Sciences, Janssen Pharmaceuticals, and Merck Laboratories, and The French National Institute for Health and Medical Research–France Recherche Nord & Sud Sida-HIV Hepatites (Inserm-ANRS) is the sponsor and a funder of the trial. AE-C is supported by a PFIS fellowship from Instituto de Salud Carlos III-Fondo Social Europeo (FI17/00194). RDM and JC are supported by a Río Hortega fellowship from Instituto de Salud Carlos III-Fondo Social Europeo (CM17/00064 and CM19/00059). We are grateful to all study participants, their partners, families, and caregivers, and local investigators and study coordinators from all the centres taking part in the NEAT001/ANRS143 trial.

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