Classification: BIOLOGICAL SCIENCES (Medical Sciences)

Menopause Accelerates Biological Aging

Morgan E. Levine[a,b], Ake T. Lu[a], Brian H. Chen[c], Dena G. Hernandez[d], Andrew B. Singleton[d], Luigi Ferrucci[c], Stefania Bandinelli[e], Elias Salfati[f], JoAnn E. Manson[g], Austin Quach[a], Cynthia D.J. Kusters[h], Diana Kuh[i], Andrew Wong[i], Andrew E. Teschendorff[j,k,l], Martin Widschwendter[j], Beate R. Ritz[h], Devin Absher[m], Themistocles Assimes[f], Steve Horvath[a,n]

Affiliations

[a]*Department of Human Genetics, University of California Los Angeles, Los Angeles, CA 90095, USA.*
[b]*Center for Neurobehavioral Genetics, University of California Los Angeles, Los Angeles, CA, 90095; USA.*
[c]*Longitudinal Study Section, Translational Gerontology Branch, Intramural Research Program, National Institute on Aging, National Institutes of Health, Bethesda, MD, 20892; USA.*
[d]*Laboratory of Neurogenetics, Intramural Research Program, National Institute on Aging, National Institutes of Health, Bethesda, MD, 20892; USA.*
[e]*Geriatric Unit, Azienda Sanitaria di Firenze, 50100; Florence, Italy.*
[f]*Department of Medicine, Stanford University School of Medicine, Stanford, CA, 94305; USA*
[g]*Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115; USA .*
[h]*Epidemiology, School of Public Health, University of California Los Angeles, Los Angeles, CA 90095; USA*
[i]*Medical Research Council Unit for Lifelong Health and Ageing at University College London, London, WC2B 4AN; United Kingdom*
[j]*Department of Women's Cancer & UCL Cancer Institute, University College London, London WC1 6BT; United Kingdom,*
[k]*Statistical Genomics Group at the University College London Cancer Institute, London, WC1E 6DD; United Kingdom*
[l]*CAS Key Laboratory of Computational Biology, CAS-MPG Partner Institute for Computational Biology, Chinese Academy of Sciences, Shanghai Institute for Biol, Shanghai 200031; China.*
[m]*HudsonAlpha Institute for Biotechnology, Huntsville, AL, 35806; USA*
[n]*Department of Biostatistics, University of California Los Angeles, Los Angeles, CA, 90095; USA*

Corresponding Author:
Steve Horvath (shorvath@mednet.ucla.edu)
(310) 825-9299
4357A Gonda Research Building
Department of Human Genetics
UCLA David Geffen School of Medicine
Los Angeles, CA 90095-7088

Keywords: Menopause, Aging, Epigenetics

Abstract

While epigenetic processes have been linked to aging and disease in other systems, it is not yet known whether they relate to reproductive aging. Recently, we developed a highly accurate epigenetic biomarker of age (known as epigenetic clock) which is based on DNA methylation levels. Here we carry out an epigenetic clock analysis of blood, saliva, and buccal epithelium using data from four large studies: Women's Health Initiative (n=1,864), InCHIANTI (n=200), PEG (n=256), and the United Kingdom Medical Research Council National Survey of Health and Development (NSHD) (n=790). We find that increased epigenetic age acceleration in blood is significantly associated with earlier menopause (P=0.00091), bilateral oophorectomy (P=0.0034), and a longer time since menopause (P=0.017). Conversely, buccal epithelium and saliva do not relate to age at menopause, however lower epigenetic age is exhibited in women who undergo menopausal hormone therapy (P=0.00078). Using genetic data we find evidence of co-heritability between age at menopause and epigenetic age acceleration in blood. Using Mendelian randomization analysis we find that two SNPs that are highly associated with age at menopause exhibit a significant association with epigenetic age acceleration. Overall, our Mendelian randomization approach and other lines of evidence suggest that menopause accelerates epigenetic aging of blood, but mechanistic studies will be needed to further dissect cause-and-effect relationships.

Significance Statement

Within an evolutionary framework, aging and reproduction are intrinsically linked. While both laboratory and epidemiological studies have observed associations between the timing of reproductive senescence and longevity, it is not yet known whether differences in the age of menopause are reflected in biomarkers of aging. Using our recently developed biomarker of aging,

the “epigenetic clock”, we examined whether age at menopause is associated with epigenetic age of blood, saliva, and buccal epithelium. This is the first definitive study to show an association between age of menopause and biological aging (measured using the epigenetic clock). Our results also indicate menopause may accelerate the epigenetic aging process in blood and that age at menopause and epigenetic age acceleration share a common genetic signature.

INTRODUCTION

Reproductive senescence, concluding in menopause, is a feature of all female mammals(1), yet humans are unique in that they experience exceptionally long post-reproductive lifespans. Within human populations, the timing of menopause onset has been linked to susceptibility for age-related morbidity and mortality outcomes(1). For instance, observational studies have uncovered associations between a woman’s age at menopause and her subsequent risk of mortality. Results based on 12,134 Dutch women showed that for every one year increase in the age of menopause, age-adjusted mortality rate was decreased by 2%(2).

While social/behavioral and developmental factors, such as smoking, lifetime socioeconomic circumstances , infant growth, breastfeeding, and childhood cognitive ability have been shown to influence reproductive aging, age at menopause is also considered to be highly heritable, with estimates from twin and sibling studies ranging from about 0.40-0.70(3-10). A recent large-scale genome-wide association study identified 44 genomic loci with common variants that significantly related to age at menopause(11), while a case-control study comparing centenarian women to those with average lifespans, found that individuals from families with a history of longevity also tend to exhibit delayed reproductive aging (12).

While these and other studies suggest that there might be a relationship between age at menopause and the biological aging rate, it has been difficult to test this hypothesis due to the dearth of molecular biomarkers of aging. Several recent articles describe epigenetic biomarkers of aging based on methylation levels(13-16), drawing upon the fact that chronological age has a profound effect on DNA methylation (DNAm) levels (17-26). While previous articles describe epigenetic age measures that only apply to a single tissue (saliva (13) or blood (14)), our recently developed "epigenetic clock" method (based on 353 CpGs) applies to every human tissue and cell type that contain DNA, with the exception of sperm(15). Age acceleration effects can be estimated by contrasting DNAm age with an individual's chronological age. For instance, a woman whose blood has a higher DNAm age than expected based upon her chronological age, can be said to exhibit positive age acceleration—or can be thought of as aging faster than expected. Age acceleration has also been shown to have a strong genetic basis—with heritability estimates of 40% for older subjects(15, 27). While the epigenetic clock has been shown to relate to a number of aging-related outcomes (27-30), it is not yet known whether it relates to reproductive aging.

Using data from four large observational studies—Women's Health Initiative (WHI), InCHIANTI, PEG, and NSHD—we examine the association between epigenetic age and: age at menopause, bilateral oophorectomy, and the use of menopausal hormone therapies (MHT). Given the strong heritability of both age at menopause and epigenetic age, we also estimate the genetic correlation between age at menopause and epigenetic aging, and carry out a Mendelian randomization analysis, to examine causality, using the top two SNPs shown previously to be strongly associated with age at menopause(31).

RESULTS

Age acceleration of blood versus age at menopause

In our primary analysis, we consider only women whose menopause occurred after age 30. As shown in **Table 1**, based on result from Pearson correlations in each of our three blood datasets (WHI, InCHIANTI, and PEG), meta-analysis showed that age at menopause was significantly associated with epigenetic age acceleration (p=0.00091). Similar meta-analytic p-values were obtained in our secondary analysis, which excluded women with surgical menopause (p=0.0083), and in our tertiary analysis, which used all women regardless of age or type (natural/surgical) of menopause (p=0.0061). Pearson correlation results from individual studies and stratifying by race/ethnicity can be found in **Figure S1**.

Multivariate linear models linking age acceleration with age at menopause

Using blood methylation data from WHI, InCHIANTI, and PEG we conducted multivariate regression for women who experienced menopause after age 30, and combined results using meta-analysis to determine whether covariates accounted for the association between epigenetic age acceleration and age at menopause (**Table 2**). Models were adjusted for age, race/ethnicity (in WHI and PEG), smoking status, age at menarche, and MHT use. Additionally, models run using PEG data, were also adjusted for Parkinson's disease (PD). In order to retain the moderate number of women with missing data on MHT use in PEG, those who were missing were coded as "never", and a dummy variable for missing was added to the model. After adjusting for possible confounders we find that higher epigenetic age acceleration is associated with a younger age at menopause (Meta-p=8.32×10^{-4}).

Time since Menopause

To examine whether menopause may be contributing to accelerated aging, we tested the association between epigenetic aging and time since menopause, using multivariate models that

adjust for race/ethnicity (WHI and PEG), and smoking (**Table S2**). Results showed that the variable "*time since menopause"* was associated with *AgeAccel* (β=0.038, P=0.007) in the WHI and in our meta-analysis (*AgeAccel* P=0.017).

Surgical menopause is associated with epigenetic age acceleration

The menopause of a substantial number of women was due to bilateral oophorectomy (i.e. n=127 women in WHI-white, 112 in WHI-black, 50 women in WHI-Hispanic, and 48 women in PEG). We evaluated the effect of surgical menopause on epigenetic age acceleration among women whose bilateral oophorectomy took place before age 50 (**Figure 1**). The association was highly consistent across the blood data sets (**Figure 1**) which led to a significant meta-analysis P-value for all 3 measures of age acceleration: *AgeAccel* (Stouffer's Z=3.2. p=0.0014).

Genetic correlation and Mendelian randomization between epigenetic age acceleration and age at menopause

Using the WHI data (n=1,940), we conducted a bivariate REML analysis to examine the overlap in genetic variants that accounted for the heritability of both age at menopause and epigenetic age acceleration. As shown in **Table 3**, the 10,769,392 autosomal SNPs or INDEL markers included in the analysis accounted for about 38% of the variance in age at menopause and 65% of the variance in *AgeAccel*. The genetic correlation (or pleiotropy) between *AgeAccel* and age of menopause was marginally significant with rG=-0.256 (one-sided P=0.054).

Although we clearly demonstrate that age at menopause relates to epigenetic age acceleration, our cross sectional data make it difficult to dissect causal relationships. Mendelian randomization (reviewed in (32))—the random assortment of genes from parents to offspring that occurs during gamete formation and conception—provides one method for assessing the causal

nature of associations. Here we test the hypothesis that menopause leads to an acceleration of epigenetic aging by leveraging the two most highly significant SNPs from a genome-wide association study for age at menopause (rs11668344 Replication P-value= 2.65×10^{-18}, rs16991615 Replication P-value= 7.90×10^{-21}). If menopause accelerates epigenetic aging then one would expect these two SNPs to also relate to epigenetic age acceleration, which is what was found (**Table 4**) for rs11668344 (P=0.031). Additionally, the directionality of the associations is consistent with the causal model (SNP→age at menopause→age acceleration), i.e. the minor allele is associated with earlier age at menopause and higher epigenetic age acceleration. On the other hand, rs16991615 was not associated with epigenetic age acceleration (P=0.763).

Analysis of buccal epithelium and saliva

We are not aware of any biological reason that suggests that blood tissue stands out when it comes to studying menopausal effects. Several previous data sets demonstrate that the epigenetic clock method also applies to buccal epithelium (35). As a result, we also examined epigenetic age measured in 790 buccal epithelium samples from National Survey of Health and Development (NSHD) from the United Kingdom Medical Research Council.

Unlike results in blood, MHT is associated with a significantly lower epigenetic age acceleration of buccal epithelium (p=0.00078, **Figure 2A**); whereas age at natural or surgical menopause does not correlate with epigenetic age acceleration across the n=419 postmenopausal buccal samples **(Figure 2B and 2D, respectively**); and no significant association was found between age acceleration and menopausal status at age 53—n=419 post- versus 371 pre-menopausal samples (**Figure 2C**).

We also analyzed saliva samples from 113 women from the PEG study of whom 16 had undergone surgical menopause. Similar to buccal epithelium, age at menopause and time since menopause were not associated with epigenetic age acceleration in saliva which might reflect the low sample size. However, surgical menopause was associated with increased *AgeAccel* of saliva (P=0.0079, **Figure 3**).

DISCUSSION

To the best of our knowledge, this is the first study that shows a) that the epigenetic age of blood has a negative correlation with age at menopause, b) that surgical menopause is associated with an increased epigenetic age of blood and saliva, c) that menopausal hormone therapy is inversely associated with epigenetic age acceleration of buccal epithelium, and d) that a SNP which relate to age at menopause also relates to epigenetic age acceleration. While our study demonstrates that postmenopausal women with a late onset of menopause are epigenetically younger than women with an early onset of menopause, it is challenging to dissect cause and effect relationships. In the following, we discuss several causal scenarios that could explain the reported findings (**Fig. S2**).

The first causal model (age at menopause ← Biological Age → Epigenetic age acceleration) assumes that both "age at menopause" and "epigenetic age acceleration" are indicator variables of a latent variable, which can be thought of as true biological age. There is evidence that risk factors for heart disease, which arguably accelerate biological age, are likely to contribute to an earlier age of menopause, rather than the commonly held notion that early menopause is a risk factor for heart disease(33). Consistent with evolutionary theories of aging, genetic predisposition to later menopause may coincide with an innate protection against early mortality(34). In this study, we observe a suggestive genetic correlation between age at menopause and epigenetic age

acceleration, which may suggest that increased age at menopause is genetically linked to decreased epigenetic age acceleration. This advocates for a common genetic etiology and is consistent with both variables being indirect measures of biological age—genetic variants which influence biological aging may therefore lead to both slower reproductive aging and lower epigenetic age.

Another causal model assumes that menopause leads to an increase of epigenetic age. This model is supported by the following lines of evidence. First, we find that longer time since menopause (irrespective of age at menopause) is associated with increased epigenetic age acceleration. Second, bilateral oophorectomy is associated with increased epigenetic age acceleration in both blood and saliva. This finding is congruent with a growing body of evidence suggesting that the premature loss of ovarian function caused by bilateral oophorectomy performed before natural menopause contributes to increased susceptibility to premature death, cardiovascular disease, dementia, parkinsonism, osteoporosis and bone fractures(35). This is also consistent with findings showing that transplantation of young ovaries in old mice significantly increases lifespan(36). Third, our study demonstrated that MHT, which arguably counters some of the effects of menopause, is associated with a decreased epigenetic age acceleration of buccal epithelium (but not of blood). Fourth, our Mendelian randomization analysis provided evidence of a causal pathway in which menopause accelerates epigenetic aging in blood—one of the most significant SNPs (rs11668344) for age of menopause also relates to epigenetic age acceleration in blood. Although we acknowledge that each of the above mentioned arguments in support of the causal model has pitfalls, in aggregate our results strongly support the causal model: Menopause → Epigenetic age acceleration.

In moving forward, it will be important to examine longitudinal change in epigenetic age of blood before and after women transition from pre- to post-menopausal status. Additionally, it

will be important to examine how long it takes accelerated epigenetic aging effects to become apparent following surgical menopause or whether they were apparent before. For instance, if accelerated aging is triggered by menopause, then one would expect age acceleration to increase as a function of time since bilateral oophorectomy. However, if the condition that led to a surgical removal of both ovaries (e.g. fibroids, menstrual disorders, endometriosis) is driving age acceleration, one would not see a linear increase in epigenetic age acceleration over time. The reason for bilateral oophorectomy was available from the NSHD sample. Overall, most women underwent bilateral oophorectomy due to reported fibroids, menstrual disorders, endometriosis (or some combination of these) in both groups (**Table S3**). Similar prevalence estimates apply to the blood data from PEG.

While our study detected an association between epigenetic age in blood and age at menopause, similar associations were not found for epigenetic age estimates from buccal epithelium. One potential explanation is that aging measures in the various tissues are capturing different phenomena. While we find a robust correlation between *AgeAccel* in blood and saliva (r=0.70) (**Figure S2**), when comparing the measures of *AgeAccel* in women with data from both blood and buccal epithelium, we find that the correlation is relatively weak (r=0.20) (**Figure S3**). At first sight, the low correlation of age acceleration between tissues is surprising. However, we interpret it as follows. The epigenetic clock is a strong predictor of age in multiple tissue when comparing across individuals. However, that does not mean that it will be correlated across tissues when comparing within-person differences. Stress factors and other perturbations act largely in a tissue specific manner (37). For that reason, one may not expect all tissue within an individual to age at the same rate, thus potentially accounting for the low/moderate correlation between epigenetic age acceleration in blood and buccal epithelium.

To the best of our knowledge, our study is the first to demonstrate that reproductive age, bilateral oophorectomy, and MHT relate to measures of epigenetic age acceleration—but the reported associations are specific to either blood, buccal epithelium, or saliva. Future studies are warranted for examining these effects in other tissues. Since epigenetic age captures aspects of biological age (27-30), our study strongly suggests that the hormonal changes that accompany menopause accelerate biological aging in women.

METHODS

Data set 1: WHI Sample Description (blood)

Participants were part of a subsample from the Women's Health Initiative (WHI), who were enrolled in an integrative genomics study with a primary aim of identifying novel genomic determinants of CHD. This sample included women ages 50-79, with an overrepresentation of racial/ethnic minorities. The WHI is a national study that began in 1993 which enrolled postmenopausal women. Women who were ineligible to participate in the trials or who chose not to be randomized were invited to participate in the observational arm of the study. The integrative genomics subsample employed a case-control sampling design. All cases and controls were required to have already undergone genome wide genotyping at baseline as well as profiling of seven cardiovascular biomarkers, as dictated by the aims of other ancillary WHI studies.

As shown in **Table S1**, the mean age at baseline for the 1,864 women in the WHI sample was 65.31 years (s.d.=7.1). *AgeAccel* ranged from -22.6 to 42.9, with a mean of 0.08 and a standard deviation of 5.3, while age at menopause ranged from age 25 to age 60, with a mean of 47.43 (s.d.=6.9). Overall, approximately half of our sample was non-Hispanic white (47.1%), one-third (32.2%) were African American, and about 20% were Hispanic. The majority of our sample

reported never smoking (53.4%), whereas 36.4% reported past smoking history, and about 10% reported they were current smokers at the time of blood draw.

At baseline, the majority of women did not have a history of any MHT use (61.1%), while approximately 21% were past users and 18% were current users. Of those who had a history of MHT, 17% were past users of unopposed estrogen, 12% were current users of unopposed estrogen, 6% were past users of estrogen plus progestin, and 5.5% were current users of estrogen plus progestin. Additionally, the majority of users were on MHT for less than five years (55%), while 16.7% took MHT for 5-9 years, about 10% took MHT for 10-14 years, and 18% took MHT for 15 or more years.

Data set 2: InCHIANTI Sample Description (blood)

The Invecchiare nel Chianti (InCHIANTI) Study is a population-based prospective cohort study of residents ages 30 or older from two areas in the Chianti region of Tuscany, Italy. Sampling and data collection procedures have been described elsewhere (38). Briefly, participants were enrolled between 1998-2000 and were examined at three-year intervals. Overall 1,326 participants donated a blood sample at baseline (1998-2000), of which 784 also donated a blood sample at the 9-year follow-up (2007-2009). DNA methylation was assayed using the Illumina Infinium HumanMethylation450 platform for participants with sufficient DNA at both baseline and Year 9 visits (n=499). Our study focused only on women (n=200). Ages for the 200 women in InCHIANTI ranged from 50 to 91, with a mean age of 70.64 years (Table S1), whereas baseline *AgeAccel* ranged from -12.9 to 12.05, with a mean of 0. Age at menopause ranged from age 26 to age 60, with a mean of 49.1. Overall, approximately 75.5% of the women from InCHIANTI were never smokers, 12.0% were former smoker, and about 12.5% were current smokers. Women with

no history of any MHT use made up 88.5% of the InCHIANTI sample. Finally, women from InCHIANTI averaged approximately 1.82 pregnancies over their lifetimes.

Data set 3: Women from the PEG cohort (blood and saliva)

We used two types of tissues from the Parkinson's disease, Environment, and Genes (PEG) study cohort: blood and saliva. The PEG study is a large population-based case-control study of Parkinson's disease of rural and township residents of California's central valley(39). Our blood data come from subjects from wave 1 (PEG1). Parkinson's disease status did not confound the relationship in blood because it was not associated with age at menopause, however we adjusted for it in multivariate analyses. The 256 women in PEG ranged in age from 35 to 91 years, with a mean age of 67.9 years (Table S1). Only 3 participants self-identified as non-Hispanic black, while 23 self-identified as Hispanic. Average age at menopause was 46.4 years. Overall, approximately 4% of the women from PEG were current smokers at the time of blood draw, and 38% were former smoker. Women with any history (current or former) of any MHT use made up 71% of the sample, and on average women in PEG reported having 3.4 pregnancies over their lifetimes.

The saliva methylation data were collected at a later time point than the blood data. For about half of the women, we had both blood and saliva methylation data but epigenetic age acceleration of blood tissue was not correlated with age acceleration in saliva.

Data set 4. NSHD (buccal epithelium)

These buccal samples stem from a subsample of 790 women participants in a British birth cohort, the United Kingdom Medical Research Council National Survey of Health and Development (NSHD) as described in (40). The women were all aged 53 years at the time of sample collection in 1999. At that time, 419 women were post-menopausal and 371 women were

pre-menopausal. MHT status was coded as "yes" only if MHT started before the age of sample collection (i.e. 53). Our results regarding the relationship between "age at menopause" and epigenetic age acceleration were largely unchanged after excluding women who experienced surgical menopause.

All women gave written informed consent for their samples to be used in genetic studies of health, and the Central Manchester Research Ethics Committee approved the use of these samples for epigenetic studies of health in 2012. Women were selected from those who provided a buccal and blood sample at age 53 years in 1999, who had not previously developed any cancer, and who had complete information on epidemiological variables of interest. Smoking status did not confound the reported relationships because smoking was not significantly associated with our measure of epigenetic age acceleration.

DNA methylation data

All DNA methylation data sets used the Illumina Infinium 450K platform. The Illumina BeadChips measures bisulfite-conversion-based, single-CpG resolution DNA methylation levels at 485577 different CpG sites in the human genome. These data were generated by following the standard protocol of Illumina methylation assays, which quantifies methylation levels by the β value using the ratio of intensities between methylated and un-methylated alleles. Specifically, the β value is calculated from the intensity of the methylated (M corresponding to signal A) and un-methylated (U corresponding to signal B) alleles, as the ratio of fluorescent signals $\beta = \text{Max}(M,0)/[\text{Max}(M,0)+\text{Max}(U,0)+100]$. Thus, β values range from 0 (completely un-methylated) to 1 (completely methylated). For our blood data sets (WHI, PEG) we used background corrected beta values. Consistent with the original publication, buccal samples were normalized using the BMIQ method (41). The correlation between DNAm age and chronological age is highly robust

with respect to different normalization methods because a) the epigenetic clock implements a custom normalization methods and b) it was constructed using training data that were normalized in different ways.

Epigenetic age acceleration

We used the DNAm age based biomarker of aging from (15) because a) its accurate measurement of age across tissues is unprecedented (and it applies to both blood and buccal epithelium), b) it is prognostic for all-cause mortality (27, 28), c) it correlates with measures of cognitive and physical fitness in the elderly (27, 42), and d) it has been found useful for studying aging effects in Down syndrome(43), Parkinson's disease (44), neuropathological variables (42); obesity(37), and HIV infection(45). DNAm age was defined using the 353 CpGs and coefficient values reported in (15). These CpGs and coefficient values were chosen in independent data using the elastic net penalized regression model to regress age on CpGs, resulting in a DNAm age measures defined as predicted age, in years.

Our measure of age acceleration (*AgeAccel*), which applies to all sources of DNA, was defined as residual resulting from a linear model that regressed DNAm age on chronological age.. Thus, a positive value for *AgeAccel* indicates that the observed DNAm age is higher than expected. *AgeAccel* has only a weak correlation with blood cell counts(45).

Genome-wide SNP data from the WHI

Genotyping was performed for all participants on Affymetrix 6.0, Illumina HumanOmni1-Quad v1.0, or Illumina HumanOmniExpressExome-8v1.0. Imputation was performed using MaCH with haplotypes phased in Beagle or Minimac(46, 47). The reference panel for imputation was based on the 1000 Genome haplotypes (released in June 2011). The quality of imputed

markers was assessed by MaCH $R^2 > 0.3$. Additional quality control filters that were employed included Hardy-Weinberg Equilibrium P-values $<10^{-3}$ and minor allele frequency (MAF) >0.01. Finally, to account for population structure, principal component analysis (PCA) was performed to generate sample eigenvectors, the first two of which were included as covariates in all GCTA analysis.

We related the two highly significant SNPs from the recent large-scale meta-analysis of age at menopause(31) to age acceleration using the WHI data. Association analysis was conducted in the two subsets of individuals stratified by platform. All women were of European ancestry, identified by multidimensional scaling analysis in PLINK. We combined the results into a single estimate by fixed-effects models weighted by inverse variance, as implemented in R *metafor*. For association analysis, we regressed each of the three age acceleration trait values on expected genotype dosage, adjusted for the first two principal components when necessary.

Ethics

This study was reviewed by institutional review board from UCLA (IRB#13-000671 and IRB#14-000061). Informed consent was obtained from all subjects.

Availability of data

The WHI and inCHIANTI data are available through NHLBI (https://biolincc.nhlbi.nih.gov/studies/bhs/). The PEG data are available from Gene Expression Omnibus (GSE72775).

Software Code Availability

We used the online version of the epigenetic clock software, which is freely available from the following webpage: https://dnamage.genetics.ucla.edu/. R source code is publicly available (from Additional file 20 in Horvath (2013) Genome Biology, PMID: 24138928).

ACKNOWLEDGEMENTS

This study was supported by NIH/NHLBI 60442456 BAA23 (Assimes, Absher, Horvath); National Institutes of Health NIH/NIA 1U34AG051425-01 (Horvath) and NIH/NINDS T32NS048004 (Levine). The WHI program is funded by the National Heart, Lung, and Blood Institute, National Institutes of Health, U.S. Department of Health and Human Services through contracts HHSN268201100046C, HHSN268201100001C, HHSN268201100002C, HHSN268201100003C, HHSN268201100004C, and HHSN271201100004C. Part of this work was funded by the Eve Appeal (https://www.eveappeal.org.uk/) and was done at University College London Hospital/University College London, which received a proportion of its funding from the Department of Health National Institute for Health Research Biomedical Research Centres funding scheme (MW). The NSHD is funded by the UK Medical Research Council [MC_UU_12019/1]. The PEG study was funded by R21 ES024356 (Horvath, Ritz) and RO1-ES10544 (Ritz).

REFERENCES

1. Finch CE (2014) The menopause and aging, a comparative perspective. *The Journal of steroid biochemistry and molecular biology* 142:132-141.
2. Ossewaarde ME*, et al.* (2005) Age at menopause, cause-specific mortality and total life expectancy. *Epidemiology* 16(4):556-562.
3. De Bruin J*, et al.* (2001) The role of genetic factors in age at natural menopause. *Human Reproduction* 16(9):2014-2018.
4. van Asselt KM*, et al.* (2004) Heritability of menopausal age in mothers and daughters. *Fertility and sterility* 82(5):1348-1351.
5. Murabito JM, Yang Q, Fox C, Wilson PW, & Cupples LA (2005) Heritability of age at natural menopause in the Framingham Heart Study. *The Journal of Clinical Endocrinology & Metabolism* 90(6):3427-3430.
6. Hardy R & Kuh D (2005) Social and environmental conditions across the life course and age at menopause in a British birth cohort study. *BJOG : an international journal of obstetrics and gynaecology* 112(3):346-354.
7. Hardy R & Kuh D (2002) Does early growth influence timing of the menopause? Evidence from a British birth cohort. *Human reproduction (Oxford, England)* 17(9):2474-2479.
8. Kuh D*, et al.* (2005) Childhood cognitive ability and age at menopause: evidence from two cohort studies. *Menopause* 12(4):475-482.
9. Mishra G, Hardy R, & Kuh D (2007) Are the effects of risk factors for timing of menopause modified by age? Results from a British birth cohort study. *Menopause* 14(4):717-724.
10. Richards M, Kuh D, Hardy R, & Wadsworth M (1999) Lifetime cognitive function and timing of the natural menopause. *Neurology* 53(2):308-314.
11. Day FR*, et al.* (2015) Large-scale genomic analyses link reproductive aging to hypothalamic signaling, breast cancer susceptibility and BRCA1-mediated DNA repair. *Nat Genet* 47(11):1294-1303.
12. Perls TT & Fretts RC (2001) The evolution of menopause and human life span. *Annals of human biology* 28(3):237-245.
13. Bocklandt S*, et al.* (2011) Epigenetic predictor of age. *PLoS ONE* 6(6):e14821.
14. Hannum G*, et al.* (2013) Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell* 49.
15. Horvath S (2013) DNA methylation age of human tissues and cell types. *Genome Biol* 14(R115).
16. Weidner CI*, et al.* (2014) Aging of blood can be tracked by DNA methylation changes at just three CpG sites. *Genome Biol* 15(2):R24.
17. Christensen B*, et al.* (2009) Aging and Environmental Exposures Alter Tissue-Specific DNA Methylation Dependent upon CpG Island Context. *PLoS Genet* 5(8):e1000602.
18. Bollati V*, et al.* (2009) Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech Ageing Dev* 130(4):234-239.
19. Rakyan VK*, et al.* (2010) Human aging-associated DNA hypermethylation occurs preferentially at bivalent chromatin domains. *Genome Res* 20(4):434-439.
20. Teschendorff AE*, et al.* (2010) Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. *Genome Res* 20(4):440-446.
21. Vivithanaporn P*, et al.* (2010) Neurologic disease burden in treated HIV/AIDS predicts survival. *Neurology* 75(13):1150-1158.
22. Horvath S*, et al.* (2012) Aging effects on DNA methylation modules in human brain and blood tissue. *Genome Biol.* 13:R97.

23. Numata S*, et al.* (2012) DNA Methylation Signatures in Development and Aging of the Human Prefrontal Cortex. *Am J Hum Genet.* 90(2):260-272.
24. Alisch RS*, et al.* (2012) Age-associated DNA methylation in pediatric populations. *Genome Res.* 22(4):623-632.
25. Johansson A, Enroth S, & Gyllensten U (2013) Continuous Aging of the Human DNA Methylome Throughout the Human Lifespan. *PLoS One* 8(6):e67378.
26. Day K*, et al.* (2013) Differential DNA methylation with age displays both common and dynamic features across human tissues that are influenced by CpG landscape. *Genome Biol.* 14(9):R102.
27. Marioni R*, et al.* (2015) DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol.* 16(1):25.
28. Christiansen L*, et al.* (2015) DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell*.
29. Horvath S*, et al.* (2015) Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging (Albany NY)*.
30. Marioni RE*, et al.* (2015) The epigenetic clock is correlated with physical and cognitive fitness in the Lothian Birth Cohort 1936. *Int J Epidemiol*:dyu277.
31. Stolk L*, et al.* (2012) Meta-analyses identify 13 loci associated with age at menopause and highlight DNA repair and immune pathways. *Nature genetics* 44(3):260-268.
32. Davey Smith G & Ebrahim S (2003) 'Mendelian randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int J Epidemiol* 32(1):1-22.
33. Kok HS*, et al.* (2006) Heart disease risk determines menopausal age rather than the reverse. *J Am Coll Cardiol* 47(10):1976-1983.
34. Rose MR (1991) *Evolutionary Biology of Aging* (Oxford University).
35. Shuster LT, Gostout BS, Grossardt BR, & Rocca WA (2008) Prophylactic oophorectomy in premenopausal women and long-term health. *Menopause International* 14(3):111-116.
36. Mason JB, Cargill SL, Anderson GB, & Carey JR (2009) Transplantation of young ovaries to old mice increased life span in transplant recipients. *The journals of gerontology. Series A, Biological sciences and medical sciences* 64(12):1207-1211.
37. Horvath S*, et al.* (2014) Obesity accelerates epigenetic aging of human liver. *Proc Natl Acad Sci U S A.* 111(43):15538-15543.
38. Ferrucci L*, et al.* (2000) Subsystems contributing to the decline in ability to walk: bridging the gap between epidemiology and geriatric practice in the InCHIANTI study. *Journal of the American Geriatrics Society* 48(12):1618-1625.
39. Costello S, Cockburn M, Bronstein J, Zhang X, & Ritz B (2009) Parkinson's Disease and Residential Exposure to Maneb and Paraquat From Agricultural Applications in the Central Valley of California. *American Journal of Epidemiology* 169(8):919-926.
40. Teschendorff AE, Yang Z, Wong A, & et al. (2015) Correlation of smoking-associated dna methylation changes in buccal cells with dna methylation changes in epithelial cancer. *JAMA Oncology* 1(4):476-485.
41. Teschendorff AE*, et al.* (2013) A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics* 29(2):189-196.
42. Levine M, Lu A, Bennett D, & Horvath S (2015) Epigenetic age of the pre-frontal cortex is associated with neuritic plaques, amyloid load, and Alzheimer's disease related cognitive functioning. *Aging (Albany NY)* Dec.
43. Horvath S*, et al.* (2015) Accelerated Epigenetic Aging in Down Syndrome. *Aging Cell* 14(1).
44. Horvath S & Ritz BR (2015) Increased epigenetic age and granulocyte counts in the blood of Parkinson's disease patients. *Aging (Albany NY)* 7(12):1130-1142.

45. Horvath S & Levine AJ (2015) HIV-1 infection accelerates age according to the epigenetic clock. *J Infect Dis*.
46. Browning SR & Browning BL (2007) Rapid and accurate haplotype phasing and missing-data inference for whole-genome association studies by use of localized haplotype clustering. *Am J Hum Genet* 81(5):1084-1097.
47. Howie B, Fuchsberger C, Stephens M, Marchini J, & Abecasis GR (2012) Fast and accurate genotype imputation in genome-wide association studies through pre-phasing. *Nat Genet* 44(8):955-959.

FIGURE LEGENDS

Figure 1: Epigenetic age acceleration versus surgical menopause status. The x-axis of each plot reports surgical menopause status before age 50, i.e. "yes" denotes the group of women with surgical menopause before age 50 whereas "no" corresponds to the group of women who did not undergo bilateral oophorectomy at any age before blood draw. The bar plots report mean values of *AgeAccel*, one standard error, and the p-value from a Student T test. The six panels correspond to (A) WHI (white), (B) WHI (black), (C) WHI (Hispanic), (D) InCHIANTI, and (E) PEG samples, and (F) all samples combined. A meta-analysis based on Stouffer's Z method indicates that *AgeAccel* is significantly positively associated with surgical menopause status (p=0.0018).

Figure 2: Epigenetic age analysis of buccal samples from NSHD. The measure of age acceleration was defined as the difference between DNAm age and the mean DNAm age in this birth cohort. The scatter plots also report Pearson correlation coefficients and corresponding p-values, while the bar plot report one standard error and the p-value from a non-parametric group comparison test (Kruskal Wallis). Epigenetic age acceleration (y-axis) is associated with (A) MHT (P=0.00078), but not with (B) age at menopause, (C) menopausal status, or (D) surgical menopause.

Figure 3: Epigenetic age analysis of saliva samples from PEG. The measure of age acceleration in saliva was defined in the same way as blood. The scatter plots report Pearson correlation coefficients and corresponding p-values, while the bar plot report one standard error and the p-value from a non-parametric group comparison test (Kruskal Wallis). There is a strong correlation between epigenetic age (y-axis) and chronological age (A). While the correlation between epigenetic age acceleration and age at menopause in saliva (B) is about twice that of the association in blood, the finding is not significant. However, we do observe an association between epigenetic age acceleration and (C) surgical menopause (P=0.0079).

TABLES

Table 1. Unadjusted Meta-analysis of *AgeAccel* in Blood versus Age at Menopause

	Meta-Analysis Z statistics (P-values)		
Measure	**Removed age at menopause<=30**	**Removed surgical menopause**	**All**
AgeAccel	3.3 (p=0.00091)	2.6 (p=0.0083)	2.7 (p=0.0061)

Meta-analysis based on Pearson correlations between age at menopause and AgeAccel in each sample. The first and second column correspond to analysis after excluding women who were 30 or younger at the age of menopause and women with surgical menopause, respectively. The last column reports findings with no exclusions applied.

Table 2: Multivariate Meta-analysis of *AgeAccel* in Blood versus Age at Menopause

	Beta Coefficient (P-value)			
	WHI	InCHIANTI	PEG	Meta P-Value
AgeAccel				
Age at Menopause	-0.063 (0.001)	-0.012 (0.772)	-0.060 (0.350)	8.32×10^{-4}
Chronological Age	0.012 (0.530)	-0.010 (0.825)	-0.051 (0.305)	
Non-Hispanic Black	0.008 (0.980)	--	-5.113 (0.201)	
Hispanic	-0.918 (0.008)	--	-1.905 (0. 321)	
Former Smoker	-0.312 (0.234)	0.446 (0.648)	-1.177 (0.238)	
Current Smoker	-0.189 (0.666)	-0.870 (0.394)	-1.378 (0.613)	
MHT	0.041 (0.871)	0.940 (0.368)	2.864 (0.018)	
Age at Menarche	-0.055 (0.501)	0.280 (0.179)	-0.020 (0.950)	
PD Status	--	--	1.075 (0.282)	

We did not adjust for race/ethnicity in the models run for InCHIANTI, given that all participants are non-Hispanic white. For all models, women with age at menopause <30 years were excluded.

Table 3: GCTA Bivariate REML Results

h^2 of *AgeAccel* (SE)	0.651 (0.13)
h^2 of Age at Menopause (SE)	0.384 (0.14)
rG (Pvalue)	-0.256 (0.054)
V(G) (SE)	18.71 (3.90)
C(G) (SE)	-4.70 (3.69)
V(e) (SE)	10.03 (3.61)
C(e) (SE)	1.69 (3.47)
Vp (SE)	28.74 (0.93)
Log likelihood	-9017.31

rG=Genetic correlation with age at menopause, V(G)=Genetic Variance; C(G)=Genetic Covariance with age at menopause; V(e)=Residual Variance; C(e)=Residual Covariance with age at menopause; h^2= Heritability (Proportion of Variance explained by all SNPs)

Table 4: Mendelian Randomization

CHR	SNP	bp	Minor/Major Alleles	β with respect to minor allele (P-value)
				AgeAccel
19	rs11668344	55833664	G/A	0.506 (0.031)
20	rs16991615	5948227	A/G	0.151 (0.763)