Epigenetic Clocks and Allostatic Load Reveal Potential Sex-Specific Drivers of Biological Aging

McCrory, Cathal

2020-03

McCrory , C , Fiorito , G , McLoughlin , S , Polidoro , S , Cheallaigh , C N , Bourke , N , Karisola , P , Alenius , H , Vineis , P , Layte , R & Kenny , R A 2020 , ' Epigenetic Clocks and Allostatic Load Reveal Potential Sex-Specific Drivers of Biological Aging ' , Journals of Gerontology. Series A: Biological Sciences and Medical Sciences , vol. 75 , no. 3 , pp. 495-503 . https://doi.org/10.1093/gerona/glz241

http://hdl.handle.net/10138/326477
https://doi.org/10.1093/gerona/glz241

unspecified
acceptedVersion

Epigenetic Clocks and Allostatic Load reveal potential sex-specific drivers of biological ageing

Authors: *Cathal McCrory[1] Giovanni Fiorito[2], Sinead McLoughlin[1], Silvia Polidoro[2], Cliona Ni Cheallaigh[1], Nollaig Bourke[1], Piia Karisola[3], Harri Alennius[3,4], Paolo Vineis[2,5], Richard Layte[6] & Rose Anne Kenny[1]

Affiliations

[1]The Irish Longitudinal Study on Ageing, Trinity College Dublin, Ireland

[2] Italian Institute for Genomic Medicine (IIGM, former HuGeF)

[3] Faculty of Medicine, University of Helsinki, 00014, Helsinki, Finland

[4] Institute of Environmental Medicine (IMM), Karolinska Institutet, Stockholm, Sweden

[5] MRCPHE Centre for Environment and Health, Imperial College London, United Kingdom

[6] Department of Sociology, Trinity College Dublin, Ireland

Corresponding author: Cathal McCrory

Email: mccrorc@tcd.ie

Telephone: +353 1 8964263

Declaration of Interest

The authors report no conflicts of interest.

ABSTRACT

Individuals of the same chronological age vary widely in the biological signs of ageing that they exhibit. Allostatic Load and 'Epigenetic Clock' measures both attempt to characterise the accelerated ageing of biological systems, but at present it is unclear the extent to which these measures are complementary or distinct. The present study examines the cross-sectional association of Allostatic Load (AL) burden with Epigenetic Age Acceleration (EAA) in a sub-sample of 490 community dwelling older-adults participating in The Irish Longitudinal study on Aging (TILDA). A battery of 14 biomarkers representing the activity of 4 different physiological systems: *Immunological*, *Cardiovascular*, *Metabolic*, and *Renal* was used to construct the AL score. DNA methylation age was computed according to the algorithms described by Horvath, Hannum and Levine. The 'epigenetic clock' allows for estimation of whether an individual is experiencing accelerated or decelerated ageing by defining epigenetic age acceleration (EAA) as the difference between DNA methylation age and chronological age. Horvath, Hannum and Levine EAA correlated 0.05, 0.03, and 0.16 with AL respectively. Disaggregation by sex revealed that AL was more strongly associated with EAA in men compared with women as assessed using Horvath's clock but not Hannum's or Levine's. Metabolic dysregulation was a strong driver of EAA in men as assessed using Horvath and Levine's clock, while metabolic and cardiovascular dysregulation were associated with EAA in women using Levine's clock. Results indicate that AL and the epigenetic clocks are measuring different age-related variance and implicate sex specific drivers of biological ageing.

Key words: epigenetic age acceleration; allostatic load; Horvath; Hannum; Levine; sex differences

INTRODUCTION

In its broadest sense, ageing describes all the biological changes that occur in the body from the moment of conception to the moment we die. The ageing process is characterised by the presence of high inter-individual variation between individuals of the same chronical age[1], and this has prompted a search for biomarkers that better capture this heterogeneity in the rate at which we age. A number of candidate measures have been mooted at the clinical, physiological, and molecular level for quantifying age acceleration. Frailty is a clinical state characterised by a multi-dimensional loss of reserve (diminished strength, endurance, and physiological function) across a host of physical systems that gives rise to vulnerability and dependency[2] and is commonly defined by a host of clinical deficits. Allostatic Load (AL) by contrast, is a sub-clinical composite index of cumulative biological dysregulation across multiple organ systems that is posited to quantify the physiological toll of life course stressors[3] that anticipates hard clinical endpoints[4,5]. Going deeper, there are also a large range of molecular markers of accelerated aging including metabolomic-based predictors (e.g. Metabolome); proteomic-based predictors (e.g. Ige glycosylation); markers of cellular senescence (e.g. telomere length); and methylation-based predictors (e.g. epigenetic clocks)[6].

Of these, the latter hold arguably the most promise as candidate markers of biological ageing. The epigenetic clocks correlate strongly with chronological age, and have been shown to be associated with many chronic diseases of ageing and mortality[7]. Horvath's clock[8] is a multi-tissue predictor that allows the age of most tissues and cell types to be estimated based on DNA methylation (DNAm) levels at 353 CpG sites, while Hannum's clock, which can only be measured in blood is based on levels at 71 CpG sites. The residual resulting from the regression of DNA methylation age on chronological age has been touted as a measure of biological ageing, with a positive / negative residual denoting age acceleration / deceleration

relative to chronological age respectively.[9] This is a fast moving field and the first of the second generation clocks are already beginning to appear. Levine's[10] clock was developed using clinical biomarkers (i.e. albumin, creatinine, glucose, CRP, lymphocyte percent, mean cell volume, red cell distribution width, alkaline phosphatase, white blood cell count), and optimised to capture CpG sites that exhibited differences in disease and mortality outcomes among same aged individuals; in addition to those CpG sites that exhibited changes in DNA methylation with age. The resulting DNAm Phenoage clock, which is based on DNAm at 513 CpG sites has been shown to outperform the first generation of clocks in relation to the prediction of many age-related diseases[7].

What is currently unknown is the extent to which putative measures of biological ageing at different levels are complementary or distinct. The limited evidence that does exist does not provide very compelling evidence that they are tapping the same underlying construct. For one thing, telomere length and epigenetic clock measures correlate close to zero even though both have been mooted to measure the ticking rate of the biological clock[11-13]. For example, Marioni et al.[13] found that Hannum age and telomere length correlated between -0.08 and 0.19 in the 1921 and 1936 Lothian Birth Cohorts. The fact that the epigenetic clock and telomere length were independently associated with age and with mortality risk in separate multivariable regression models suggests that these two measures are tapping different age-related processes.

Similarly, Belsky et al.[11] examined the inter-relationships between 4 measures of molecular ageing (telomere length, Horvath, Hannum and Weidner epigenetic clocks), and 3 clinical-biomarker composites (Klemera-Doubal method (KDM) biological age[14], age-related homeostatic dysregulation, and pace of ageing[15]), among a sample of 800 participants in the

Dunedin longitudinal Study. Telomere length correlated near zero with the 3 epigenetic clock measures and with the 3 clinical biomarker composites measured cross-sectionally at 38 years of age. The 3 epigenetic clock measures were moderately inter-correlated (r = 0.32 - 0.52), as were the 3 clinical biomarker composites (r= 0.39 - 0.56) but the overlap between the epigenetic clocks and the clinical composites did not exceed 0.15. Breitling et al.[12] also observed a non-significant association between Horvath's EAA residual and telomere length in a large cohort of German participants (n=1820), but they did report a significant association between Horvath's EAA and a 34 deficit frailty index. Adjusting for age, sex and leukocyte distribution, the frailty index increased by 0.25% for each additional year of EAA.

Thus we have a number of candidate biological age predictors that seemingly share little in common. In a recent commentary, Shiels et al.[1] speculated that: *"The epigenetic component, which has the capacity to transmit both intergenerationally and transgenerationally, is particularly intriguing, as it may act as the body's hamartia in facilitating the spread of allostatic load across the whole organism"*. If so, then one might expect to see substantial correlations between measures of epigenetic ageing and AL. To the best of our knowledge, no study has yet examined the extent to which these two measures are related or distinct, so the present study will help fill this deficit.

METHOD

Sample

The Irish Longitudinal Study on Ageing (TILDA) is a large prospective cohort study examining the social, economic, and health circumstances of 8,175 community-dwelling older-adults aged 50 years of age and older resident in the Republic of Ireland. The sample was generated using a 3-stage selection process and the Irish Geodirectory as the sampling

frame. A detailed description of study design is available elsewhere.[16] Briefly, respondents completed a computer-assisted personal interview (n = 8,175) in the home and a separate self-completion paper and pencil questionnaire (n = 6,915) that collected information on sensitive topics. All participants were invited to undergo a comprehensive clinical health assessment at one of two national centers using trained nursing staff and standard operating protocols.

Epigenetic Sub-Sample

This analysis uses a sub-sample of the TILDA cohort for whom we have DNA methylation data. The sample was selected on the basis of respondents' life course social class trajectory using the cross-classification of father's and respondent's own social class. We purposefully selected 4 groups (stable low, stable high, upwardly mobile, downwardly mobile) comprising 125 cases per group with random selection within each group in order to investigate the impact of life course (dis)advantage and mobility on later life health. The selection of the sample is described in detail elsewhere[17].

Measurement of Allostatic Load

A battery of 14 biomarkers representing the activity of 4 different physiological systems: *Immunological* (C Reactive Protein (CRP), Interleukin 1 receptor-antagonist (IL1ra), Interleukin 6 (IL6), Interleukin 8 (IL8)), *Cardiovascular* (Systolic Blood Pressure (SBP), Diastolic Blood Pressure (DBP), Resting Heart Rate (RHR)*)*, *Metabolic* (High Density Lipoprotein (HDL), Total cholesterol (TC), Waist-hip ratio (WHR), Body Mass Index (BMI), Glycated haemoglobin (HbA1c)), and *Renal* (Creatinine, Cystatin C) was used to construct the AL score. An overall AL score was calculated by computing the number of parameters for which a respondent fell within the highest risk quartile using sex-specific cut-

offs. Following Seeman et al. (2014), we incorporated medication data into the calculation of our AL score. Medication use was recorded during the course of the household interview. Participants were classified as high risk in SBP if they were taking anti-hypertensive medication (C02, C03, C09), high risk in RHR if they were taking beta-blockers (C07) or calcium channel blockers (C08), high risk in glycated haemoglobin if taking any diabetes medications, including insulin (A10), and high risk in cholesterol if taking statins (C10AA, C10BA, C10BX). The overall level of missingness with respect to the AL biomarkers was very small. Only five people were missing on any biomarker and the maximum number of biomarkers for which any one person was missing was three or less. Following Castagne et al. (2018), a conservative approach (maximum bias imputation) was taken to those missing on any biomarker; by systematically classifying them as 'not at risk' if missing on the biomarker.

CRP was measured using ELISA Kit (Cat No CYT298 Millipore), with sensitivity of 0.20 ng/ml. A control serum from one donor and commercially available LiCheck control (Bio-Rad, Ref. 591-596) were used as controls on all the plates. The inflammatory markers, *IL1ra*, *IL6* and IL8 were measured by Luminex (Bio-Plex 200, Bio-Rad), and spiked serum as well as two concentrations of known samples were used as controls on each plate. Two measurements of seated SBP, DBP and RHR were obtained separated by a 1-min interval using an automatic digital BP monitor (OMRONTM, M10-IT). The means of the two readings were averaged to derive SBP, DBP and RHR. Respondents provided a non-fasting blood sample during the course of the health assessment and these were sent for immediate analysis (within 24 hrs) to derive a detailed lipid profile which included HDL, and TC. BMI was calculated from measured height and weight. Height was measured using a SECA 240 wall mounted measuring rod and weight was measured using a SECA electronic floor scales.

WHR is a measure of distribution of body fat (both subcutaneous and intra-abdominal). The waist was defined as the point midway between the iliac crest and the costal margin (lower rib). The hip circumference was defined as being the widest circumference over the buttocks and below the iliac crest. HbA1c was analysed by reversed-phase cation exchange chromatography using an ADAMS HA-8180V analyser which is traceable to the internationally agreed standard developed by the International Federation of Clinical Chemistry. Cystatin C and Creatinine were measured simultaneously from frozen plasma. Cystatin C was measured using a second generation particle enhanced immunoturbidimetric assay (Roche Tina-quant™) on a Roche Cobas 701 analyzer. This assay has a measuring range of 0.40 – 6.80 mg/L and is traceable to the European reference standard material (ERM-DA471/IFCC) for Cystatin C. Creatinine was measured using an enzymatic method traceable to isotope-dilution mass spectrometry (Roche Creatinine plus ver.2, Roche Diagnostics, Basel Switzerland).

Measurement of Epigenetic Age Acceleration

For the microarray, DNA samples were extracted from buffy coats using the QIAGEN GENTRA AUTOPURE LS (Qiagen, Crawley, UK). Bisulphite conversion of 500 ng of each sample was performed using the EZ DNA Methylation-Lightning™ Kit according to the manufacturer's protocol (Zymo Research, Orange, CA). Then, bisulfite-converted DNA was used for hybridization on the Infinium HumanMethylation 850k BeadChip, following the Illumina Infinium HD Methylation protocol. Briefly, a whole genome amplification step was followed by enzymatic end-point fragmentation and hybridization to HumanMethylation EPIC Chip at 48°C for 17 h, followed by single nucleotide extension. The incorporated nucleotides were labeled with biotin (ddCTP and ddGTP) and 2,4-dinitrophenol (DNP) (ddATP and ddTTP). After the extension step and staining, the BeadChip was washed and

scanned using the Illumina HiScan SQ scanner. The intensities of the images were extracted using the GenomeStudio (v.2011.1) Methylation module (1.9.0) software, which normalizes within-sample data using different internal controls that are present on the HumanMethylation 850k BeadChip and internal background probes. The methylation score for each CpG was represented as a β-value according to the fluorescent intensity ratio representing any value between 0 (unmethylated) and 1 (completely methylated).

DNA methylation age was computed according to the algorithm described by Horvath [18], based on a set of 353 age-associated CpG sites, the one based on 71 blood-specific age-associated CpG sites described by Hannum *et al*.(2013), and the one based on the 513 CpG sites described by Levine et al.[10]. Out of the 889 age-related CpGs, (6 are in common between Horvath and Hannum, 41 between Horvath and Levine, and 6 between Levine and Hannum) we detected 867 (more than 96%). The CpGs missing are those that are not present in the new *Illumina 850k methylation BeadChip*. Briefly, the DNA methylation age is computed as a weighted average of the age-related CpGs, with weights defined using a penalized regression model (Elastic-net regularization) [18]. The few missing values were imputed using the k-nearest neighboring (KNN) imputation algorithm implemented in the R Bioconductor package *impute*[19]. Epigenetic Age Acceleration (EAA) was defined as the difference between epigenetic and chronological age. Positive values of EAA (that is, epigenetic age is higher than the chronological age) indicate accelerated ageing and *vice versa*. Since EAA could be correlated with chronological age and white blood cell (WBC) percentage, we computed the so-called 'intrinsic' EAA[20], defined as the residuals from the linear regression of EAA with chronological age and WBC percentages[20]. The latter were estimated using the Houseman[21] algorithm. Intrinsic EAA is not dependent on age and WBC

by definition. Ten individuals were missing on epigenetic age and are excluded from the analysis resulting in a final case base of 490 individuals.

COVARIATES

We control for a number of demographic variables in the minimally adjusted models including: age (years), sex (male, female) and life course socio-economic trajectory (stable high, downwardly mobile, upwardly mobile, stable low) in order to control for characteristics associated with selection into the sample. We adjust additionally for smoking (never smoked, past smoker, current smoker), physical activity level (low, medium, high), and frequency of alcohol consumption (non-heavy vs heavy drinker) in the full multivariable adjusted models to take account of differences in lifestyle-related behaviours that may confound results by sex. Physical activity was assessed using the eight-item short form of the International Physical Activity Questionnaire (IPAQ)[22]. It measures the amount of time (mins) spent walking and engaged in moderate and vigorous physical activity, and the amount of time spent sedentary. We use a categorical variable representing low, medium and high levels of physical activity as per the IPAQ protocol (www.ipaq.ki.se). Frequency of alcohol consumption was assessed by asking respondents how often they have drunk alcohol in the past six months (almost every day, five or six times a week, three or four days a week, once or twice a week, once or twice a month, less than once a month, not at all in the past six months). Individuals who drank more than four days per week on average were classified as heavy drinkers. Six individuals were missing on the physical activity measure and 38 individuals were missing on the alcohol consumption measure so we coded these as 'missing' using dummy variables so that they would not be lost to the analysis.

Statistical Analysis

We used Stata, version 15.0 (Stata, College Station, TX) for all analyses. We examined the bivariate associations between AL and the EAA measures using Pearson's product-moment correlation coefficients in the overall sample and separately by sex. As AL represents a count of deficits, we also calculated the non-parametric (Spearman's) and polyserial correlations, but these were very similar to Pearson's. We used the Fisher r-to-z transformation to assess the significance of the difference between the correlation coefficients for men and women. We estimated a series of multivariable linear regression models where each measure of EAA was regressed on AL adjusting for age, sex, and life course socio-economic trajectory in the basic adjusted model (model 1), and additionally for smoking history, physical activity, and alcohol consumption in the full multivariable adjusted model (model 2). We tested for effect modification by fitting a sex*AL interaction term which was significant with respect to Horvath's, but not Hannum's or Levine's clock. We therefore report results for the overall sample and stratified by sex. The next step in the analysis involved estimating a series of multivariable linear regression models in which we regressed each measure of EAA separately on each of the 4 system components comprising the AL index (i.e. inflammatory, cardiovascular, metabolic, and renal burden), to determine whether some systems were more strongly associated with EAA than others. The final step in decomposing the relationship between AL and EAA involved estimating a series of linear regression models to examine the impact of being biologically dysregulated (i.e. highest quartile of clinical risk) in each of the 14 biomarkers comprising the AL index on each measure of EAA**.**

RESULTS

Table 1 describes the characteristics of the sample. The mean age of the sample was 62.2 years (SD = 8.3), and 49.8% were male. Table 2 shows the bivariate associations between chronological age, AL, and the three measures of EAA. In the overall sample, the correlation

between AL and the age acceleration residuals for the epigenetic clocks were close to zero: Horvath EAA (r=0.05), Hannum EAA (r = 0.03), and Levine (r=0.16). Disaggregation by sex revealed that AL was more strongly associated with EAA in men using Horvath's (r=0.16 vs -0.05; Z=2.33; p=.002 two-tailed) but not Hannum's (r=0.08 vs -0.02; Z=1.10; p=.271 two tailed), or Levine's clocks (0.15 vs 0.16; Z= -0.11; p=0.91 two-tailed).

Table 3 shows the results of the regression of each measure of EAA on AL burden adjusting for age, sex, and socio-economic trajectory in model 1, and additionally for smoking history, physical activity, and alcohol consumption in model 2. In the minimally adjusted models, a one unit increase in AL burden was associated with increased EAA using Levine's clock, but not with Horvath's or Hannum's. Specifically, a one unit increase in AL was associated with 0.35 (CI=0.16, 0.54; p<.001) years of EAA in the full sample using Levine's clock; 0.31 years for men (CI=0.03, 0.59; p=.029) and 0.39 years for women (CI=0.12, 0.66; p=.005). The significant association in men was rendered non-significant (B=0.23, CI=-0.05, 0.52) after adjustment for lifestyle factors in model 2.

As AL is a multi-system composite index of physiological dysregulation, we decided to investigate further to establish whether there were system-specific components that were more strongly linked with EAA than others. Table 4 shows that metabolic dysregulation was closely associated with EAA in men with a one unit increase in metabolic burden associated with 0.99 (CI=0.23, 1.74; p=.011), 0.43 (CI= -0.34, 1.20; p>.05) and 0.59 (CI=0.05, 1.14; p=.033) years of EAA using Horvath's, Hannum's and Levine's clocks respectively in the minimally adjusted models. Metabolic dysregulation continued to be strongly linked with EAA as assessed using Horvath's clock when adjusted additionally for lifestyle factors in model 2 (B=1.03, CI=0.27, 1.80; p=0.008.). None of the other AL system components were

significantly associated with EAA in men. Women were characterised by a different pattern across the 3 epigenetic clocks (Table 4). In general, increases in inflammatory, cardiovascular and metabolic burden were unrelated to EAA in women when assessed using the Horvath and Hannum clocks in both the minimally and full multivariable adjusted models, but cardiovascular and metabolic dysregulation were associated with 0.87 (CI=0.15, 1.59; p=.019) and 0.61 (CI=0.05, 1.17; p=.032) years of EAA respectively using Levine's clock in the minimally adjusted model (model 1); and these associations were not appreciably affected when adjusted additionally for lifestyle factors (model 2).

Finally, figures 1a-1c display the EAA residual associated with being biologically dysregulated (i.e. highest risk quartile) in each of the 14 AL biomarkers separately for men and women across each of the 3 clocks in the full multivariable adjusted models. In general, we see that physiological dysregulation in the metabolic biomarkers carries a heavier EAA penalty for men compared with women as assessed using Horvath and Hannum's clocks, and this effect was particularly pronounced for Hba1c. Dysregulation in HbA1c was associated with significant EAA in men across all 3 clocks: Horvath (B=3.29, CI=1.11, 5.48; p=0.003), Hannum (B=2.53, CI=0.30, 4.76; p=0.026) and Levine (B=2.36, CI=0.80, 3.92; p=0.003) while dysregulation in CRP was associated with significant EAA in men using Levine's clock (B=1.91, CI=0.36, 3.45; p=0.016), and at the 10% level using Horvath (B=2.03, CI=-0.15, 4.20; p=0.068) and Hannum's clocks (B=2.09, CI=-0.11, 4.29; p=0.062). By contrast, none of the individual biomarkers were associated with EAA in women as assessed using Horvath's or Hannum's clocks. Dysregulation in SBP (B=1.60, CI=0.07, 3.12; p=0.040), DBP (B=2.48, CI=-0.88, 4.08; p=0.002) and WHR (B=2.51, CI=0.90, 4.12; p=0.002) were all associated with significant EAA in women using Levine's clock. We tested formally for sex differences by fitting separate sex*biomarker interaction terms with respect to each of the 3 clocks;

however only two of the contrasts were statistically significant. Dysregulation in Hba1c was associated with significantly higher EAA in men compared with women using Horvath's clock and dysregulation in DBP was associated with significantly higher EAA in women compared with men using Levine's clock.

DISCUSSION

We found that the correlation between AL and the various measures of EAA were of small magnitude overall, and were higher for men compared with women, as assessed using Horvath's clock. The small amount of overlap between AL and EAA suggests that the measures are tapping distinct rather than related biological ageing processes. Although one might intuitively expect that two putative measures of age acceleration would be positively correlated, it is consistent with the results of other studies that have shown minimal overlap between EEA and telomere length[11-13], and between EEA and other clinical biomarker composites[11,12]. There is little doubt that the epigenetic clock tracks chronological age better than any other candidate biomarker. In this study the epigenetic clocks estimated from blood plasma correlated in the range 0.74 – 0.84 with chronological age, while AL was much more modestly correlated ($r = 0.32$) with chronological age; and was comparable in magnitude to the estimated correlation of 0.30 – 0.38 between telomere length and chronological age indicated by a recent systematic review[23]. What is more doubtful is whether the EAA residual resulting from the regression of DNA methylation age on chronological age represents the *sine qua non* of biological ageing.

Marioni et al.[24] failed to find an association between Horvath's clock and walking speed in the 1936 Lothian Birth Cohort, although they did report a significant association with grip strength and lung function cross-sectionally. Notably, EAA did not predict change in function

in any of the measures over a 6-year follow-up. Maddock et al. (in submission) noted that Horvath and Hannum's clocks were not associated with walking speed, grip strength, lung function, chair rises, or measures of cognition either cross-sectionally or at follow-up in a meta-analysis of the National Survey of Health and Development, the National Child Development Study, and Twins UK study. In a recent article, Horvath and Raj[7] conceded that the first generation clocks exhibit only weak associations with clinical measures and that the second generation clocks may perform better in this regard. Consistent with this interpretation, McCrory et al[17]. (2019) found that Levine EAA was associated with slower performance on the timed-up-and-go task (TUG) and the Fried frailty phenotype, but not with activity limitations in cross-section, while Horvath and Hannum EAA were not associated with any of the outcome measures. By contrast, AL was associated with each of the 3 age-related outcome variables under investigation, and more strongly than Levine's clock. Thus while the first generation epigenetic clocks may well be tapping the ticking rate of the biological clock, which is seemingly strongly determined by early life factors[25]; they may not yet been fully capturing the biological age acceleration that is caused by extrinsic factors such as life course stressors.

What explains the different pattern for men and women?

AL burden was associated with EEA in men and women using Levine's clock, but not with either Horvath or Hannum. The higher correlations of AL with Levine's clock in all likelihood arises because Levine used some clinical biomarkers (CRP, creatinine, glucose) in the development of her clock that are also components of the AL index. Nevertheless, a striking feature of the data was the different results for men and women with respect to Horvath's clock, where a one unit increase in AL burden was associated with a significantly heavier EAA for men compared with women in the minimally and full multivariable adjusted

models. Disaggregation of the AL index into its system specific components was instructive as it showed that metabolic dysregulation carried a heavier EAA penalty for men compared with women as assessed using Horvath's clock, where a one unit increase in metabolic burden was associated with a ~1 year of EAA in men in the full multivariable adjusted models, while it was unrelated to EAA in women. By contrast, metabolic dysregulation was associated with EAA in men (~0.5 years) and women (~0.6 years) using Levine's clock. We noted another important sex difference in that cardiovascular dysregulation was associated with EAA (~0.9 years) in women as assessed using Levine's clock, but not in men. Indeed, the only consistent trend to emerge was that CRP and Cystatin C were in general associated with positive EAA in men and women across all 3 clocks.

These differential findings with respect to men and women raise an important theoretical question as to whether there are different drivers of biological ageing in men and women, or whether the epigenetic clock estimators are confounded by other biological processes correlated with ageing such as differences in endogenous hormone levels, experience of menopause, or use of prescribed medications (e.g. hormone replacement therapy). Previous studies have shown that synthetic hormones (HRT) can affect the epigenetic clock[26,27], that HRT is associated with reductions in fasting glucose, insulin resistance, and type 2 diabetes risk[28] and that DNAm is increased in breast tissue[29] (i.e. a very hormone responsive tissue). Women also have about 4 times the amount of endogenous estrogens compared with men[30], which is important because estrogens possess potent anti-inflammatory and anti-oxidative properties which may help buffer host resistance against many age-related diseases.

Unfortunately, we do not have any measures of endogenous hormone levels for participants in our sample, but it is entirely plausible that differences in hormone levels help account for

the sex differences we have observed. Less discussed but still plausible is that changes in androgen levels (i.e. testosterone) accelerate ageing related processes in men. Men have about 25 times the amount of testosterone compared with women and beginning around the age of 35-40 years testosterone decreases by approximately 1-3% per year.[30] Moreover, testosterone deficiency is associated with insulin resistance and may predispose older men to the metabolic syndrome or type 2 diabetes[31,32]. Future studies should be designed to address these possibilities by examining which genes the CpG sites are located at, and whether they have a link to metabolism near estrogen or androgen regulated genes.

The finding that metabolic dysregulation is associated with EAA in men and women using Levine's clock adds to a growing corpus of research implicating adiposity as a major determinant of cellular ageing. Horvath[33] has previously reported that BMI correlated 0.42 with EAA in liver tissue but not in blood, which may help explain the null finding with respect to Hannum's clock. Moreover, there is accumulating evidence that adiposity may drive epigenetic changes in DNA methylation patterns rather than the reverse[34-36], but it remains to be established whether these epigenetic changes represent an intermediate stage between adiposity and age-related diseases[37] or whether they merely serve as a marker of cumulative biological insults at the cellular level without necessarily being a cause of disease per se.

Limitations

The sample design was selective, which may have led us to underestimate the potential association between AL and EAA that we might observe in the full sample. We feel this is unlikely however, as previous studies that were less selective also showed minimal overlap between the epigenetic clocks and a range of clinical biomarker composites that bear a close

resemblance to AL[11]. A further limitation is that our measure of AL does not include measures of sympathetic-adrenal medullary (e.g. epinephrine, norepinephrine) or hypothalamic-pituitary-adrenal axis (e.g. cortisol, DHEAS) activation which are hypothesised to play a primary role in the physiological cascade that drives secondary organ damage (i.e. immune, cardiovascular, metabolic), that in turn is hypothesised to precipitate earlier disease and morbidity. Indeed, some recent studies suggest DNAm levels may help regulate gene expression of cortisol in particular stress related genes[38,39]. Perhaps the correlation with AL would have been higher if these measures were part of our AL index.

Strengths

These limitations are balanced by a number of strengths. We have measures of AL burden and 3 epigenetic clocks for a relatively large sample of community-dwelling older persons which allowed us to examine the empirical overlap between different biomarkers of ageing. Each participant also completed a comprehensive clinic-based health assessment administered by trained nurses using standard operating protocols which allowed us to examine the association of each of the biological age measures with indices of physical health and functioning in later life that anticipate hard clinical end-points.

CONCLUSIONS

In a recent review of biological age predictors, the epigenetic clock was mooted to represent the most promising candidate biomarker of ageing that we currently have[6]. The results of this study, while not conclusive, calls for some re-evaluation of whether the age acceleration residual from the epigenetic clocks actually represents "biological ageing" and further investigation of the extent to which they are confounded by sex.

Acknowledgements

Cathal McCrory is supported by the Health Research Board (HRB) of Ireland under an Emerging Investigator Award (EIA-2017-012). Giovanni Fiorito is supported by the Italian Institute for Genomic Medicine (IIGM). This work was also supported by the Lifepath grant to Paolo Vineis at Imperial College London (European Commission H2020 grant, Grant number: 633666). Funding for the TILDA project was supported by the Irish Government, the Atlantic Philanthropies, and Irish Life plc. The funders had no involvement in the study design, collection, analysis and interpretation of data, or authorship of the submitted work. The authors declare no conflicts of interest.

REFERENCES

1. Shiels PG, Stenvinkel P, Kooman JP, McGuinness D. Circulating markers of ageing and allostatic load: A slow train coming. *Practical Laboratory Medicine.* 2017;7:49-54.
2. Morley JE, Vellas B, van Kan GA, et al. Frailty Consensus: A Call to Action. *Journal of the American Medical Directors Association.* 2013;14(6):392-397.
3. Seeman T, Epel E, Gruenewald T, Karlamangla A, McEwen BS. Socio-economic differentials in peripheral biology: Cumulative allostatic load. *Annals of the New York Academy of Sciences.* 2010;1186(1):223-239.
4. Castagné R, Garès V, Karimi M, et al. Allostatic load and subsequent all-cause mortality: which biological markers drive the relationship? Findings from a UK birth cohort. *European Journal of Epidemiology.* 2018;33(5):441-458.
5. Robertson T, Beveridge G, Bromley C. Allostatic load as a predictor of all-cause and cause-specific mortality in the general population: Evidence from the Scottish Health Survey. *PLOS ONE.* 2017;12(8):e0183297.
6. Jylhava J, Pedersen NL, Hagg S. Biological Age Predictors. *EBioMedicine.* 2017;21:29-36.
7. Horvath S, Raj K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nature reviews Genetics.* 2018;19(6):371-384.
8. Horvath S. DNA methylation age of human tissues and cell types. *Genome biology.* 2013;14(10):3156.
9. Horvath S, Gurven M, Levine ME, et al. An epigenetic clock analysis of race/ethnicity, sex, and coronary heart disease. *Genome Biol.* 2016;17(1):171.
10. Levine ME, Lu AT, Quach A, et al. An epigenetic biomarker of aging for lifespan and healthspan. *bioRxiv.* 2018.

11. Belsky DW, Moffitt TE, Cohen AA, et al. Eleven Telomere, Epigenetic Clock, and Biomarker-Composite Quantifications of Biological Aging: Do They Measure the Same Thing? *American Journal of Epidemiology.* 2018;187(6):1220-1230.

12. Breitling LP, Saum K-U, Perna L, Schöttker B, Holleczek B, Brenner H. Frailty is associated with the epigenetic clock but not with telomere length in a German cohort. *Clinical Epigenetics.* 2016;8:21.

13. Marioni RE, Harris SE, Shah S, et al. The epigenetic clock and telomere length are independently associated with chronological age and mortality. *International Journal of Epidemiology.* 2016;45(2):424-432.

14. Bouchard TJ, Jr., McGue M. Genetic and environmental influences on human psychological differences. *Journal of neurobiology.* 2003;54(1):4-45.

15. Belsky DW, Caspi A, Houts R, et al. Quantification of biological aging in young adults. *Proc Natl Acad Sci U S A.* 2015;112(30):E4104-4110.

16. Whelan BJ, Savva GM. Design and methodology of the Irish Longitudinal Study on Ageing. *Journal of the American Geriatrics Society.* 2013;61 Suppl 2:S265-268.

17. McCrory C, Fiorito G, Ni Cheallaigh C, et al. How does socio-economic position (SEP) get biologically embedded? A comparison of allostatic load and the epigenetic clock(s). *Psychoneuroendocrinology.* 2019;104:64-73.

18. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol.* 2013;14(10):R115.

19. Troyanskaya O, Cantor M, Sherlock G, et al. Missing value estimation methods for DNA microarrays. *Bioinformatics.* 2001;17(6):520-525.

20. Chen BH, Marioni RE, Colicino E, et al. DNA methylation-based measures of biological age: meta-analysis predicting time to death. *Aging (Albany NY).* 2016;8(9):1844-1865.

21. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC bioinformatics.* 2012;13:86.

22. Craig CL, Marshall AL, Sjostrom M, et al. International physical activity questionnaire: 12-country reliability and validity. *Medicine and science in sports and exercise.* 2003;35(8):1381-1395.

23. Muezzinler A, Zaineddin AK, Brenner H. A systematic review of leukocyte telomere length and age in adults. *Ageing research reviews.* 2013;12(2):509-519.

24. Marioni RE, Shah S, McRae AF, et al. The epigenetic clock is correlated with physical and cognitive fitness in the Lothian Birth Cohort 1936. *International Journal of Epidemiology.* 2015;44(4):1388-1396.

25. Li S, Wong EM, Dugué P-A, et al. Genome-wide average DNA methylation is determined in utero. *International Journal of Epidemiology.* 2018;47(3):908-916.

26. Bahl A, Pöllänen E, Ismail K, et al. Hormone Replacement Therapy Associated White Blood Cell DNA Methylation and Gene Expression are Associated With Within-Pair Differences of Body Adiposity and Bone Mass. *Twin Research and Human Genetics.* 2015;18(6):647-661.

27. Levine ME, Lu AT, Chen BH, et al. Menopause accelerates biological aging. *Proceedings of the National Academy of Sciences.* 2016;113(33):9327-9332.

28. Mauvais-Jarvis F, Manson JE, Stevenson JC, Fonseca VA. Menopausal Hormone Therapy and Type 2 Diabetes Prevention: Evidence, Mechanisms, and Clinical Implications. *Endocrine reviews.* 2017;38(3):173-188.

29. Sehl ME, Henry JE, Storniolo AM, Ganz PA, Horvath S. DNA methylation age is elevated in breast tissue of healthy women. *Breast cancer research and treatment.* 2017;164(1):209-219.

30. Horstman AM, Dillon EL, Urban RJ, Sheffield-Moore M. The Role of Androgens and Estrogens on Healthy Aging and Longevity. *The Journals of Gerontology: Series A.* 2012;67(11):1140-1152.

31. Traish AM, Saad F, Guay A. The dark side of testosterone deficiency: II. Type 2 diabetes and insulin resistance. *Journal of andrology.* 2009;30(1):23-32.

32. Zitzmann M. Testosterone deficiency, insulin resistance and the metabolic syndrome. *Nature reviews Endocrinology.* 2009;5(12):673-681.

33. Horvath S, Erhart W, Brosch M, et al. Obesity accelerates epigenetic aging of human liver. *Proc Natl Acad Sci U S A.* 2014;111(43):15538-15543.

34. Geach T. Methylation a consequence not a cause. *Nature Reviews Endocrinology.* 2017;13:127.

35. Mendelson MM, Marioni RE, Joehanes R, et al. Association of Body Mass Index with DNA Methylation and Gene Expression in Blood Cells and Relations to Cardiometabolic Disease: A Mendelian Randomization Approach. *PLOS Medicine.* 2017;14(1):e1002215.

36. Wahl S, Drong A, Lehne B, et al. Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. *Nature.* 2017;541(7635):81-86.

37. Campanella G, Gunter MJ, Polidoro S, et al. Epigenome-wide association study of adiposity and future risk of obesity-related diseases. *International journal of obesity (2005).* 2018.

38. Kometani M, Yoneda T, Demura M, et al. Cortisol overproduction results from DNA methylation of CYP11B1 in hypercortisolemia. *Scientific reports.* 2017;7(1):11205-11205.

39. Wrigglesworth J, Ancelin M-L, Ritchie K, Ryan J. Association between DNA methylation of the KITLG gene and cortisol levels under stress: a replication study. *Stress.* 2019;22(1):162-168.

Table 1: Descriptive characteristics of the Sample

	All Sample (n=490)	**Men (n=244)**	**Women (n=246)**
	Mean (SD) or n (%)	*Mean (SD) or n (%)*	*Mean (SD) or n (%)*
Age	62.2 (8.3)	61.9 (8.1)	62.5 (8.5)
Horvath EAA	0.0 (7.4)	0.12 (7.2)	-0.12 (7.5)
Hannum EAA	0.0 (7.3)	0.07 (7.2)	-0.07 (7.4)
Levine EAA	0.0 (5.2)	0.25 (5.1)	-0.25 (5.3)
Allostatic Load	4.06 (2.58)	4.10 (2.43)	4.02 (2.71)
- Inflammatory	0.97 (1.03)	0.96 (1.00)	0.99 (1.05)
- Cardiovascular	1.11 (0.97)	1.16 (0.97)	1.07 (0.98)
- Metabolic	1.50 (1.23)	1.51 (1.21)	1.50 (1.26)
- Renal	0.47 (0.72)	0.47 (0.70)	0.47 (0.73)
Life course trajectory			
- Stable high	123 (24.7)	64 (26.2)	59 (24.0)
- Upwardly mobile	125 (25.1)	62 (25.4)	63 (25.6)
- Downwardly mobile	121 (24.7)	58 (23.8)	63 (25.6)
- Stable low	121 (25.5)	60 (24.6)	61 (24.8)
Smoking history			
- Never smoked	193 (39.4)	91 (37.3)	102 (41.5)
- Past smoker	211 (43.1)	109 (44.7)	102 (41.5)
- Current smoker	86 (17.6)	44 (18.0)	42 (17.1)
Physical Activity			
- Lowest	142 (29.0)	55 (22.5)	87 (35.4)
- Intermediate	175 (35.7)	85 (34.8)	90 (36.6)
- Highest	167 (34.1)	101 (41.4)	66 (26.8)
- Missing	6 (1.2)	3 (1.2)	3 (1.2)
Heavy drinker			
- No	393 (80.2)	189 (77.5)	204 (82.9)
- Yes	59 (12.0)	36 (14.8)	23 (9.4)
- Missing	38 (7.8)	19 (7.8)	19 (7.7)

Table 2: Zero-order correlations between chronological age and the biological age predictors estimated using Pearson's product-moment correlation

All Sample	Age	Horvath's clock	Horvath EAA	Hannum's clock	Hannum EAA	Levine's clock	Levine EAA
Horvath's clock	0.74***						
Horvath EAA	0.00	0.67***					
Hannum's clock	0.74***	0.92***	0.56***				
Hannum EAA	0.00	0.55***	0.83***	0.67***			
Levine's clock	0.84***	0.66***	0.05	0.66***	0.04		
Levine EAA	0.00	0.06	0.10	0.06	0.09	0.54***	
Allostatic Load	0.32***	0.26***	0.05	0.25***	0.03	0.35***	0.16***

Men	Age	Horvath's clock	Horvath EAA	Hannum's clock	Hannum EAA	Levine's clock	Levine EAA
Horvath's clock	0.78***						
Horvath EAA	0.13	0.72***					
Hannum's clock	0.77***	0.92***	0.62***				
Hannum EAA	0.08	0.57***	0.81***	0.69***			
Levine's clock	0.85***	0.72***	0.20**	0.70***	0.15*		
Levine EAA	0.04	0.14*	0.18**	0.13*	0.16*	0.56***	
Allostatic Load	0.23***	0.26***	0.16*	0.22**	0.08	0.27***	0.15*

Women	Age	Horvath's clock	Horvath EAA	Hannum's clock	Hannum EAA	Levine's clock	Levine EAA
Horvath's clock	0.70***						
Horvath EAA	-0.11	0.63***					
Hannum's clock	0.72***	0.92***	0.51***				
Hannum EAA	-0.09	0.54***	0.85***	0.64***			
Levine's clock	0.83***	0.59***	-0.09	0.61***	-0.06		
Levine EAA	-0.03	-0.02	0.02	0.00	0.03	0.52***	
Allostatic Load	0.39***	0.27***	-0.05	0.29***	-0.02	0.42***	0.16*

*** significant at the 0.001 level; **significant at the 0.01 level; *significant at the 0.05 level

Table 3: Epigenetic age acceleration (years) associated with a one unit increase in allostatic load burden in baseline and multivariable adjusted models

	HORVATH EAA		HANNUM EAA		LEVINE EAA	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
	B (95% CI)	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*
All Sample	0.11 (-0.16, 0.38)	0.15 (-0.13, 0.43)	0.06 (-0.21, 0.33)	0.09 (-0.19, 0.37)	0.35*** (0.16, 0.54)	0.32*** (0.13, 0.51)
Men	0.33 (-0.06, 0.71)	0.36 (-0.05, 0.76)	0.12 (-0.27, 0.52)	0.20 (-0.22, 0.61)	0.31* (0.03, 0.59)	0.23 (-0.05, 0.52)
Women	-0.03 (-0.43, 0.36)	0.03 (-0.36, 0.43)	0.04 (-0.35, 0.42)	0.08 (-0.32, 0.47)	0.39** (0.12, 0.66)	0.40** (0.13, 0.68)

Model 1: Adjusted for age, sex, and socio-economic trajectory

Model 2: Model 1 + smoking, physical activity, alcohol consumption

*** significant at the 0.001 level; **significant at the 0.01 level; *significant at the 0.05 level

Table 4: Epigenetic age acceleration (years) associated with being dysregulated in each of the four systems comprising the allostatic load index in baseline and multivariable adjusted models

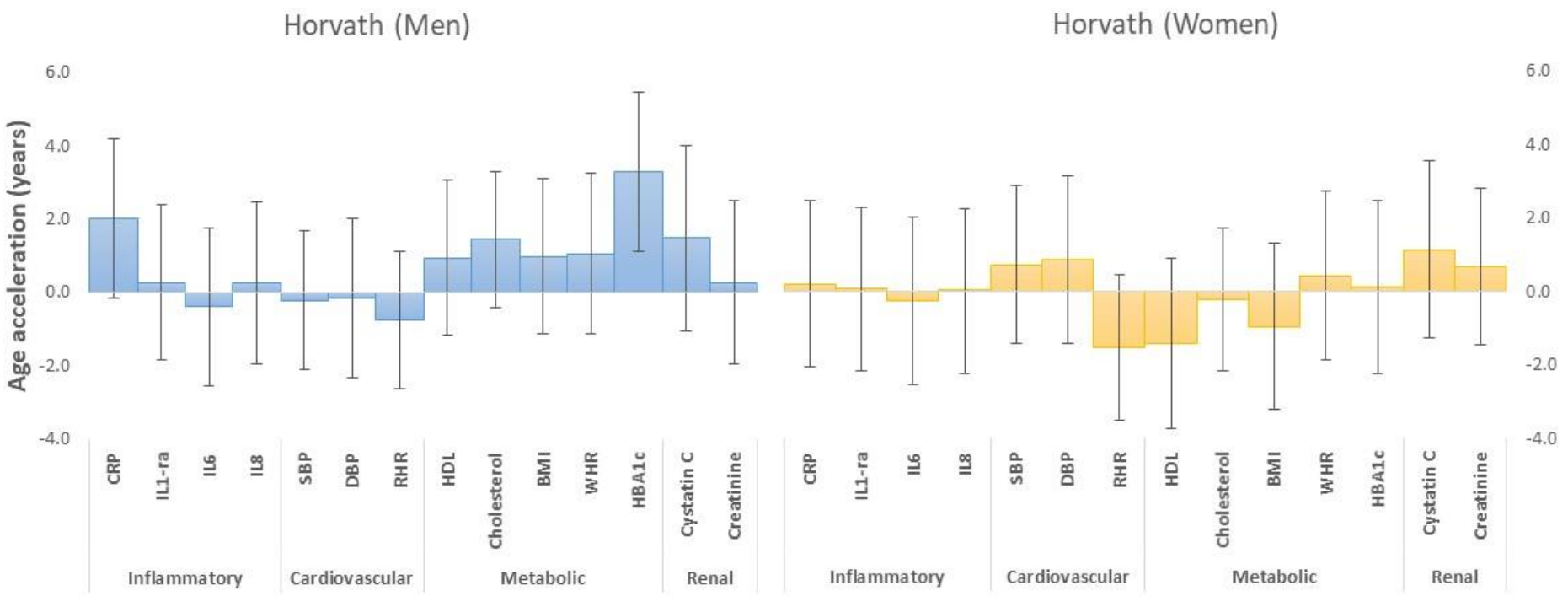
	HORVATH EAA		HANNUM EAA		LEVINE EAA	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
All Sample	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*	*B (95% CI)*
Inflammatory	0.11 (-0.53, 0.76)	0.20 (-0.45, 0.86)	0.15 (-0.49, 0.80)	0.24 (-0.41, 0.89)	0.41 (-0.04, 0.87)	0.33 (-0.13, 0.79)
Cardiovascular	-0.29 (-0.99, 0.41)	-0.29 (-1.00, 0.41)	-0.22 (-0.92, 0.47)	-0.21 (-0.92, 0.49)	0.52* (0.03, 1.01)	0.44 (-0.05, 0.94)
Metabolic	0.42 (-0.13, 0.97)	0.43 (-0.12, 0.97)	0.19 (-0.35, 0.74)	0.20 (-0.34, 0.75)	0.60** (0.22, 0.99)	0.56** (0.18, 0.95)
Renal	0.39 (-0.60, 1.38)	0.66 (-0.34, 1.67)	0.21 (-0.77, 1.20)	0.39 (-0.62, 1.39)	0.58 (-0.12, 1.28)	0.54 (-0.17, 1.25)
Men						
Inflammatory	0.45 (-0.47, 1.36)	0.41 (-0.54, 1.35)	0.41 (-0.51, 1.33)	0.53 (-0.43, 1.48)	0.43 (-0.23, 1.08)	0.29 (-0.39, 0.97)
Cardiovascular	-0.26 (-1.21, 0.69)	-0.28 (-1.25, 0.68)	-0.37 (-1.32, 0.58)	-0.31 (-1.29, 0.67)	0.21 (-0.48, 0.89)	0.06 (-0.63, 0.75)
Metabolic	0.99* (0.23, 1.74)	1.03** (0.27, 1.80)	0.43 (-0.34, 1.20)	0.51 (-0.27, 1.29)	0.59* (0.05, 1.14)	0.51 (-0.04, 1.05)
Renal	0.40 (-0.99, 1.79)	0.60 (-0.86, 2.07)	-0.01 (-1.41, 1.39)	0.17 (-1.31, 1.65)	0.55 (-0.45, 1.55)	0.40 (-0.64, 1.45)
Women						
Inflammatory	-0.11 (-1.03, 0.82)	0.01 (-0.93, 0.94)	-0.03 (-0.95, 0.89)	0.04 (-0.89, 0.97)	0.41 (-0.24, 1.06)	0.36 (-0.31, 1.02)
Cardiovascular	-0.18 (-1.21, 0.86)	-0.06 (-1.10, 0.99)	0.05 (-0.97, 1.07)	0.12 (-0.92, 1.16)	0.87* (0.15, 1.59)	0.91* (0.18, 1.65)
Metabolic	-0.08 (-0.87, 0.72)	-0.04 (-0.83, 0.76)	0.01 (-0.77, 0.80)	0.03 (-0.77, 0.82)	0.61* (0.05, 1.17)	0.66* (0.10, 1.23)
Renal	0.36 (-1.04, 1.77)	0.64 (-0.77, 2.06)	0.41 (-0.97, 1.80)	0.59 (-0.83, 2.01)	0.61 (-0.38, 1.60)	0.64 (-0.37, 1.65)

Model 1: Adjusted for age, sex, and socio-economic trajectory

Model 2: Model 1 + smoking, physical activity, alcohol consumption

*** significant at the 0.001 level; **significant at the 0.01 level; *significant at the 0.05 level

Figures 1a: Horvath epigenetic age acceleration (years) associated with being in the highest quartile of clinical risk across each of the 14 allostatic load biomarkers separately by sex

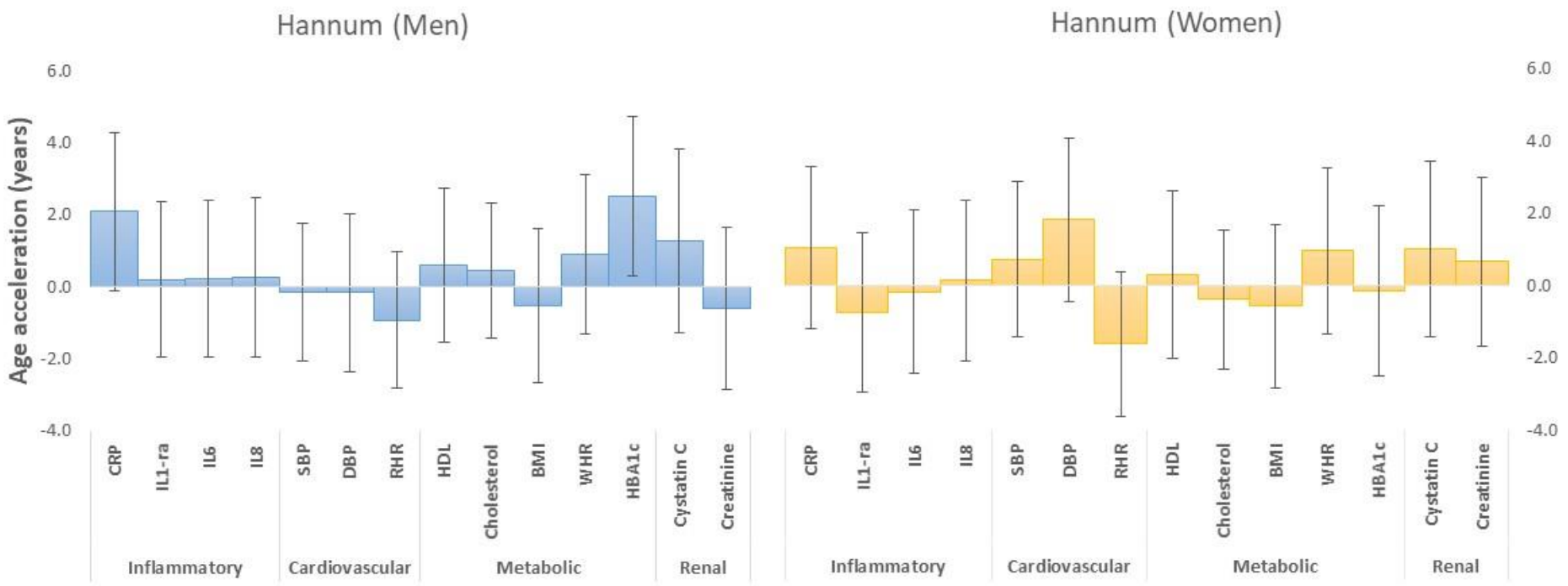


Error bars represent the 95% confidence intervals

Model 1: Adjusted for age, and socio-economic trajectory

Model 2: Model 1 + smoking, physical activity, and alcohol consumption

Figures 1b: Hannum epigenetic age acceleration (years) associated with being in the highest quartile of clinical risk across each of the 14 allostatic load biomarkers separately by sex

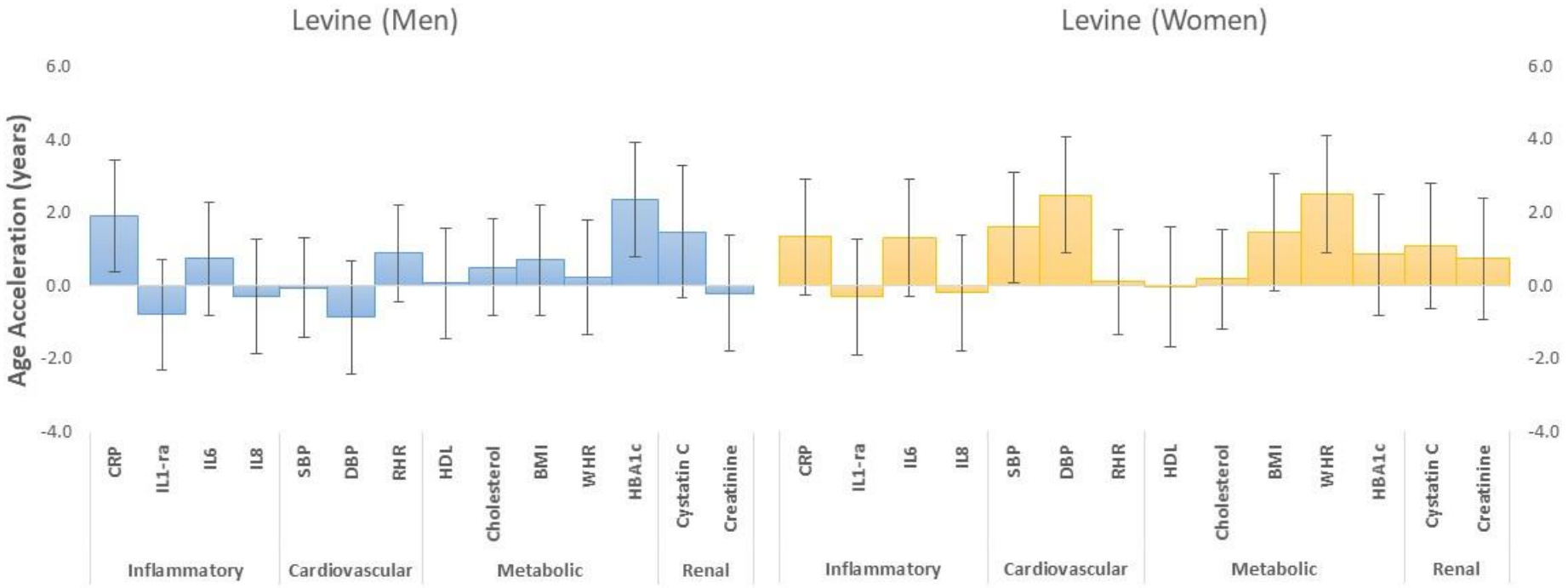


Error bars represent the 95% confidence intervals

Model 1: Adjusted for age, and socio-economic trajectory

Model 2: Model 1 + smoking, physical activity, and alcohol consumption

Figures 1c: Levine epigenetic age acceleration (years) associated with being in the highest quartile of clinical risk across each of the 14 allostatic load biomarkers separately by sex



Error bars represent the 95% confidence intervals

Model 1: Adjusted for age, and socio-economic trajectory

Model 2: Model 1 + smoking, physical activity, and alcohol consumption