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### DNA methylation age is associated with an altered hemostatic profile in a multi-ethnic meta-analysis

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## **Title**

DNA methylation age is associated with an altered hemostatic profile in a multi-ethnic meta-analysis

**Running Title:** Epigenetic aging and altered hemostatic factors

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- Elevated epigenetic age is associated with an altered hemostatic factor profile and lower clotting time (aPTT)

- DNA methylation age is associated with mRNA levels of fibrinogen in multiple tissues

## **Abstract**

Many hemostatic factors are associated with age and age-related diseases, however much remains unknown about the biological mechanisms linking aging and hemostatic factors. DNA methylation is a novel means by which to assess epigenetic aging, which is a measure of age and the aging processes as determined by altered epigenetic states.

We used a meta-analysis approach to examine the association between measures of epigenetic aging and hemostatic factors, as well as a clotting time measure. For fibrinogen, we used European and African-ancestry participants who were meta-analyzed separately and combined via a random effects meta-analysis. All other measures only included participants of European-ancestry. We found that 1-year higher extrinsic epigenetic age as compared to chronological age was associated with higher fibrinogen (0.004 g/L per year; 95% CI: 0.001, 0.007; P = 0.01) and plasminogen activator inhibitor 1 (PAI-1; 0.13 U/mL per year; 95% CI: 0.07, 0.20; P =  $6.6 \times 10^{-5}$ ) concentrations as well as lower activated partial thromboplastin time, a measure of clotting time. We replicated PAI-1 associations using an independent cohort. To further elucidate potential functional mechanisms we associated epigenetic aging with expression levels of the PAI-1 protein encoding gene (*SERPINE1*) and the three fibrinogen subunit-encoding genes (*FGA*, *FGG*, and *FGB*), in both peripheral blood and aorta intima-media samples. We observed associations between accelerated epigenetic aging and transcription of *FGG* in both tissues. Collectively, our results indicate that accelerated epigenetic aging is associated with a pro-coagulation hemostatic profile, and that epigenetic aging may regulate hemostasis in part via gene transcription.

## Introduction

Hemostatic factors are circulating proteins that are critical factors in, or indicators of the blood clotting/coagulation process. They include, but are not limited to, fibrinogen, factor VII (FVII), factor VIII (FVIII), von Willebrand factor (vWF), plasminogen activator-inhibitor 1 (PAI-1), and D-dimer. Dysregulation and/or deficiency of hemostatic factors can cause bleeding events and thrombotic disorders,<sup>1-5</sup> and elevated levels of some hemostatic factors are associated with cardiovascular outcomes such as coronary heart disease<sup>6,7</sup>, incident and recurrent myocardial infarction<sup>8-10</sup>, peripheral arterial disease,<sup>9,11,12</sup> and venous thrombosis.<sup>13,14</sup>

Advancing age is often associated with an altered hemostatic factor profile typified by heightened plasma concentrations of hemostatic factors such as fibrinogen and coagulation factor VII (FVII).<sup>15-17</sup> This age-associated “prothrombotic” hemostatic profile may contribute to associations between age and cardiovascular disease.<sup>18,19</sup> Typically, studies of aging are performed by comparing the hemostatic/clinical profiles of patients with their chronological age. Though effective, this approach does not inform on the underlying biological changes linking aging and altered hemostatic profiles.

Biomarkers of molecular aging have been developed in recent years using epigenetic<sup>20,21</sup> and transcriptomic<sup>22</sup> data. In the case of epigenetics, these biomarkers appear to be weakly correlated with telomere length,<sup>23-25</sup> a traditional molecular biomarker of aging, and independently associated with both age and mortality.<sup>24</sup> Epigenetic age acceleration effects are often tissue specific<sup>20,26</sup> and associated with a number of clinical outcomes including: mortality,<sup>24,27</sup> obesity,<sup>26</sup> cancer,<sup>28,29</sup> cardiovascular disease,<sup>29</sup> HIV-1 infection,<sup>30</sup> and traumatic stress.<sup>31</sup> However, the association between epigenetic aging biomarkers and hemostatic factors has yet to be investigated.

Here, we present the first study into the relationship between epigenetic aging biomarkers and hemostatic factors. Accelerated epigenetic aging may underlie the association between alterations in the hemostatic profile and aging, and reveal novel mechanisms of hemostatic regulation.

## **Methods:**

### ***Participating Cohorts:***

A total of 11 studies from the Cohorts for Heart & Aging Research in Genomic Epidemiology (CHARGE) Hemostasis Working Group participated in these analyses (**Supplemental Table 1**).<sup>32</sup> The Framingham Heart Study (FHS) and Cardiovascular Health Study (CHS) contributed multiple sets of data which were analyzed separately. Datasets from CHS were non-overlapping in participants, whereas data from FHS was longitudinal and collected over the course of 3 consecutive examinations of the Offspring cohort (1991-1995, 1995-1998, & 1998-2001). Plasma levels of five hemostatic factors and one measure of clotting time were examined (units used for analysis given in parentheses): fibrinogen (g/L), PAI-1 (U/mL), D-dimer (ng/mL), FVII (%), vWF (%), and activated partial thromboplastin time (aPTT; s); selected based on their availability in at least three independent cohorts. Fibrinogen and PAI-1 were also assessed in an independent sample of 1,427 individuals from the FHS Generation 3 (FHS Gen3) cohort, which were held out of discovery analyses to be used for replication. These samples were treated and analyzed in an identical manner as the discovery FHS samples. A complete description of each study and the methods used to measure each hemostatic factor are given in the Supplemental Materials.

### ***Epigenetic Aging Assessment:***

All cohorts assessed DNA methylation via the Illumina Infinium HumanMethylation450 array using methods described in the Supplemental Materials. In order



to assess epigenetic aging, an online calculator was used, <https://dnamage.genetics.ucla.edu/>, which takes as input methylation beta values, chronological age, sex, and tissue type and estimates multiple epigenetic aging measures.<sup>20</sup> We primarily used two epigenetic measures for this analysis, both of which assess deviations of epigenetic age from chronological age: Extrinsic Epigenetic Age Acceleration Difference (EEAD) and Intrinsic Epigenetic Age Acceleration Difference (IEAD). IEAD is a blood-specific measure of aging which adjusts the “standard” tissue agnostic measure<sup>20</sup> for the following blood immune cell counts imputed from methylation data: naïve CD8+ T cells, exhausted CD8+ T cells, plasma B cells, CD4+ T cells, natural killer cells, monocytes, and granulocytes.<sup>33</sup> EEAD is calculated by weighting the global epigenetic age measure by imputed blood immune cell counts, and is thus designed to track aging of the immune system as assessed by DNA methylation changes. The term “difference” in IEAD and EEAD refers to taking the difference between the epigenetic aging measures and chronological age. Differences between epigenetic and chronological age are associated with outcomes such as mortality,<sup>34</sup> and may indicate accelerated aging. Cell counts for IEAD and EEAD were estimated based on methylation data following the Houseman<sup>35</sup> and Horvath<sup>36</sup> methods.

As EEAD and IEAD are blood-specific measures, we used age acceleration difference (AAD) to assess differences between epigenetic and chronological age in non-blood tissues, i.e. aorta intima-media. AAD is calculated as the difference between the original, tissue agnostic DNA methylation age measure<sup>20</sup> and chronological age. AAD and IEAD are correlated in blood.<sup>27</sup> For AAD, EEAD, and IEAD the cohorts performed a common filtering approach used in previous meta-analyses of epigenetic aging measures.<sup>27</sup> This approach removed individuals whose sex from their epigenetic profile differed from their reported sex and samples where the estimated cell type was not peripheral blood, and samples whose correlation with internal standards used by the online calculator was  $\leq 0.80$ .

### *Statistical Analyses:*

We used two models to understand the relationship between our measures of epigenetic aging and hemostatic factors. For each model the hemostatic factor was the outcome while the epigenetic aging measure was the predictor. The first model was a basic model which adjusted for chronological age, (chronological age)<sup>2</sup>, and sex. The second (full) model adjusted for the basic model terms plus body mass index (BMI, kg/m<sup>2</sup>), physical activity (active vs inactive), and smoking status (current, former, never). The terms in the full model were chosen to provide an adjustment for potential confounders from lifestyle without including alternative cardiovascular outcomes of interest, such as blood pressure or history of cardiovascular disease, as including these colliders could introduce bias.<sup>37</sup> For studies that did not have a specific variable (e.g. physical activity), it was left out of the model. The basic model was our primary model and all cohorts had the complete covariate information necessary for this model.

In FHS, CHS, and ARIC the epigenetic age measures were based on methylation assessed at a different examination than the hemostatic factor measurement thus an age difference term was calculated as the difference between the chronological age at methylation assessment and chronological age when the hemostatic factors were assessed. Linear and quadratic age difference terms were added to the models. We evaluated the effect of this approach on the observed associations by comparing the association between epigenetic age at a single time point and the same hemostatic factor assessed over multiple time points in FHS. Since FHS had hemostatic measures taken at multiple time points but DNA methylation data only at a single time point (FHS exam 8), we used the assessment closest to the methylation assessment. For CHS and ARIC there was a single assessment of DNA methylation and a single assessment of the hemostatic factors available, each done at a different time point. For cohorts with family data only independent probands were used. The

exception to this is the French-Canadian Family study on Factor V Leiden Thrombophilia (F5L) which is a study sample composed of 5 large multigenerational families. For this cohort family structure was adjusted for in the models via a variance components models implemented in the *pedigreemm* package in R.<sup>38</sup>

We used the *metafor* package<sup>39</sup> in R<sup>40</sup> to perform meta-analyses. We required that hemostatic factors have at least 3 cohorts reporting for the meta-analysis. For all outcomes except PAI-1 we had both European and African ancestry cohorts (**Supplemental Table 1**), however fibrinogen was the only outcome with at least 3 cohorts reporting for both ethnicities, thus fibrinogen was the only hemostatic factor for which we performed race-specific meta-analyses. Initial analyses suggested there was some heterogeneity in the European ancestry fibrinogen estimates (Cochran's Q P-value < 0.05), thus for fibrinogen we used a random-effects meta-analysis for the European-ancestry and combined ethnicity meta-analyses. For all other analyses a fixed-effects meta-analysis was used as little evidence of heterogeneity was observed. To facilitate comparison with the fixed effects models for other outcomes, we also performed a fixed effects meta-analysis for fibrinogen as a sensitivity analysis. We considered associations statistically significant after a Bonferroni correction but also report associations that achieved a nominal statistical significance level of  $P < 0.05$  as these may also be of interest. Previous analyses have observed sex-specific associations with epigenetic aging.<sup>27</sup> We used a multiplicative interaction model to determine if there was an interaction between sex and epigenetic aging measures.

#### ***Associations with peripheral blood gene expression***

To better understand the role of transcriptional regulation in our associations, we used data from the Cooperative Health Research in the Region of Augsburg S4 examination (KORA S4) and the Rotterdam Study (RS) to examine the association between our epigenetic aging measures and the peripheral blood expression of genes for the significant hemostatic

factors. For KORA<sup>41</sup> and RS gene expression was assessed on the Illumina HumanHT-12v3 and Illumina HT-12v4 Expression BeadChip arrays, respectively. Both studies isolated RNA from whole blood using PAXGene Blood RNA kits (Qiagen; Hilden, Germany). For both cohorts, samples with a RNA quality score < 7 were excluded from amplification and analysis, and the final gene expression values were log<sub>2</sub>-transformed. A total of 731 gene expression samples passed quality control and had epigenetic aging assessed in RS, while 439 samples were included in the analysis for KORA S4. Full details are available in the Supplemental Materials. In addition to the cross-sectional gene expression analyses done using gene expression from KORA S4 and RS, we performed a prospective gene expression analysis using KORA F4, since prospective analyses are less influenced by reverse confounding. In this analysis we examined the association between epigenetic aging measures assessed in KORA S4 samples and gene expression from the same individuals measured during the follow-up KORA F4 study.

We used the same variables for confounder adjustment, in basic and full models, as previously described for all gene expression analyses, and additionally included terms for RNA integrity number and amplification plate. The log<sub>2</sub>-transformed gene expression was the outcome in all models. For the cross-sectional analyses in KORA S4 and RS we examined the results from a fixed-effects meta-analysis. Given the limited number of genes assessed and the high correlation between probes for the same gene, associations were considered significant at the  $P < 0.05$  level. We refer to the gene expression results according to the study in which the gene expression was assessed, thus KORA F4 gene expression results refer exclusively to the prospective gene expression analyses, while KORA S4 gene expression results refer exclusively to the cross-sectional analyses using KORA S4 gene expression and epigenetic aging measures.

### ***Aortic intima-media expression***

We also examined the association between epigenetic aging and gene expression in aortic intima-media samples to 1) validate peripheral blood associations and 2) evaluate tissue-specific associations. We used samples from 22 patients with overlapping methylation and gene expression from the Advanced Study of Aortic Pathology biobank (ASAP). Methylation was assessed on the Illumina Infinium HumanMethylation450 array, and gene expression was assayed using the Affymetrix GeneChip® Human Exon 1.0 ST array from RNA isolated using the RNeasy Mini Kit (QIAGEN).<sup>42</sup> Full details for ASAP are available in the Supplemental Materials. The same adjustment models as before were used with the exception that amplification plate and RNA Integrity Number were not included, smoking was assessed as current, former, or never, and physical activity was assessed as the number of days exercised per week. Smoking and physical activity were included as linear variables. Clinical covariates for all gene expression analyses are given in **Supplemental Table 2**.

## **Results**

Baseline characteristics of all studies are given in Supplementary Table 1. For the discovery analyses, each of the 5 hemostatic factors examined we had between 3 and 11 cohorts participating in the meta-analyses. PAI-1 was the only hemostatic factor not measured in any of the African-ancestry studies. Distributions of the hemostatic factors were comparable across the ancestries except for the African Americans in ARIC who had higher D-dimer values and lower FVII values. The age range of participating studies was from 36.9 y to 79.1 y and studies varied from 28.6 to 60.7 in percentage of male participants. The sample size for each of the assessed outcomes ranged from 3,875 (aPTT) to 16,545 (fibrinogen). For the replication in the 3<sup>rd</sup> Generation Framingham Heart Study (FHS Gen3) the mean age was 45.5 y and 48.6% of the cohort were males. The two hemostatic factors assessed in FHS Gen3 were fibrinogen (n = 1,388) and PAI-1 (n = 1,402). In using FHS to evaluate the assessment

of epigenetic age at different time points from the hemostatic factor, exam closest in time to the hemostatic measurement had the strongest (by magnitude of the effect estimate) association, but the confidence intervals for all estimates largely overlapped (**Supplemental Figure 1**).

### *Associations between epigenetic aging and hemostasis measures*

We examined six outcomes and two measures of epigenetic aging in the analyses, thus, the Bonferroni cutoff was set at  $P < 0.0042$  ( $0.05/12$ ). Results for all outcomes are given in **Supplemental Tables 3 and 4**, which also contain summary statistics of regressions of the hemostatic factors on AAD for reference purposes. The basic model was considered the primary model on which we based determinations of statistical significance.

PAI-1 was significantly associated with both epigenetic age acceleration measures in both the basic and full models (**Table 1, Figure 1**). Fibrinogen was the most widely available hemostatic factor with both European (8 cohorts,  $n = 13,183$ ) and African-ancestry (3 cohorts,  $n = 3,362$ ) cohorts represented. We observed significant associations between both measures of blood DNA methylation age and fibrinogen in the African-ancestry cohorts (**Supplemental Tables 3, 4**). In the combined-ethnicity analyses we observed a significant association for EEAD and fibrinogen in the basic model which was attenuated slightly in the full model. (**Figure 2, Table 1**). To facilitate comparison with fixed-effects models used for the other hemostatic factors we also performed fixed-effects meta-analysis for fibrinogen for both EEAD and IEAD (**Supplemental Table 5**). Results mirrored the random effects meta analyses with slightly smaller standard errors and evidence of heterogeneity in some models. The clotting time measure aPTT was significantly associated with EEAD in the basic model. This association was somewhat attenuated in the full model (**Table 1**).

A summary of all associations is given in **Table 2**. Overall, accelerated epigenetic aging was associated with higher clotting factor concentration (fibrinogen and PAI-1) and decreased clotting time (aPTT). For fibrinogen and aPTT, associations were attenuated in the confounder-adjusted full model. As a sensitivity analysis, we removed all cohorts with hemostatic measures and DNA methylation arrays not performed on the same sample. This restriction removed all the African-American ancestry cohorts and left 7 European-ancestry cohorts with fibrinogen measured (N = 6,461) and three European-ancestry cohorts with PAI-1 measured (N = 767). The results were basically unchanged in this sensitivity analysis (**Supplemental Figures 2 and 3**). Despite ARIC having higher values of D-dimer and lower values of FVII than the other cohorts, little to no heterogeneity was seen in the meta-analyses of these outcomes (**Supplemental Tables 3 and 4**). Though all epigenetic aging measures were strongly associated with sex (**Supplemental Table 6**), there were no significant sex-interactions in the meta-analysis after a multiple test correction (**Supplemental Table 7**).

### ***Replication in FHS Gen3***

We used an independent cohort of individuals from FHS Gen3 to attempt to replicate associations with PAI-1 and fibrinogen. We successfully replicated the PAI-1 associations with IEAD and EEAD, but fibrinogen was not associated with epigenetic aging in FHS Gen3 (**Table 3**).

### ***Gene expression analyses***

Given the observed associations with fibrinogen and PAI-1, we examined associations between epigenetic aging measures and whole blood gene expression for the three genes (*FGA*, *FGB*, *FGG*) which encode the fibrinogen subunits, as well as *SERPINE1*, which encodes PAI-1. In a meta-analysis of cross-sectional associations from KORA S4 and RS, we observed associations for *FGA* and *SERPINE1*. EEAD was associated with *FGA* gene

expression for both the basic and full models while *SERPINE1* was negatively associated with IEAD in the basic model (**Table 4a**).

Given the potential for reverse causation when examining epigenetics and gene expression, we examined, in a prospective model, the association between epigenetic aging assessed in KORA S4 with gene expression measured in KORA F4, a follow-up survey of KORA S4 participants. We did not observe associations with *FGA* or *SERPINE1* in the prospective model but did observe an association between IEAD and *FGG* (**Table 4b**).

To understand the tissue specificity of the associations, we examined associations between gene expression and epigenetic aging in aortic intima-media, a second tissue type we had access to that had the necessary DNA methylation and hemostatic factor measurements. All of the genes considered are primarily expressed in tissues other than whole blood as indicated by data from the human genotype tissue expression consortium,<sup>43,44</sup> **Supplemental Figure 4**. We only examined AAD in these tissue samples since EEAD and IEAD are blood-specific measures due to their dependence on blood immune cell counts. We observed an association between AAD and both *FGG* and *FGB* gene expression, though the association with *FGB* was negative. The *FGG* association was similar to prospective KORA F4 associations with IEAD (**Table 4b**).

## Discussion

Hemostatic factors, such as fibrinogen and PAI-1, are risk factors for multiple adverse cardiovascular outcomes.<sup>7,9,10,14</sup> As hemostatic factors measures are associated with age, it is possible that age-related biological mechanisms influence the regulation and thus concentration of hemostatic factors. Here, our results show that higher epigenetic age as compared with chronological age is associated with higher concentrations of fibrinogen and PAI-1 and decreased clotting time. These associations mirror the alterations in the hemostatic profile seen with advancing age. Although coagulation and thrombosis is a balance between



pro- and anti-coagulation, as well as pro- and anti-thrombolysis, higher fibrinogen and PAI-1, along with lower clotting time, may indicate a shift towards a “prothrombotic” hemostatic profile.

### ***PAI-1***

PAI-1 is a serine protease inhibitor which slows the cleavage of plasminogen to plasmin by inhibiting urokinase plasminogen activator. PAI-1 is a risk factor for cardiovascular disease and thrombosis<sup>45</sup>. In mice PAI-1 deficiencies are protective against liver fibrosis and carotid artery atherosclerosis progression.<sup>46,47</sup> However, in humans, individuals homozygous for a *PAI-1* frame-shift mutation experienced abnormal bleeding but no other abnormalities. Heterozygous individuals did not experience abnormal bleeding events.<sup>48</sup> Like other hemostatic factors, PAI-1 is strongly associated with age, and may partly underlie associations between advanced age and cardiovascular outcomes<sup>18</sup>. All epigenetic biomarkers of accelerated aging we examined were associated with higher PAI-1 concentrations. We did not find evidence that the epigenetic regulation of PAI-1 is influenced by the regulation of mRNA levels of *SERPINE1*, its coding gene, as none of the accelerated epigenetic aging measures were associated with *SERPINE1* gene expression in peripheral blood or in aortic intima-media. However, this may be due to *SERPINE1* being only moderately expressed in blood as compared to other tissues such as endothelial cells, and arteries, and thus blood transcriptomic levels may not properly reflect PAI-1 regulation.

### ***Fibrinogen***

Fibrinogen is an essential hemostatic factor and primary phase inflammation marker. As such, fibrinogen dysregulation plays a causal role in multiple bleeding disorders<sup>49</sup>, is a risk factor for cardiovascular disease,<sup>6,7</sup> and is associated with mortality.<sup>7,50</sup> Fibrinogen was associated with multiple measures of accelerated epigenetic aging, most strongly and consistently with EEAD, which strongly correlates with epigenetic aging of the immune

system. We also found evidence that accelerated epigenetic aging was associated with transcription of the fibrinogen gene cluster (*FGA*, *FGB*, *FGG*). As with PAI-1, the associations observed here would be proxies as fibrinogen is primarily expressed in the liver (**Supplemental Figure 4**). In a study of liver biopsy samples from German individuals with non-alcoholic fatty liver disease and controls (biopsy samples from exclusion of liver malignancy) there was no association between *FGA*, *FGB*, or *FGG* gene expression and Horvath epigenetic age acceleration.<sup>26</sup> However, this should still be examined in a population free of disease as non-alcoholic fatty liver disease is known to affect hemostasis.<sup>51,52</sup> In a prospective analysis, higher baseline IEAD was associated with higher levels of *FGG* transcription at follow-up. This association mirrored cross-sectional associations in aortic tissue where higher AAD, a tissue agnostic epigenetic aging measure correlated with IEAD, was also associated with higher *FGG* transcription. Given that mortality and other health outcomes have associations with both accelerated epigenetic aging and fibrinogen, future studies should evaluate if associations between epigenetic aging and health outcomes are partially mediated by fibrinogen.

### ***Strengths and Limitations***

A strength of these analyses is the large sample size of the discovery datasets. For the analysis of fibrinogen, 11 cohorts participated, which included both European and African-ancestry individuals. For the other outcomes we were limited to European-ancestry individuals. Future meta-analyses should include cohorts representing a diverse array of ethnicities, particularly underrepresented ancestry groups. Another limitation is the cross-sectional design, which is not robust to reverse confounding like a prospective design would be. However, we demonstrated associations between epigenetic aging and the transcription of *FGG* using a prospective design that is robust to reverse confounding, suggesting that our observations are not entirely driven by bias introduced from reverse confounding effects.

Estimates of the association between epigenetic aging and the hemostatic factors were in many cases modest. This is to be expected given other modest associations between DNA methylation age and outcomes such as mortality,<sup>27</sup> though it does not eliminate the possibility of substantial increased population health burden from modest individual effects, or large individual effects for persons with severe epigenetic aging.

Another strength of this study was our ability to evaluate associations between accelerated epigenetic aging and gene expression in multiple human tissues. This yielded important clues into the links between accelerated epigenetic aging and transcriptomic regulation of the genes for PAI-1 and fibrinogen. Notably, we were also able to use prospective analyses, which are robust to reverse confounding, to show that associations in blood are similar to those observed in aortic intima-media tissue. We were limited in our analysis of epigenetic aging in aorta intima-media tissue as we had only a few samples (N = 22). Expanding both the number of samples and the diversity of tissues should be a key focus for future studies, particularly given the tissue-specific nature of both DNA methylation and gene expression. These studies should particularly collect tissue samples where the hemostatic measures are primarily expressed, such as liver for fibrinogen.

### ***Conclusion***

In conclusion, we observed multiple associations between epigenetic markers of accelerated aging and hemostatic factors, which together suggest that accelerated epigenetic aging is associated with a prothrombotic hemostatic profile. Advanced age has long been associated with a prothrombotic state, which may underlie associations between age and clinical outcomes, and these analyses suggest that DNA methylation may play an important role in understanding associations between aging, hemodynamics, and clinical outcomes. In the case of fibrinogen, we found evidence that accelerated aging drives gene transcription in multiple tissue types. Given these observations, as the overlap between the outcomes associated with

hemostatic factors and those associated with epigenetic aging increase, researchers should evaluate if altered hemostatic regulation is a means by which epigenetic aging associates with adverse health outcomes.

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### **Authorship contributions**

Authors appear according to their initials. In the case of authors with the same initials the first two letters of the name are used.

Study concept and design: NLS, CKW-C, AP, SLRK, JAS, DAT, PEM, JBJvanM, AGU, OHF, AD, FG

Data acquisition: XG, BMP, NS, MW, PW, WK, DL, CL, SLRK, JAS, DAT, PEM, DIB, GW, LL, EJC, FG, PW, WT, ACM, EB, JSP, HMB, AFC, PE, HP

Drafting manuscript: CKW-C, JEH, PSdeV

Critical revision: JAB, KLW, BMcK, XG, BMP, NS, NLS, CKW-C, JEH, ADJ, CJO'D, MAJ, WZ, EBW, JAS, WDH, IJD, JMS, DAT, PEM, NSR, WT, ACM, EB, JSP

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Data analysis / statistics: JAB, KLW, BMcK, CKW-C, JEH, CL, MAJ, WZ, EBW, WDH, MaGe, PEM, JvanD, MoGh, AD, VT, KE, NSR, OB, HMB

### **Disclosure of Conflicts of Interest:**

BMP serves on the DSMB for a clinical trial funded by the manufacturer (Zoll LifeCor) and on the Steering Committee of the Yale Open Data Access Project funded by Johnson & Johnson. AD has received consultancy and research support from Metagenics Inc. (outside the scope of submitted work). Metagenics Inc. had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; or preparation, review, or approval of the manuscript.

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**Table 1.** Meta-analysis associations between accelerated epigenetic aging and hemostatic factors.

Basic and full model associations for hemostatic factor – epigenetic aging associations which were Bonferroni significant,  $P < 4.16 \times 10^{-3}$ , in the basic model associations. Effect estimates (Beta) are given per one year higher epigenetic age as compared to chronological age. Basic model adjusted for age, age<sup>2</sup>, and sex. Full model adjusted for age, age<sup>2</sup>, sex, body mass index, physical activity, and smoking. For fibrinogen estimates are given for the random effects meta-analysis. aPTT = accelerated partial thromboplastin time; Beta = effect estimate; CI = 95% confidence interval; EA = European ancestry population; EEAD = extrinsic epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; PAI-1 = plasminogen activator inhibitor-1; P(Q) = heterogeneity (Cochran’s Q) P-value; SE = standard error \* = combined-race meta-analysis

Table 1. Significant associations											
Outcome	Aging Measure (y)	Basic					Full				
		Beta	SE	CI	P	P(Q)	Beta	SE	CI	P	P(Q)
PAI-1 (U/mL)	EEAD	0.18	0.04	0.11, 0.25	<b>2.4x10<sup>-7</sup></b>	0.10	0.13	0.03	0.07, 0.20	<b>6.6x10<sup>-5</sup></b>	0.18
PAI-1 (U/mL)	IEAD	0.18	0.05	0.09, 0.27	<b>1.4x10<sup>-4</sup></b>	0.78	0.16	0.04	0.07, 0.25	<b>2.6x10<sup>-4</sup></b>	0.79
Fibrinogen* (g/L)	EEAD	0.01	0.002	0.002, 0.009	<b>0.002</b>	0.004	0.004	0.002	9.5E-04, 0.007	0.01	0.03
aPTT (s)	EEAD	-0.02	0.01	-0.04, -0.007	<b>0.003</b>	0.08	-0.02	0.01	-0.03, -0.006	0.005	0.12

**Table 2.** Summary of associations with  $P < 0.05$ .

Table 2 gives an overall summary of associations with those Bonferroni significant in either the basic or full model marked. Blank cells indicate outcomes which did not achieve Bonferroni significance in either the basic or full model. Associations which were Bonferroni significant in the basic model but only nominally ( $P < 0.05$ ) significant in the full model are marked with a single “+” or “-“. A blank cell indicates that the observed association had  $P > 0.05$ . Effect estimates and p-values for all associations are given in **Supplemental Tables 3** and **4**. AA = African ancestry population; aPTT = activated partial thromboplastin time; EA = European ancestry; EEAD = extrinsic epigenetic age acceleration difference; FVII = Factor VII; IEAD = intrinsic epigenetic age acceleration difference; PAI-1 = plasminogen activator inhibitor-1; RE = combined ancestry random effects model; vWF = von Willebrand Factor ++ (-) = bonferonni significant positive (negative) association; + (-) = nominal ( $P < 0.05$ ) significant positive (negative) association; \* = combined race random effects meta-analysis

	Basic		Full	
	EEAD	IEAD	EEAD	IEAD
Fibrinogen*	++		+	
PAI-1	++	++	++	++
D-dimer				
FVII				
vWF				
aPTT	--		-	

**Table 3.** Results from replication of fibrinogen and PAI-1 in FHS Generation 3 cohort. All aging measures were assessed in years while fibrinogen was measured in g/L and PAI-1 in U/mL. Replicated associations are given in bold. Basic model adjusted for age, age<sup>2</sup>, and sex. Full model adjusted for age, age<sup>2</sup>, sex, body mass index, smoking, and physical activity (where available). Additional linear and quadratic time difference terms were included in both models to represent the time between sample collection for the hemostatic factor assessment and assessment of DNA methylation. Effect estimates (Beta) are given per one year higher epigenetic age as compared to chronological age. AAD = age acceleration difference; Beta = effect estimate; EEAD = extrinsic epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; LCI = lower 95% confidence interval; PAI-1 = plasminogen activator inhibitor-1; SE = standard error; UCI = upper 95% confidence interval

Aging measure	Hemostatic Factor	Model	Beta	SE	LCI	UCI	P
EEAD	Fibrinogen	Basic	6.4x10 <sup>-3</sup>	3.4x10 <sup>-3</sup>	-3.0x10 <sup>-4</sup>	1.3x10 <sup>-2</sup>	0.06
IEAD	Fibrinogen	Basic	4.0x10 <sup>-3</sup>	4.5x10 <sup>-3</sup>	-4.7x10 <sup>-3</sup>	1.3x10 <sup>-2</sup>	0.37
EEAD	Fibrinogen	Full	-8.6x10 <sup>-5</sup>	3.2x10 <sup>-3</sup>	-6.4x10 <sup>-3</sup>	6.2x10 <sup>-3</sup>	0.98
IEAD	Fibrinogen	Full	-8.6x10 <sup>-5</sup>	4.2x10 <sup>-3</sup>	-8.3x10 <sup>-3</sup>	8.1x10 <sup>-3</sup>	0.98
<b>EEAD</b>	<b>PAI-1</b>	<b>Basic</b>	<b>0.17</b>	<b>0.05</b>	<b>0.07</b>	<b>0.28</b>	<b>0.001</b>
<b>IEAD</b>	<b>PAI-1</b>	<b>Basic</b>	<b>0.16</b>	<b>0.07</b>	<b>0.03</b>	<b>0.29</b>	<b>0.01</b>
EEAD	PAI-1	Full	0.05	0.05	-0.04	0.14	0.27
IEAD	PAI-1	Full	0.09	0.06	-0.03	0.20	0.16

**Table 4.** Association between epigenetic aging and gene expression. We examined the association between epigenetic age acceleration and gene expression using both cross-sectional (4a, 4c) and prospective (4b) models in both peripheral blood (4a, 4b) and aorta intima-media samples (4c). Participating cohort abbreviated names are given in parentheses in the sub-table headings. In table 4a we give estimates from the meta-analysis of the cross-sectional associations between epigenetic age acceleration and log<sub>2</sub>-transformed gene expression for KORA and RS. Both cohorts used different version of the same gene expression microarray which contained identical gene expression probe designs allowing for direct comparisons. For the prospective associations (4b), accelerated epigenetic aging was estimated in baseline (KORA S4) samples and gene expression measured in samples collected during follow-up 4-5 years later (KORA F4). Only associations with P < 0.05 in the full (age, age<sup>2</sup>, sex, body mass index, smoking, and physical activity) adjusted model are shown. Effect estimates (Beta) are given per one year higher epigenetic age as compared to chronological age. AAD = age acceleration difference; EEAD = extrinsic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; P(Q) = P-value for heterogeneity; SE = standard error

a. Cross-sectional meta-analysis (KORA F4 + RS)						
Gene	Aging Measure	Estimate	SE	P	Adjustment	P(Q)
<i>FGA</i>	EEAD	0.002	0.001	0.05	Full	0.10
<i>SERPINE1</i>	IEAD	-0.002	0.001	0.04	Full	0.57
b. Prospective associations (KORA S4)						
Gene	Aging Measure	Estimate	SE	P	Adjustment	
<i>FGG</i>	IEAD	0.004	0.002	0.04	Full	
c. Aorta intima-media (ASAP)						
Gene	Aging Measure	Estimate	SE	P	Adjustment	
<i>FGG</i>	AAD	0.009	0.004	0.046	Full	

**Figure 1.** Association between epigenetic aging and PAI-1. Sample sizes are given in parentheses next to the abbreviated cohort names.

Effect estimates (Estimate) represent the association per one year higher epigenetic age as compared to chronological age as observed in the full model. AAD = epigenetic age acceleration difference; EEAD = extrinsic epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; FE = fixed effects.

**Figure 2.** Association between epigenetic aging and fibrinogen. Sample sizes are given in parentheses next to the abbreviated cohort names.

Effect estimates (Estimate) represent the association per one year higher epigenetic age as compared to chronological age as observed in the full model. AAD = epigenetic age acceleration difference; EEAD = extrinsic epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; FE = fixed effects; RE = random effects.

## PAI-1, IEAD

Estimate [95% CI]

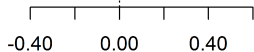
F5L (201) 0.07 [-0.29, 0.44]

MARTHA (337) 0.10 [-0.09, 0.28]

KORA F3 (229) 0.19 [-0.05, 0.43]

FHS Gen6 (2385) 0.19 [0.08, 0.30]

FE Meta-Analysis 0.16 [0.07, 0.25]



## PAI-1, EEAD

Estimate [95% CI]

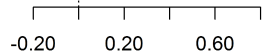
F5L (201) 0.37 [0.12, 0.62]

MARTHA (337) 0.05 [-0.09, 0.19]

KORA F3 (229) 0.16 [0.01, 0.32]

FHS Gen6 (2385) 0.13 [0.04, 0.22]

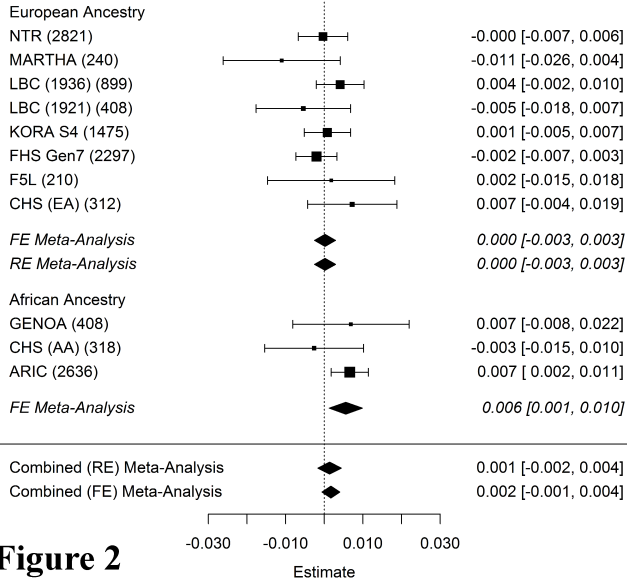
FE Meta-Analysis 0.13 [0.07, 0.20]



**Figure 1**

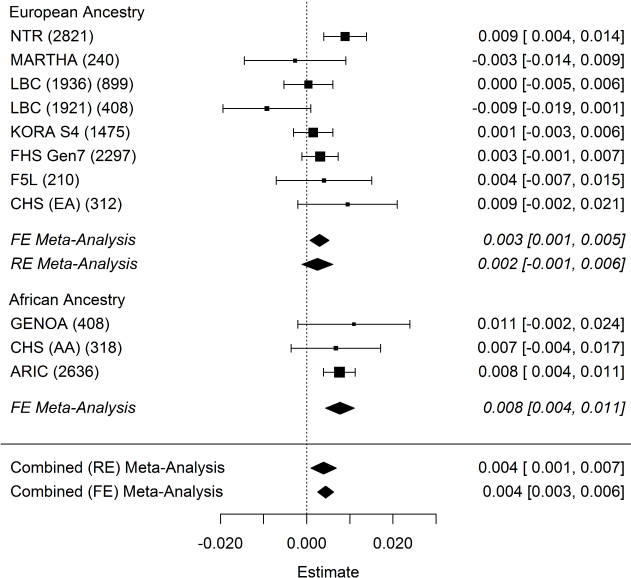
## Fibrinogen, IEAD Diff (Full)

Estimate [95% CI]



## Fibrinogen, EEAD Diff (Full)

Estimate [95% CI]



**Figure 2**