Regular Article

THROMBOSIS AND HEMOSTASIS

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

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KEY POINTS

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

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

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 performed European and African ancestry–specific meta-analyses which were then combined via a random effects meta-analysis. For all other measures we could not estimate ancestry-specific effects and used a single fixed effects meta-analysis. We found that 1-year higher extrinsic epigenetic age as compared with chronological age was associated with higher fibrinogen (0.004 g/L/y; 95% confidence interval, 0.001-0.007; $P = .01$) and plasminogen activator inhibitor 1 (PAI-1; 0.13 U/mL/y; 95% confidence interval, 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 3 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 procoagulation hemostatic profile, and that epigenetic aging may regulate hemostasis in part via gene transcription. (*Blood*. 2018;132(17):1842-1850)

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,[1-5] and elevated levels of some hemostatic factors are associated with cardiovascular outcomes such as coronary heart disease,[6,7] incident and recurrent myocardial infarction,[8-10] peripheral arterial disease,[9,11,12] and venous thrombosis.[13,14]

Advancing age is often associated with an altered hemostatic factor profile typified by heightened plasma concentrations of hemostatic factors such as fibrinogen and coagulation FVII.[15-17] This age-associated "prothrombotic" hemostatic profile may contribute to associations between age and cardiovascular disease.[18,19] Typically, studies of aging are performed by comparing the hemostatic/clinical profiles of patients with their chronological age. Although 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[20,21] and transcriptomic[22] data. In the case of epigenetics, these biomarkers appear to be weakly correlated with telomere length,[23-25] a traditional molecular biomarker of aging, and independently associated with both age and mortality.[24] Epigenetic age acceleration effects are often tissue specific[20,26] and associated with a number of clinical outcomes including mortality,[24,27] obesity,[26] cancer,[28,29] cardiovascular disease,[29] HIV-1 infection,[30] and traumatic stress.[31] 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, available on the *Blood* Web site).[32] The Framingham Heart Study (FHS) and Cardiovascular Health Study (CHS) contributed multiple sets of data that were analyzed separately. Data sets from CHS were nonoverlapping in participants, whereas data from FHS were longitudinal and collected over the course of 3 consecutive examinations of the Offspring cohort (1991-1995, 1995-1998,

and 1998-2001). Plasma levels of 5 hemostatic factors and 1 measure of clotting time were examined (units used for analysis are given in parentheses): fibrinogen (g/L), PAI-1 (U/mL), D-dimer (ng/mL), FVII (%), vWF (%), and activated partial thromboplastin time (aPTT; seconds), selected according to their availability in at least 3 independent cohorts. Fibrinogen and PAI-1 were also assessed in an independent sample of 1427 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 Methods.

Epigenetic aging assessment

All cohorts assessed DNA methylation via the Illumina Infinium HumanMethylation450 array, using methods described in the supplemental Materials. To assess epigenetic aging, an online calculator was used (https://dnamage.genetics.ucla.edu/), which takes as input methylation β values, chronological age, sex, and tissue type and estimates multiple epigenetic aging measures.[20] We primarily used 2 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 that adjusts the "standard" tissue agnostic measure[20] for the following blood immune cell counts imputed from methylation data: naive CD8+ T cells, exhausted CD8+ T cells, plasma B cells, CD4+ T cells, natural killer cells, monocytes, and granulocytes.[33] 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[34] and may indicate accelerated aging. Cell counts for IEAD and EEAD were estimated on the basis of methylation data after the Houseman[35] and Horvath[36] methods.

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

Statistical analyses

We used 2 models to understand the relationship between our measures of epigenetic aging and hemostatic factors. For each model, the hemostatic factor was the outcome, whereas the epigenetic aging measure was the predictor. The first model was a basic model that adjusted for chronological age, chronological age squared, and sex. The second (full) model adjusted for the basic model terms plus body mass index (kg/m²), 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.[37] For studies that did not have a specific variable (eg, 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 point and the same hemostatic factor assessed over multiple points in FHS. Because FHS had hemostatic measures taken at multiple points but DNA methylation data only at a single 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 performed at a different 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 model implemented in the *pedigreemm* package in R.[38]

We used the *metafor* package[39] in R[40] 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 ancestry 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 < .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 < .05, as these may also be of interest. Previous analyses have observed sex-specific associations with epigenetic aging.[27] We used a multiplicative interaction model to determine whether 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[41] 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 lower than 7 were excluded from amplification and analysis, and the final gene expression values were log₂-transformed. A total of 731 gene expression samples passed quality control and had epigenetic aging assessed in RS, whereas 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 performed using gene expression from KORA S4 and RS, we performed a prospective gene expression analysis using KORA F4, as 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 as included terms for RNA integrity number and amplification plate. The log₂-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* < .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, whereas 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 both validate peripheral blood associations and evaluate tissue-specific associations. We used samples from 22 patients with overlapping methylation and gene expression from the Advanced Study of Aortic Pathology biobank. 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

Table 1. Significant meta-analysis associations between accelerated epigenetic aging and hemostatic factors

Outcome	Aging measure, y	Basic					Full				
		β	SE	CI	P	P(Q)	β	SE	CI	P	P(Q)
PAI-1, U/mL	EEAD	0.18	0.04	0.11-0.25	2.4×10^{-7}	0.10	0.13	0.03	0.07-0.20	6.6×10^{-5}	.18
PAI-1, U/mL	IEAD	0.18	0.05	0.09-0.27	1.4×10^{-4}	0.78	0.16	0.04	0.07-0.25	2.6×10^{-4}	.79
Fibrinogen, g/L*	EEAD	0.01	0.002	0.002-0.009	.002	0.004	0.004	0.002	9.5E-04 to 0.007	.01	.03
aPTT, s	EEAD	−0.02	0.01	−0.04 to −0.007	.003	0.08	−0.02	0.01	−0.03 to −0.006	.005	.12

Basic and full model associations for hemostatic factor – epigenetic aging associations that were Bonferroni significant, $P < 4.16 \times 10^{-3}$, in the basic model associations. Effect estimates (β) are given per 1 year higher epigenetic age as compared with chronological age. Basic model adjusted for age, age squared, and sex. Full model adjusted for age, age squared, sex, body mass index, physical activity, and smoking. For fibrinogen estimates are given for the random effects meta-analysis.

β, effect estimate; CI, 95% confidence interval; EA, European ancestry population; P(Q), heterogeneity (Cochran's Q) P value; SE, standard error.

*Combined-race meta-analysis

RNeasy Mini Kit (QIAGEN).[42] Full details for the Advanced Study of Aortic Pathology biobank 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 supplemental Table 1. For the discovery analyses, for 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 to 79.1 years, 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 3875 (aPTT) to 16 545 (fibrinogen). For the replication in the 3rd Generation Framingham Heart Study (FHS Gen3), the mean age was 45.5 years, and 48.6% of the cohort were men. The 2 hemostatic factors assessed in FHS Gen3 were fibrinogen (n = 1388) and PAI-1 (n = 1402). In using FHS to evaluate the assessment of epigenetic

age at different 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).
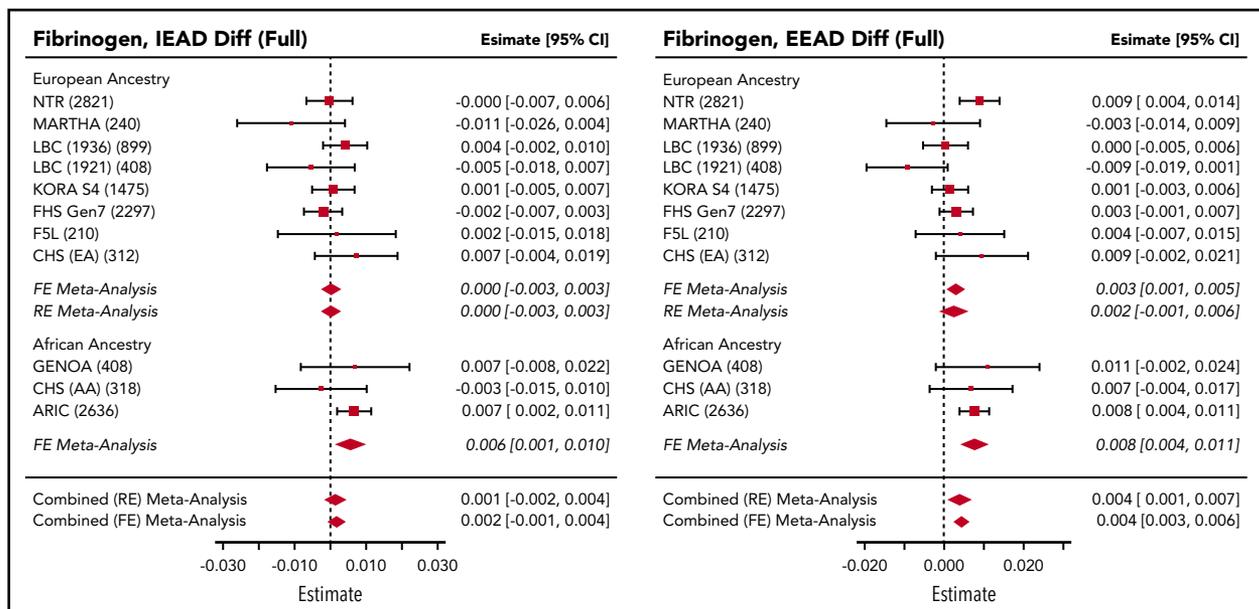
Associations between epigenetic aging and hemostasis measures

We examined 6 outcomes and 2 measures of epigenetic aging in the analyses; thus, the Bonferroni cutoff was set at P < .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 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 ancestry (8 cohorts, n = 13 183) and African ancestry (3 cohorts, n = 3362) cohorts represented. We observed significant associations between both measures of blood DNA methylation age and fibrinogen in the African ancestry cohorts (supplemental Tables 3 and 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
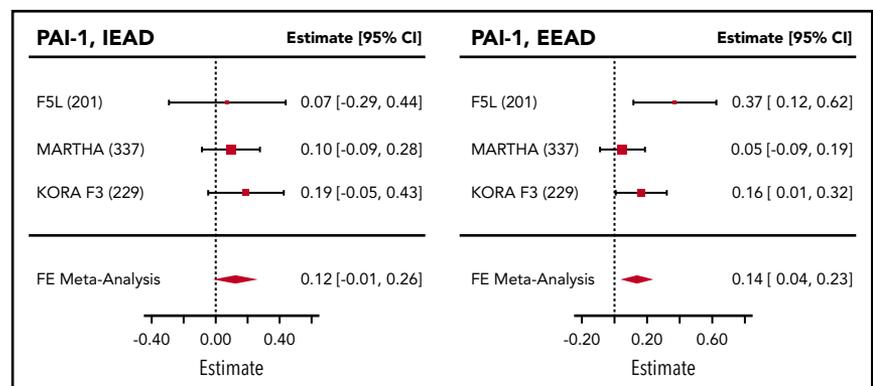
Figure 1. Association between epigenetic aging and PAI-1. Sample sizes are given in parentheses next to the abbreviated cohort names. Effect estimates (Estimate) represent the association per 1 year higher epigenetic age as compared with chronological age, as observed in the full model. FE, fixed effects.

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 1 year higher epigenetic age as compared with chronological age, as observed in the full model. RE, random effects.

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 = 6461) and 3 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). Although 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 3 genes (*FGA*, *FGB*, *FGG*) that 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, whereas *SERPINE1* was negatively associated with IEAD in the basic model (Table 4).

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

Table 2. Summary of associations with P < .05

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

Overall summary of associations with those Bonferroni significant in either the basic or full model marked. Blank cells indicate outcomes that did not achieve Bonferroni significance in either the basic or full model. Associations that were Bonferroni significant in the basic model but only nominally (P < .05) significant in the full model are marked with a single "+" or "−". A blank cell indicates that the observed association had P > .05. Effect estimates and P values for all associations are given in supplemental Tables 3 and 4.

AA, African ancestry population; ++(−−), Bonferroni significant positive (negative) association. +(−), nominal (P < .05) significant positive (negative) association.

*Combined race random effects meta-analysis.

Table 3. Results from replication of fibrinogen and PAI-1 in FHS Gen3 cohort.

Aging measure	Hemostatic factor	Model	β	SE	LCI	UCI	P
EEAD	Fibrinogen	Basic	6.4×10^{-3}	3.4×10^{-3}	-3.0×10^{-4}	1.3×10^{-2}	.06
IEAD	Fibrinogen	Basic	4.0×10^{-3}	4.5×10^{-3}	-4.7×10^{-3}	1.3×10^{-2}	.37
EEAD	Fibrinogen	Full	-8.6×10^{-5}	3.2×10^{-3}	-6.4×10^{-3}	6.2×10^{-3}	.98
IEAD	Fibrinogen	Full	-8.6×10^{-5}	4.2×10^{-3}	-8.3×10^{-3}	8.1×10^{-3}	.98
EEAD	**PAI-1**	**Basic**	**0.17**	**0.05**	**0.07**	**0.28**	**.001**
IEAD	**PAI-1**	**Basic**	**0.16**	**0.07**	**0.03**	**0.29**	**.01**
EEAD	PAI-1	Full	0.05	0.05	-0.04	0.14	.27
IEAD	PAI-1	Full	0.09	0.06	-0.03	0.20	.16

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 squared, and sex. Full model adjusted for age, age squared, 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 (β) are given per 1 year higher epigenetic age as compared with chronological age.

LCI, lower 95% confidence interval; UCI, upper 95% confidence interval.

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 4).

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 the genes considered are primarily expressed in tissues other than whole blood, as indicated by data from the human genotype tissue expression consortium[43,44] (supplemental Figure 4). We only examined AAD in these tissue samples, as EEAD and IEAD are blood-specific measures because of their dependence on blood immune cell counts. We observed an association between AAD and both *FGG* and *FGB* gene expression, although the association with *FGB* was negative. The *FGG*

association was similar to prospective KORA F4 associations with IEAD (Table 4).

Discussion

Hemostatic factors, such as fibrinogen and PAI-1, are risk factors for multiple adverse cardiovascular outcomes.[7,9,10,14] As hemostatic factor 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, as well as 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,

Table 4. Association between epigenetic aging and gene expression

Gene	Aging measure	Estimate	SE	P	Adjustment	P(Q)
Cross-sectional meta-analysis (KORA F4 + RS)*						
FGA	EEAD	0.002	0.001	.05	Full	0.10
SERPINE1	IEAD	-0.002	0.001	.04	Full	0.57
Prospective associations (KORA S4)†						
FGG	IEAD	0.004	0.002	.04	Full	
Aorta intima-media (ASAP)						
FGG	AAD	0.009	0.004	.046	Full	

We examined the association between epigenetic age acceleration and gene expression using both cross-sectional and prospective models in both peripheral blood and aorta intima-media samples. Participating cohort abbreviated names are given in parentheses.

*Estimates from the meta-analysis of the cross-sectional associations between epigenetic age acceleration and $\log_2$-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, accelerated epigenetic aging was estimated in baseline (KORA S4) samples and gene expression measured in samples collected during follow-up 4 to 5 years later (KORA F4). Only associations with $P < .05$ in the full (age, age squared, sex, body mass index, smoking, and physical activity) adjusted model are shown. Effect estimates (β) are given per 1 year higher epigenetic age as compared with chronological age. ASAP, Advanced Study of Aortic Pathology biobank.

higher fibrinogen and PAI-1, along with lower clotting time, may indicate a shift toward a "prothrombotic" hemostatic profile.

PAI-1

PAI-1 is a serine protease inhibitor that slows the cleavage of plasminogen to plasmin by inhibiting urokinase plasminogen activator. PAI-1 is a risk factor for cardiovascular disease and thrombosis.[45] In mice, PAI-1 deficiencies are protective against liver fibrosis and carotid artery atherosclerosis progression.[46,47] However, in humans, individuals homozygous for a *PAI-1* frameshift mutation experienced abnormal bleeding, but no other abnormalities. Heterozygous individuals did not experience abnormal bleeding events.[48] Similar to other hemostatic factors, PAI-1 is strongly associated with age and may partly underlie associations between advanced age and cardiovascular outcomes.[18] 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 a result of *SERPINE1* being only moderately expressed in blood as compared with 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,[49] is a risk factor for cardiovascular disease,[6,7] and is associated with mortality.[7,50] 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 nonalcoholic 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.[26] However, this should still be examined in a population free of disease, as nonalcoholic fatty liver disease is known to affect hemostasis.[51,52] 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 whether 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 data sets. For the analysis of fibrinogen, 11 cohorts participated, including both European- and African-ancestry cohorts, allowing us to estimate ancestry-specific associations. For the other outcomes, we were limited and could not estimate ancestry-specific effects. 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 as 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,[27] although 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 in which the hemostatic measures are primarily expressed, such as liver for fibrinogen.

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 whether altered hemostatic regulation is a means by which epigenetic aging associates with adverse health outcomes.

Authorship

Contribution: C.K.W.-C., D.-A.T., S.L.R.K., J.B.J.V.M., A.G.U., O.H.F., F.G., A.D., P.-E.M., J.A.S., N.L.S., and A.P. provided the study concept and design; M.W., P.W., H.P., W.K., D.L., C.L., P.S.W., D.-A.T., W.T., A.C.M., E.B., X.G., B.M.P., N.S., D.I.B., G.W., L.L., S.L.R.K., P.E., A.F.-C., J.S.P., F.G., P.-E.M., E.J.C.d.G., J.A.S., and H.M.B. provided data acquisition; C.K.W.-C., J.E.H., and P.S.d.V. drafted the manuscript; C.K.W.-C., J.E.H., W.D.H., M.A.J., J.A.B., N.S.R., C.J.O., D.-A.T., W.T., A.C.M., E.B., K.L.W., B.M., X.G., B.M.P., N.S., I.J.D., W.Z., E.B.W., J.S.P. A.D.J., P.-E.M., J.M.S., J.A.S., and N.L.S., provided critical revision; C.G., H.P., W.K., C.J.O., D.L., D.-A.T., D.I.B., I.J.D., S.L.R.K., J.B.J.V.M., A.G.U., O.H.F., P.E., A.D.J., F.G., P.-E.M., E.J.C.d.G., J.M.S., J.A.S., and A.P. provided funding; and C.K.W.-C., J.E.H., K.E., M. Germain, J.v.D., W.D.H., M.A.J., J.A.B., M. Ghanbari, L.D., N.S.R., C.L., V.T., K.L.W., B.M., W.Z., E.B.W., P.-E.M., A.D., and H.M.B. provided data analysis/statistics.

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Footnotes

REFERENCES

1. Ginsburg D. von Willebrand Disease. Williams Hematology. Philadelphia, PA: McGraw-Hill; 2001:1813-1828.

2. Acharya SS, Coughlin A, Dimichele DM; North American Rare Bleeding Disorder Study Group. Rare Bleeding Disorder Registry: deficiencies of factors II, V, VII, X, XIII, fibrinogen and dysfibrinogenemias. J Thromb Haemost. 2004;2(2):248-256.

3. Antonarakis SE. Molecular genetics of coagulation factor VIII gene and hemophilia A. Thromb Haemost. 1995;74(1):322-328.

4. Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. N Engl J Med. 2011; 365(2):147-156.

5. Dahlbäck B. Blood coagulation. Lancet. 2000; 355(9215):1627-1632.

6. Koenig W. Fibrin(ogen) in cardiovascular disease: an update. Thromb Haemost. 2003; 89(4):601-609.

7. Danesh J, Lewington S, Thompson SG, et al; Fibrinogen Studies Collaboration. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. JAMA. 2005;294(14):1799-1809.

8. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC; European Concerted Action on Thrombosis and Disabilities Angina Pectoris Study Group. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. N Engl J Med. 1995;332(10):635-641.

9. Tzoulaki I, Murray GD, Lee AJ, Rumley A, Lowe GD, Fowkes FGR. Relative value of inflammatory, hemostatic, and rheological factors for incident myocardial infarction and

stroke: the Edinburgh Artery Study. *Circulation.* 2007;115(16):2119-2127.

10. Wiman B, Andersson T, Hallqvist J, Reuterwall C, Ahlbom A, deFaire U. Plasma levels of tissue plasminogen activator/plasminogen activator inhibitor-1 complex and von Willebrand factor are significant risk markers for recurrent myocardial infarction in the Stockholm Heart Epidemiology Program (SHEEP) study. *Arterioscler Thromb Vasc Biol.* 2000; 20(8):2019-2023.

11. Smith FB, Lee AJ, Hau CM, Rumley A, Lowe GD, Fowkes FG. Plasma fibrinogen, haemostatic factors and prediction of peripheral arterial disease in the Edinburgh Artery Study. *Blood Coagul Fibrinolysis.* 2000;11(1):43-50.

12. Tzoulaki I, Murray GD, Price JF, et al. Hemostatic factors, inflammatory markers, and progressive peripheral atherosclerosis: the Edinburgh Artery Study. *Am J Epidemiol.* 2006;163(4):334-341.

13. Lane DA, Grant PJ. Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. *Blood.* 2000;95(5):1517-1532.

14. Cushman M, Folsom AR, Wang L, et al. Fibrin fragment D-dimer and the risk of future venous thrombosis. *Blood.* 2003;101(4): 1243-1248.

15. Mari D, Coppola R, Provenzano R. Hemostasis factors and aging. *Exp Gerontol.* 2008;43(2): 66-73.

16. Kamath S, Lip GY. Fibrinogen: biochemistry, epidemiology and determinants. *QJM.* 2003; 96(10):711-729.

17. Balleisen L, Bailey J, Epping PH, Schulte H, van de Loo J. Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population: I. Baseline data on the relation to age, gender, body-weight, smoking, alcohol, pill-using, and menopause. *Thromb Haemost.* 1985;54(2):475-479.

18. Wilkerson WR, Sane DC. Aging and thrombosis. New York: Thieme Medical Publishers; 2002:555-568.

19. Cesari M, Pahor M, Incalzi RA. Plasminogen activator inhibitor-1 (PAI-1): a key factor linking fibrinolysis and age-related subclinical and clinical conditions. *Cardiovasc Ther.* 2010; 28(5):e72-e91.

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

21. Hannum G, Guinney J, Zhao L, et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell.* 2013; 49(2):359-367.

22. Peters MJ, Joehanes R, Pilling LC, et al; NABEC/UKBEC Consortium. The transcriptional landscape of age in human peripheral blood. *Nat Commun.* 2015;6(1):8570.

23. Nwanaji-Enwerem JC, Colicino E, Trevisi L, et al. Long-term ambient particle exposures and blood DNA methylation age: findings from the VA normative aging study. *Environ Epigenet.* 2016;2(2):dvw006.

24. Marioni RE, Harris SE, Shah S, et al. The epigenetic clock and telomere length are independently associated with chronological age and mortality. *Int J Epidemiol.* 2016;45(2): 424-432.

25. Chen BH, Carty CL, Kimura M, et al. Leukocyte telomere length, T cell composition and DNA methylation age. *Aging (Albany NY).* 2017; 9(9):1983-1995.

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

27. 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.

28. Zheng Y, Joyce BT, Colicino E, et al. Blood epigenetic age may predict cancer incidence and mortality. *EBioMedicine.* 2016;5:68-73.

29. Perna L, Zhang Y, Mons U, Holleczek B, Saum K-U, Brenner H. Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clin Epigenetics.* 2016;8(1):64.

30. Horvath S, Levine AJ. HIV-1 infection accelerates age according to the epigenetic clock. *J Infect Dis.* 2015;212(10):1563-1573.

31. Boks MP, van Mierlo HC, Rutten BP, et al. Longitudinal changes of telomere length and epigenetic age related to traumatic stress and post-traumatic stress disorder. *Psychoneuroendocrinology.* 2015;51: 506-512.

32. Psaty BM, O'Donnell CJ, Gudnason V, et al; CHARGE Consortium. Design of prospective meta-analyses of genome-wide association studies from 5 cohorts. *Circ Cardiovasc Genet.* 2009;2(1):73-80.

33. Levine ME, Hosgood HD, Chen B, Absher D, Assimes T, Horvath S. DNA methylation age of blood predicts future onset of lung cancer in the women's health initiative. *Aging (Albany NY).* 2015;7(9):690-700.

34. Marioni RE, Shah S, McRae AF, et al. DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol.* 2015; 16(1):25.

35. Houseman EA, Accomando WP, Koestler DC, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinformatics.* 2012;13(1):86.

36. Horvath S, Ritz BR. Increased epigenetic age and granulocyte counts in the blood of Parkinson's disease patients. *Aging (Albany NY).* 2015;7(12):1130-1142.

37. Cole SR, Platt RW, Schisterman EF, et al. Illustrating bias due to conditioning on a collider. *Int J Epidemiol.* 2010;39(2):417-420.

38. Vazquez AI, Bates DM, Rosa GJ, Gianola D, Weigel KA. Technical note: an R package for fitting generalized linear mixed models in animal breeding. *J Anim Sci.* 2010;88(2): 497-504.

39. Viechtbauer W. Conducting meta-analyses in R with the metafor package. *J Stat Softw.* 2010;36(3):1-48.

40. R Core Team. R: A language and environment for statistical computing. Vol. 3.1.0. Vienna: R Foundation for Statistical Computing; 2015.

41. Schramm K, Marzi C, Schurmann C, et al. Mapping the genetic architecture of gene regulation in whole blood. *PLoS One.* 2014; 9(4):e93844.

42. Folkersen L, Wågsäter D, Paloschi V, et al. Unraveling divergent gene expression profiles in bicuspid and tricuspid aortic valve patients with thoracic aortic dilatation: the ASAP study. *Mol Med.* 2011;17(11-12):1365-1373.

43. Battle A, Brown CD, Engelhardt BE, et al; eQTL manuscript working group. Genetic effects on gene expression across human tissues. *Nature.* 2017;550(7675):204-213.

44. GTEx Consortium. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science.* 2015;348(6235):648-660.

45. Vaughan DE. PAI-1 and atherothrombosis. *J Thromb Haemost.* 2005;3(8):1879-1883.

46. Eitzman DT, Westrick RJ, Xu Z, Tyson J, Ginsburg D. Plasminogen activator inhibitor-1 deficiency protects against atherosclerosis progression in the mouse carotid artery. *Blood.* 2000;96(13):4212-4215.

47. Wang H, Zhang Y, Heuckeroth RO. PAI-1 deficiency reduces liver fibrosis after bile duct ligation in mice through activation of tPA. *FEBS Lett.* 2007;581(16):3098-3104.

48. Fay WP, Parker AC, Condrey LR, Shapiro AD. Human plasminogen activator inhibitor-1 (PAI-1) deficiency: characterization of a large kindred with a null mutation in the PAI-1 gene. *Blood.* 1997;90(1):204-208.

49. Acharya SS, Dimichele DM. Rare inherited disorders of fibrinogen. *Haemophilia.* 2008; 14(6):1151-1158.

50. Woodward M, Lowe GD, Rumley A, Tunstall-Pedoe H. Fibrinogen as a risk factor for coronary heart disease and mortality in middle-aged men and women. The Scottish Heart Health Study. *Eur Heart J.* 1998;19(1): 55-62.

51. Targher G, Chonchol M, Miele L, Zoppini G, Pichiri I, Muggeo M. Nonalcoholic fatty liver disease as a contributor to hypercoagulation and thrombophilia in the metabolic syndrome. *Seminars in thrombosis and hemostasis.* Semin Thromb Hemost. 2009:277-287

52. Yeung ENW, Treskes P, Martin SF, et al. Fibrinogen production is enhanced in an in-vitro model of non-alcoholic fatty liver disease: an isolated risk factor for cardiovascular events? *Lipids Health Dis.* 2015;14(1):86.