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## DNA methylation analysis identifies novel genetic loci associated with circulating fibrinogen levels in blood

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### Abstract

**Background:** Fibrinogen plays an essential role in blood coagulation and inflammation. Circulating fibrinogen levels may be determined by inter-individual differences in DNA methylation at CpG sites, and vice versa.

**Methods:** We performed an epigenome-wide association study (EWAS) of circulating fibrinogen levels in 18,037 White, Black, American Indian, and Hispanic participants representing 14 studies from the CHARGE consortium. Circulating leukocyte DNA methylation was measured in 12,904 participants using the Illumina 450K array, and in 5,133 participants using the EPIC array. Each study performed an EWAS of fibrinogen using linear mixed models adjusted for potential confounders. Study-specific results were combined using array-specific meta-analysis, followed by cross-replication of epigenome-wide significant associations. We compared models with and without C-reactive protein (CRP) adjustment to examine the role of inflammation.

**Results:** We identified 208 and 87 significant CpG sites associated with fibrinogen from the 450K ( $p\text{-value} < 1.03 \times 10^{-7}$ ) and EPIC arrays ( $p\text{-value} < 5.78 \times 10^{-8}$ ), respectively. There were 78 associations from the 450K array that replicated in the EPIC array and 26 vice versa. After accounting for the overlapping sites, there were 83 replicated CpG sites located in 61 loci, of

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#### Conflict of Interest

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which only 4 have been previously reported for fibrinogen. Examples of genes located near these CpG sites were *SOCS3* and *AIM2*, which are involved in inflammatory pathways. The associations for all 83 replicated CpG sites were attenuated after CRP adjustment, although many remained significant.

**Conclusion:** We identified 83 CpG sites associated with circulating fibrinogen levels. These associations are partially driven by inflammatory pathways shared by both fibrinogen and CRP.

### Keywords

DNA methylation; Fibrinogen; Epigenome-wide association study; Inflammation; Mendelian randomization

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### Introduction

Fibrinogen plays an essential role in blood coagulation and inflammation. (1, 2) These two processes contribute to the development of thrombotic and atherosclerotic diseases, such as coronary artery disease, ischemic stroke, and venous thromboembolism. (3–5) Previously, in the context of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium, a number of genome-wide association studies (GWAS) were conducted to identify genetic loci involved in circulating fibrinogen levels, and to date, 42 loci associated with fibrinogen were identified. (6, 7) However, these loci account for only approximately 3% of the variability in fibrinogen levels. (6) Thus, an overall variance of fibrinogen levels is incompletely explained, and other factors likely also drive this variation, including epigenetic changes.

Epigenetic modifications are potentially heritable biochemical alterations to the genome that do not change the underlying genetic makeup, but they can alter the phenotypic expression. (8, 9) As one of the most stable epigenetic mechanisms, DNA methylation is one of the most well-studied epigenetic mechanisms that regulate gene expression. (8, 10) It is the mechanism whereby a methyl group is attached to the DNA sequence, usually to a cytosine nucleotide followed by a guanine nucleotide, also referred to as CpG sites. DNA methylation can be modified by both genetic and environmental factors, and, as such, investigating the variation in DNA methylation may further explain the phenotypic variation in fibrinogen levels.

Recently, epigenome-wide association studies (EWAS) have successfully identified associations between numerous phenotypes and DNA methylation at cytosine-phosphate-guanine (CpG) sites in circulating leukocytes. (8, 11, 12) Studying leukocyte DNA methylation levels from whole blood in relation to fibrinogen has several advantages compared to using other tissues: whole blood samples are readily available, fibrinogen is present in the circulation, and leukocytes within whole blood are intimately involved in inflammatory pathways that regulate fibrinogen production. An association between DNA methylation and fibrinogen levels may be caused by DNA methylation which has an impact on fibrinogen levels, or fibrinogen itself may directly or indirectly influence DNA methylation. Thus, analysis of these associations may provide insight into the regulation of fibrinogen as well as identify downstream effects of fibrinogen that may mediate its role in

inflammation and thrombosis. The aim of this study was to examine the association between circulating leukocyte DNA methylation levels at CpG sites and circulating fibrinogen levels by performing the first EWAS for fibrinogen in 18,037 individuals within the setting of the CHARGE consortium.

## Methods

### Study population and design

This study was performed within the context of the CHARGE Hemostasis and Epigenetics Working Groups. A total of 18,037 participants from the following 14 studies were included: the Airwave Health Monitoring Study (Airwave), Atherosclerosis Risk in Communities (ARIC) study, the Cardiovascular Health Study (CHS), the Framingham Heart Study (FHS), Genetic Epidemiology Network of Arteriopathy (GENOA), Lothian Birth Cohort 1921 (LBC 1921), Lothian Birth Cohort 1936 (LBC 1936), the LUdwigshafen RiSk and Cardiovascular Health (LURIC) study, MARseille Thrombosis Association study (MARTHA), Multi-Ethnic Study of Atherosclerosis (MESA), Netherlands Twin Register (NTR), the Study of Health in Pomerania Independent cohort (SHIP-Trend), the Strong Heart Study (SHS) and the TwinsUK cohort (TwinsUK). Our study population was divided into two analysis groups determined by the array type used to measure DNA methylation. A total of 12,904 participants from eight studies were included in the Illumina 450K Human Methylation microarray (450K array) group which included individuals from White, Black and Hispanic population groups. The Illumina EPIC Human Methylation microarray (EPIC array) group consisted of a total of 5,133 participants from five studies which included individuals from White, Black, and American Indian population groups. Details for each study are provided in the Supplementary Methods. Participants using anticoagulation therapy at the time of phlebotomy were excluded from all analyses. All study participants provided written informed consent to participate, and all study sites received approval to conduct this research from their local Institutional Review Boards.

### Fibrinogen measurements

Fibrinogen levels were measured from blood samples in g/L using the Clauss method. (13) Airwave, CHS, LBC, LURIC, MARTHA, NTR, SHIP-Trend, and SHS measured fibrinogen at the same time point as DNA methylation. Other studies measured fibrinogen prior to DNA methylation, including ARIC (White: mean [SD] time difference = 3.68 [1.35] years, Black: 3.3 (1.08) years), FHS (UMN: 13.58 [1.05] years, GEN3: 6.18 [0.59] years), and MESA (9.52 [0.51] years). GENOA measured fibrinogen after DNA methylation (2.3 [0.5] years). In the TwinsUK study the fibrinogen was measured prior to DNA methylation in some participants and after DNA methylation in others (0.11 [1.96] years). Fibrinogen levels were natural log-transformed to approximate the normal distribution, and outliers with fibrinogen values greater than 3 standard deviations from the study-specific log-transformed mean were removed. Study-specific methods are described in the Supplementary Methods.

### DNA methylation quantification

DNA methylation levels for all studies were measured utilizing multiple cell types isolated from whole blood or buffy coat samples, except for MESA which extracted from CD14+

monocytes and CD4+ T cells. DNA methylation levels were analyzed using the Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA, USA) or Infinium MethylationEPIC BeadChip array (Illumina). CpG probes included in more than one of the arrays are consistently named, facilitating the combination of data from different arrays. Greater than 90% of probes on the Infinium HumanMethylation450 BeadChip array are also present on the Infinium MethylationEPIC BeadChip array, but the latter also includes an additional 330,000 probes targeting enhancer regions. (14) The degree of DNA methylation was expressed using  $\beta$  values, which ranged from 0–1. The  $\beta$  value of a specific CpG site was interpreted as the proportion of copies of the CpG site that are methylated. DNA methylation pre-processing and  $\beta$  value normalization were conducted independently in different studies using study-specific methods. Further study-specific DNA methylation methods are described in the Supplementary Methods.

### Study-level association analyses

An EWAS of circulating fibrinogen levels was performed for each study. Ancestry-stratified linear regression models were used to evaluate the association of inter-individual variation in DNA methylation assessed as  $\beta$  values (outcome variable) and fibrinogen levels (independent variable). All models were adjusted for age, sex, smoking status, body mass index (BMI), cell type proportions, technical covariates to control for batch effects, and ancestry informative principal components. Additionally, if blood samples used for measuring fibrinogen were obtained at a different time point than the blood samples used for measuring DNA methylation, the time difference between the fibrinogen and DNA methylation measurements was used as an additional covariate in the regression models. Study-specific analytic modeling details can be found in the Supplementary Methods.

### Meta-analyses and cross-replication

Meta-analyses were performed separately for the 450K array and EPIC array, followed by cross-replication. Study-specific results were first meta-analyzed within ancestry groups, and the resulting ancestry-specific results were then meta-analyzed to produce array-specific results for the 450K and EPIC array. All meta-analyses were performed using fixed effect inverse-variance weighting as implemented in METAL. (15) In the 450K meta-analysis, a Bonferroni correction was applied to correct for multiple testing with a significance threshold of  $1.03 \times 10^{-7}$  (0.05 / 485,436 CpG sites). With the EPIC array meta-analysis, a Bonferroni corrected significance threshold of  $5.78 \times 10^{-8}$  (0.05 / 865,052 CpG sites) was used. Significant CpG sites that were present on both arrays were then cross-replicated: the results from the 450K array meta-analysis were replicated in the EPIC array meta-analysis results, and vice versa. Lastly, a meta-analysis of the 450K and EPIC array results was also performed for epigenome-wide significant and cross-replicated CpG sites in order to estimate their overall associations with fibrinogen levels.

### Sensitivity analyses

In ARIC, a sensitivity analysis was performed adjusting additionally for C-reactive protein (CRP), a marker of inflammation, to examine the role of inflammation. CRP was specifically chosen because it is phenotypically correlated with fibrinogen, an established marker of inflammation, a stable clinical readout as well as has a relatively stable half-life.

(16) CRP was measured in mg/L using a high-sensitivity assay from the blood samples drawn at visit 2, which is the same visit at which DNA methylation was assessed. The analysis was restricted to individuals with CRP measurements, and CRP was incorporated into the model as natural log-transformed values. Initial analyses were performed for each population group (White and Black), and they were then meta-analyzed. We evaluated the changes in beta values of the significant and replicated CpG sites from the main analysis and considered genetic loci to be attenuated after CRP adjustment when the absolute values of betas were reduced by more than 10%.

Another sensitivity analysis was performed which evaluated the effect of differences in the timing of the fibrinogen and DNA methylation measures. We performed meta-analyses restricted to 1) studies that measured fibrinogen and DNA methylation at the same time, 2) studies that measured DNA methylation prior to fibrinogen measurement, and 3) studies that measured fibrinogen prior to DNA methylation. We then compared the associations of significant and replicated results from the main analysis across the three groups. The results from TwinsUK were excluded in this sensitivity analysis as they included samples using both directions in temporality.

Within the ARIC study, a sex-specific meta-analysis was performed to evaluate whether there is heterogeneity by sex in the significant and replicated CpG sites.

### Bi-directional Mendelian randomization analysis

We performed bi-directional two-sample Mendelian randomization (MR) analyses to provide evidence for causal effects underlying the associations between fibrinogen and DNA methylation at significant and replicated CpG sites. By using a bidirectional approach, we explored whether genetically determined DNA methylation levels alter fibrinogen levels or whether genetically determined fibrinogen levels influence DNA methylation levels. For both directions of MR, the primary analyses combined evidence from multiple genetic instruments using fixed-effect inverse variance-weighted meta-analysis, as implemented in the R package *TwoSampleMR*. (17) When there was only one genetic instrument available, the Wald ratio test was instead evaluated. For three or more genetic instruments, the inverse variance-weighted meta-analysis was supplemented by sensitivity analyses, including MR Egger, weighted median, and weighted mode. The Bonferroni corrected p-value threshold was calculated using the total number of primary MR analyses performed.

We examined the association between genetically determined DNA methylation levels at each CpG site with fibrinogen levels. For significantly associated and replicated CpG sites from our study, genetic instruments were identified using a publicly available database of methylation quantitative trait loci (meQTLs) from the Genetics of DNA Methylation Consortium (GoDMC). (18) Genetic variants associated with each CpG site at  $p < 0.05$  were distance-pruned to select the most significant variant at each associated locus to use as genetic instruments ( $\pm 1$ Mb). We obtained effect estimates for these instruments on fibrinogen levels from summary statistics of the largest published GWAS of fibrinogen. (6)

We also examined the association between genetically determined fibrinogen levels with DNA methylation levels at each CpG site. We used the largest published fibrinogen GWAS

to identify genetic variants associated with fibrinogen according to the same significance threshold that de Vries et al. applied in their analysis ( $p\text{-value} < 2.5 \times 10^{-8}$ ). (6) Genetic variants associated with fibrinogen were then distance-pruned to select the most significant variant at each associated locus to use as genetic instruments ( $\pm 1\text{Mb}$ ). Betas and standard errors for the association of these instruments with DNA methylation levels at the significantly associated and replicated CpG sites were obtained from association analyses within the ARIC study that are further described in the Supplementary Methods.

### Annotation of associated CpG sites

For the epigenome-wide significant and cross-replicated CpG sites, the nearest genes were identified and annotated using the genomic coordinates provided by Illumina (GRCh37/hg19). They were also checked to see whether any of the probes were cross-reactive, any CpG sites were single nucleotide polymorphisms (SNPs) and whether a SNP exists in the probe binding sites. A CpG site was determined to be polymorphic when a SNP with a minor allele frequency  $> 0.01$  in the 1000 Genomes Project (Phase 1) resided at the position of the cytosine or guanine on either strand, or within 10 base pairs from the CpG within the probe binding site. (19) Additionally, the results were looked up in the EWAS Catalog to gain insights on existing findings on those associations present in the literature. (20) They were also compared to the results from the largest fibrinogen GWAS published (6), and overlapping genetic loci were identified based on distance ( $\pm 1\text{Mb}$ ). The associations of methylation at epigenome-wide significant and cross-replicated CpG sites with gene expression levels, also known as expression quantitative trait methylation sites (eQTM), were identified using published results from the BIOS consortium. (21)

Lastly, to evaluate the correlation structure of the significant and replicated CpG sites, their pairwise Pearson correlation coefficients were calculated within the ARIC study to evaluate difference in correlation between population groups. Correlation coefficients were computed separately in the White and Black population groups, and then combined by computing the sample-size weighted mean correlation coefficient. All 83 significant and replicated CpG sites were ranked by array-specific meta-analysis  $p$ -values in ascending order. Then, any CpGs that were correlated with the top CpG site ( $r^2 > 0.1$ ) were removed iteratively until a set of independent CpG sites were identified.

### Percent Variance Explained

Percent variance explained was calculated in the ARIC White and Black population groups that were included in the meta-analysis and cross-replication. We utilized the identified independent CpG sites based on pairwise Pearson correlation  $R^2$ . Race-stratified linear regression models were used to estimate the percent variance in fibrinogen levels. The null model included fibrinogen levels as the dependent variable, and all of the covariates used in the meta-analysis. The adjusted model additionally included independent CpG sites as independent variables. Then, the difference in adjusted  $R^2$  values for each race was calculated to estimate the percent variance explained by the statistically independent CpG sites.

## Results

### Study characteristics

Descriptive characteristics of studies in the 450K array (n = 12,904) and EPIC array studies (n = 5,133) are described in Table 1. The mean age at fibrinogen measurement ranged from 37.6 years (SD = 10.43) in the Netherlands Twin Register (NTR) to 79.1 years (SD = 0.58) in the LBC 1921. Mean fibrinogen level ranged from 2.74 g/L (SD = 0.66) from the NTR study to 3.88 g/L (SD = 0.87) in the Airwave study. Among all study participants, 58.4% were females (n = 10,531).

### 450K array meta-analysis

An overall summary of the meta-analyses and cross-replication results is shown in Figure 1. A total of 208 CpG sites were significantly associated (Bonferroni corrected p-value <  $1.03 \times 10^{-7}$ ) with fibrinogen levels in the meta-analysis of 450K array data (Table S1, Figure S1). Fibrinogen level was positively associated with 109 CpG sites and negatively associated with 99 CpG sites (Figure 2a).

### EPIC array meta-analysis

In the EPIC array meta-analysis, we identified 87 CpG sites that were significantly associated with fibrinogen (Bonferroni corrected p-value =  $5.78 \times 10^{-8}$ ) (Table S2). A Manhattan plot depicting the associations between all CpG sites and fibrinogen is shown in Figure S2. Fibrinogen was positively associated with 31 CpG sites and negatively associated with 56 CpG sites (Figure 2b).

### Cross replication

Of the 208 CpG sites significantly associated with circulating fibrinogen levels in the 450K array analysis, 196 CpG sites were available on the EPIC array and 78 replicated in the EPIC array meta-analysis results (Table S3). For the significant associations from the EPIC array, out of 87 CpG sites, 33 were available on the 450K array and 26 replicated in the 450K array meta-analysis results (Table S4). When these results were combined, removing 21 overlapping CpG sites, 83 CpG sites remained significant (Table 2). The most significant CpG site from the 450K array, cg18181703 in *SOCS3*, (effect size =  $-0.023$ ; p-value =  $1.07 \times 10^{-32}$ ) was also the strongest association in the EPIC array (effect size =  $-0.034$ , p-value =  $6.32 \times 10^{-24}$ ). When the 450K and EPIC array meta-analyses were combined for a meta-analysis, all 83 significant and cross-replicated CpG sites were significant in this meta-analysis (Table S5).

### Sensitivity analyses

In the setting of the ARIC study, the original model was additionally adjusted for CRP to identify a potential confounding effect of inflammation. The effects of all 83 significantly replicated loci were attenuated after adjustment (mean percent change in effect sizes [SD]: 35.41 [18.30] %) (Table S6, Figure S3–S4). In the sensitivity analysis addressing the time difference in measurements for fibrinogen and DNA methylation, the effect sizes of the 83 significant and replicated results from the main meta-analysis were generally consistent



across three subsets of studies grouped according to the timing of the fibrinogen and DNA methylation measurements: 1) fibrinogen measurement predating DNA methylation measurement, 2) DNA methylation measurement predating fibrinogen measurement and 3) fibrinogen and DNA methylation measured at the same time (Table S7).

Also, in the setting of the ARIC study, we performed a sex-specific analysis, meta-analyzing sex-specific EWAS results for the White and Black population groups in the ARIC study. The effect sizes of the 83 significant and replicated results from the main meta-analysis were generally consistent with the effect sizes of the sex-specific analyses (Table S8). These effect sizes for male and female appear to be highly positively correlated with a Pearson correlation coefficient of 0.9 (Figure S5).

### Bidirectional Mendelian randomization

MR analysis evaluating a causal effect of DNA methylation on fibrinogen levels yielded 25 CpG sites that had one instrument, and 27 CpG sites with two or more instruments (Table S9). A Bonferroni corrected significance threshold of  $9.62 \times 10^{-4}$  ( $0.05/\text{number of CpG sites that had one or more instruments}$ ) was applied. This analysis provided evidence for a causal effect of DNA methylation on fibrinogen levels at only one site: based on two genetic instruments, genetically determined levels of DNA methylation at cg02650017 in *PHOSPHO1* were associated with fibrinogen levels ( $\beta_{\text{InverseVarianceWeighted}} = -0.02$ ,  $p\text{-value} = 8.50 \times 10^{-5}$ ) (Tables S9–S10). Because there were only two genetic instruments available for this CpG site, we were not able to perform sensitivity analyses that require three or more genetic instruments for this association. MR analyses did not provide any evidence for a causal effect of fibrinogen levels on DNA methylation, using 38 genetic instruments, as there were no significant associations between genetically determined fibrinogen levels and DNA methylation at CpG sites (Tables S11–S12).

### Annotation

None of the 83 significant and cross-replicated CpG sites had cross-reactive probes, were a SNP, or harbored a SNP existing in the probe binding sites (Table S5). According to the EWAS Catalog, 16 distinct CpG sites out of the 83 significant and cross-replicated CpG sites identified 69 associations in previous EWAS of various phenotypes including CRP, BMI, smoking and inflammatory bowel disease, among many (Table S13). When the significant and cross-replicated CpG sites were checked against the findings from the fibrinogen GWAS (6), there were 6 CpG sites that were located near four significant loci from the fibrinogen GWAS (Table S14). These included cg24678869 near *IL6R* gene, cg25653947 near *PLEC* gene, cg26663590 near *ATXN2L* gene, and cg09349128 near *SHANK3* gene. We identified 37 CpG sites that were associated with altered gene expression levels of one or more genes in the eQTM database from the BIOS Consortium (Table S15). In 5 cases, DNA methylation and gene expression were positively associated, and in 32 cases, they were inversely associated. There was widespread correlation amongst the significant and replicated CpG sites, as shown in Figure S6, including both positive and negative correlations. After removing correlated CpG sites according to  $r^2 \geq 0.1$ , there were 10 independent CpG sites (cg01059398, cg07830855, cg07573872, cg05316065, cg14162417, cg00607627, cg20692268, cg00138407, cg17501210, and cg19459791) (Table S16). The

10 independent CpG sites identified above, explained 2.56% and 2.41% of the variance of fibrinogen in the White and Black population groups in the ARIC study, respectively.

## Discussion

In this meta-analysis of fibrinogen EWAS, we identified and cross-replicated 83 CpG sites located in or near 70 loci that were significantly associated with circulating fibrinogen levels. After accounting for the correlation structure, there were 10 statistically independent CpG sites. Many of these associations remained significant, but were attenuated when additionally adjusted for CRP levels, suggesting that the associations are at least partially driven by inflammation.

Fibrinogen plays an important role in inflammatory pathways, and it is well-established that fibrinogen expression is upregulated in response to inflammatory stimuli during the acute phase response. Indeed, most inflammatory diseases, including both acute and chronic settings are associated with cytokine-mediated elevation of fibrinogen. (3, 22) Thus, it is indeed expected that the current EWAS would uncover this relationship. Notably, the sensitivity analysis adjusting additionally for CRP showed that the associations for all 83 replicated CpG sites were attenuated after CRP adjustment, although many of them remained nominally significant. Additionally, 59 of the 83 CpG sites that were significantly associated with fibrinogen in our study were also reported in a previous EWAS of CRP. (8) Thus, our findings suggest that this relationship may also be due to methylation events that have an effect on both fibrinogen and inflammation, in addition to previously defined synthetic mechanisms (IL-6, IL-6 receptor and JAK/STAT signaling pathways).

Several of the loci associated with both fibrinogen and CRP harbor important inflammatory genes, including *AIM2*, *BCL3*, and *SOCS3*. DNA methylation at cg10636246 near *AIM2* was inversely associated with fibrinogen levels as well as with *AIM2* mRNA expression. *AIM2* is associated with secretion of IL-18, a proinflammatory cytokine that leads to activation of the STAT3 pathway and upregulation of fibrinogen expression. (23, 24) Another CpG site, cg26470501, was located near the *BCL3* gene. *B-cell leukemia 3 (BCL3)* is a member of the I $\kappa$ B family, and I $\kappa$ B proteins constitute an important element of the NF- $\kappa$ B signal transduction pathway. (25) Specifically, *BCL3* is a nuclear protein that is heavily involved in regulating NF- $\kappa$ B activity. (26) Activation of the NF- $\kappa$ B pathway leads to the production of inflammatory cytokines, such as tumor necrosis factor alpha and IL-1 $\beta$ . IL-1 $\beta$  has been demonstrated to bind to fibrinogen to facilitate inflammatory and hemostatic responses at sites of injury. (27) DNA methylation at this CpG site was inversely associated with both fibrinogen levels and *BCL3* mRNA expression. Since fibrinogen is highly expressed during an acute-phase inflammatory response and activation of the NF- $\kappa$ B pathway, it is biologically plausible that the association between fibrinogen and DNA methylation at this CpG site is driven by inflammation. (3) Hypomethylation at cg18181703, located in the *SOCS3* gene, was associated with increased fibrinogen levels as well as increased *SOCS3* mRNA expression in whole blood. *SOCS3* suppresses proinflammatory cytokine signaling by downregulating STAT3, which upregulates the expression of fibrinogen and other acute-phase proteins. (28, 29) Thus, based on this known biology, we would expect hypomethylation of *SOCS3* to be associated with decreased, not

increased levels of fibrinogen. It is unclear why hypomethylation of *SOCS3* was associated with increased levels of fibrinogen in our study, but it is possible that this can be explained by reverse causation, or a third variable associated with both *SOCS3* methylation and fibrinogen. Notably, hypomethylation at this CpG site was also associated with increased CRP levels, (8) and CpG sites near *SOCS3* have been reported in association with several other phenotypes related to inflammation, including BMI (12, 30–32), inflammatory bowel disease (33) including Crohn's disease (34), and smoking (35–38).

Our MR found that genetically determined DNA methylation at cg02650017 was associated with fibrinogen levels. In addition, meQTLs for cg02650017 were associated with expression of *PHOSPHO1*, indicating that *PHOSPHO1* may regulate fibrinogen levels. *PHOSPHO1* has an established function in biomineralization, (39, 40) and genomic association studies also suggest a role in glycemia. Variants in *PHOSPHO1* are associated with fasting insulin levels, and DNA methylation at *PHOSPHO1* is associated with decreased risk of future type 2 diabetes mellitus. (41, 42) Furthermore, DNA methylation levels at this CpG site was shown to be associated with levels of coagulation factor VIII, a co-factor for factor IX-mediated activation of factor X, which leads to production of thrombin and ultimately, conversion of fibrinogen to fibrin, in the Jackson Heart Study. (2, 43) The association of methylation at *PHOSPHO1* with both fibrinogen and factor VIII suggests that this locus may regulate coagulation more broadly.

Our study had several strengths. First, the use of a large consortium-based study population, allowed for the identification of more generalizable associations. While different ethnic compositions may have attributed to decreased replication rate, this conversely also implies that the CpG sites that cross-replicated were more generalizable across different population groups. Second, our use of a cross-replication approach ensures the robustness of the 83 reported associations.

This study also had limitations. First, not all included studies used concurrent blood samples for the measurement of fibrinogen and DNA methylation. Our sensitivity analysis found consistent results regardless of temporal differences, suggesting the impact of this limitation is likely to be minor. Second, DNA methylation levels are tissue-specific, and we only measured DNA methylation in circulating leukocytes. Consequently, our study was not designed to identify associations with CpG sites where DNA methylation in other tissues regulates fibrinogen levels, such as in liver where fibrinogen is produced. Third, although we adjusted for cell type proportions in our analyses, there may be residual confounding due to cell subtype proportion heterogeneity. (44, 45) Fourth, we had limited statistical power to assess the association between genetically determined fibrinogen levels and DNA methylation in our MR analyses. The 38 genetic instruments for fibrinogen only explain approximately 3% of the variance in fibrinogen levels (6), and our sample size to assess the association of these instruments with DNA methylation was relatively modest. Finally, the estimates of the variance in fibrinogen levels explained by the 10 statistically independent CpG sites may be overestimated because the ARIC study that was used for this analysis was also included in our EWAS.

In conclusion, we performed the first meta-analysis of EWAS of circulating fibrinogen levels, using multi-ethnic data from participants in a large consortium of epidemiological cohort studies. We identified 83 CpG sites where circulating leukocyte DNA methylation is robustly associated with circulating fibrinogen levels. Inflammation appears to play an important role in the relationship between DNA methylation and fibrinogen levels. These observations support the need to define the complex interplay between fibrinogen and inflammatory pathways, and to functionally and causally connect DNA methylation and fibrinogen levels.

## Supplementary Material

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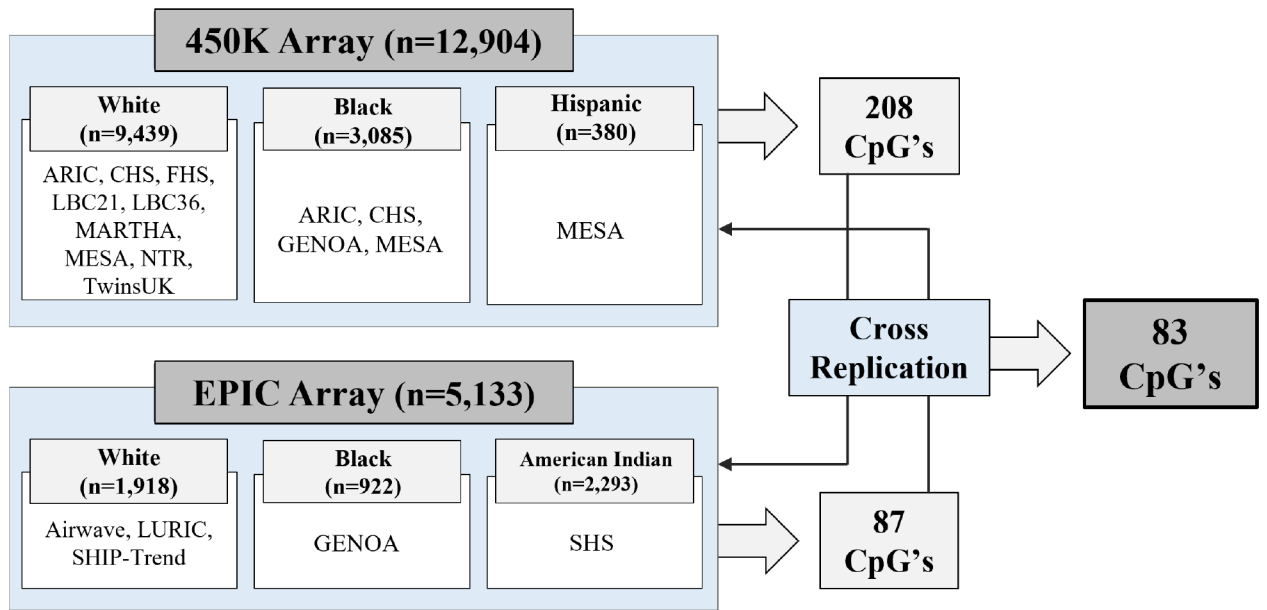
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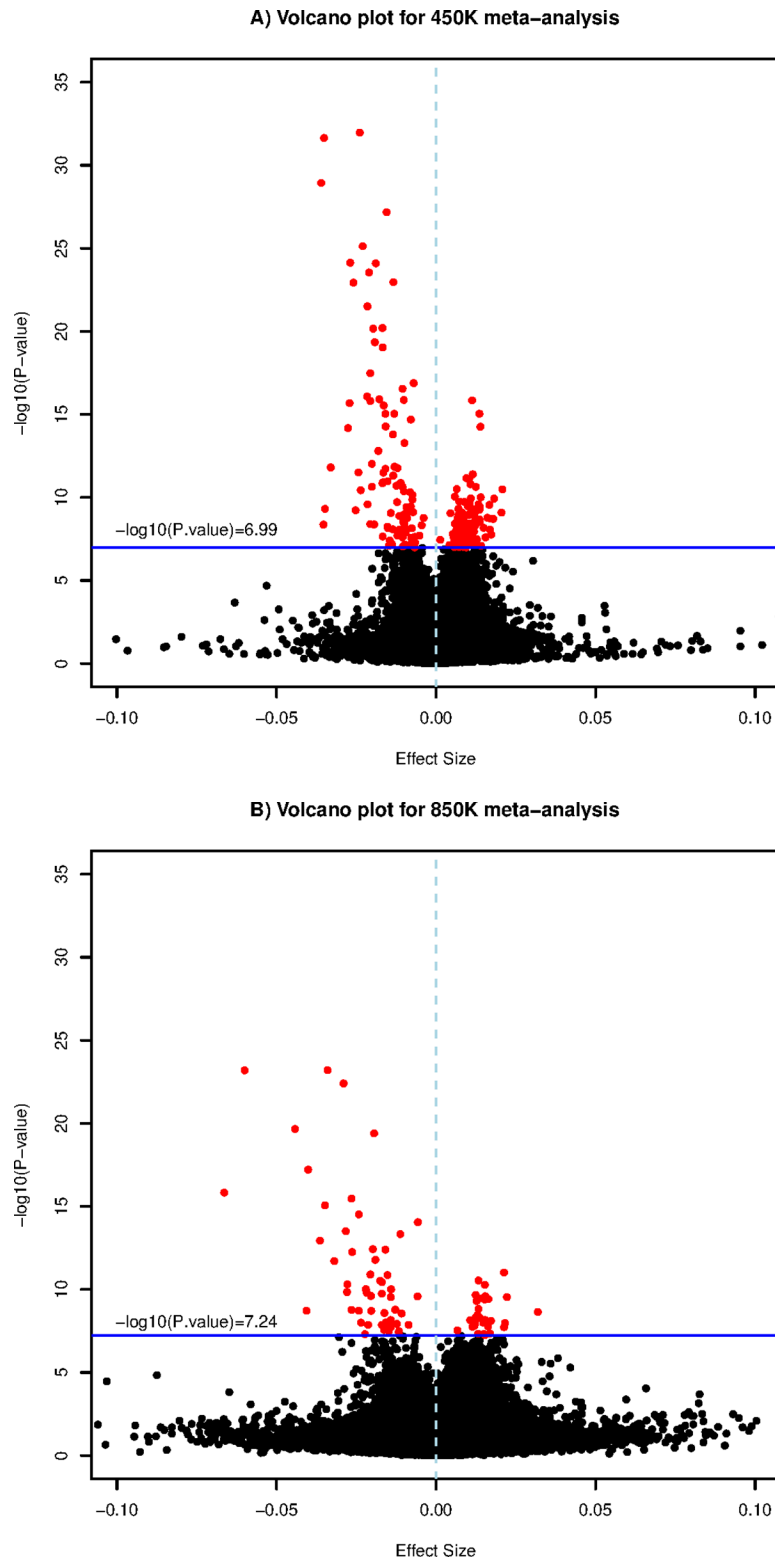
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### Essentials

- Circulating fibrinogen levels may be determined by inter-individual differences in DNA methylation at CpG sites, and vice versa.
- An epigenome-wide association study of circulating fibrinogen levels in 18,037 participants was performed.
- There were 83 replicated CpG sites located in 61 loci that were associated with circulating fibrinogen levels.
- Associations found in the analyses are partially driven by inflammatory pathways shared by both fibrinogen and C-reactive protein.



**Figure 1.** Summary of the analysis approach and findings of the array-specific meta-analyses and cross-replication



**Figure 2.**

Volcano plots of A) 450K and B) EPIC array meta-analyses showing the effect size and p values of the included cytosine-phosphate-guanine sites. The horizontal blue line indicates the significance threshold.

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**Table 1.**

Characteristics of the 450K and EPIC array studies

Study	Race*	N	Sex (% Female)	Smoking (% Current smoker)	Age (Mean, SD)		BMI, kg/m <sup>2</sup> (Mean, SD)		Fibrinogen, g/L (Mean, SD)	
					Mean	SD	Mean	SD	Mean	SD
<b>450K array (n = 12,904)</b>										
ARIC	Black	2271	62.97%	28.80%	53.25	5.72	29.72	6.05	3.19	0.66
ARIC	White	753	60.96%	22.21%	55.69	5.14	25.81	4.32	2.93	0.58
CHS	Black	312	61.50%	16.70%	73.1	5.36	28.5	5.06	3.38	0.71
CHS	White	368	60.50%	0.92%	75	4.98	26.78	4.87	3.27	0.71
FHS_gen3	White	1443	52.18%	14.94%	45.39	7.9	27.72	5.62	3.34	0.65
FHS_umn	White	1820	60.16%	23.68%	65.31	8.75	28.05	5.29	3.71	0.67
GENOA_450K	Black	271	71.59%	10.33%	66.79	6.54	31.4	6.56	3.73	0.84
LBC21	White	407	60.14%	7.11%	79.14	0.58	26.2	4.06	3.6	0.6
LBC36	White	872	49.55%	11.35%	69.59	0.83	27.76	4.38	3.57	0.84
MARTHA	White	336	79.00%	28.00%	43.69	14.07	24.24	4.45	3.33	0.64
MESA	Black	231	56.28%	16.59%	60.62	9.14	30.92	5.75	3.61	0.79
MESA	White	574	47.91%	12.20%	60.99	9.6	28.33	4.95	3.34	0.62
MESA	Hispanic	380	49.47%	13.42%	58.81	9.29	29.46	4.87	3.54	0.68
NTR	White	2796	66.20%	21.10%	37.6	12.7	24.3	4	2.74	0.66
TwinsUK	White	70	100%	10%	55.73	10.43	24.93	3.63	3.16	0.8
<b>EPIC array (n = 5,133)</b>										
Airwave	White	894	41.83%	9.17%	41.05	9.21	27.17	4.42	3.881	0.865
GENOA_EPIC	Black	922	71.27%	12.63%	62.71	9.63	31.8	6.72	3.68	0.81
LURIC	White	829	29.60%	19.80%	62.6	10.5	27.5	4.05	3.77	0.55
SHIP-Trend	White	195	57.44%	20%	48.2	12.48	26.89	4.05	2.94	0.68
SHS	AI	2293	58.48%	38.33%	56.19	8.1	30.31	6.1	2.92	0.75

\* AI: American Indian

**Table 2.**

Significant and cross-replicated DNA methylation sites associated with circulating fibrinogen levels

CpG site	Chr	Position	Effect size (450K)	P-value (450K)	Effect size (EPIC)	P-value (EPIC)	Gene
cg18181703	17	76354621	-0.0239	1.07E-32	-0.034	6.32E-24	<i>SOCS3</i>
cg16936953	17	57915665	-0.0351	2.26E-32	-0.0364	1.16E-13	<i>VMP1</i>
cg12054453	17	57915717	-0.036	1.16E-29	-0.0401	6.14E-18	<i>VMP1</i>
cg09349128	22	50327986	-0.0155	6.79E-28	-0.0194	4.02E-20	<i>CRELD2</i>
cg17501210	6	166970252	-0.023	7.47E-26	-0.0265	3.40E-16	<i>RPS6KA2</i>
cg18942579	17	57915773	-0.0269	7.42E-25	-0.0319	1.95E-12	<i>VMP1</i>
cg18608055	19	1130866	-0.0189	8.08E-25	-0.0157	9.75E-09	<i>SBNO2</i>
cg12992827	3	101901234	-0.021	2.86E-24	-0.0217	1.59E-10	<i>ZPLD1</i>
cg12170787	19	1130965	-0.0134	1.09E-23	-0.0103	6.66E-08	<i>SBNO2</i>
cg07573872	19	1126342	-0.0259	1.17E-23	-0.0198	3.74E-13	<i>SBNO2</i>
cg01409343	17	57915740	-0.0215	3.15E-22	-0.0192	2.70E-07	<i>VMP1</i>
cg02716826	9	33447032	-0.0168	6.29E-21	-0.0143	5.12E-07	<i>AQP3</i>
cg25325512	6	37142220	-0.0197	6.90E-21	-0.0235	9.96E-09	<i>PIM1</i>
cg10552523	11	313478	-0.0192	4.52E-20	-0.0203	1.95E-09	<i>IFITM2</i>
cg01971407	11	313624	-0.0167	9.27E-20	-0.0146	4.72E-06	<i>IFITM2</i>
cg10636246	1	159046973	-0.0206	3.32E-18	-0.0263	5.60E-13	<i>AIM2</i>
cg27469606	19	1154485	-0.007	1.31E-17	-0.0041	1.30E-04	<i>SBNO2</i>
cg18513344	3	195531298	-0.0105	2.87E-17	-0.0108	2.80E-09	<i>MUC4</i>
cg01059398	3	172235808	-0.0178	1.23E-16	-0.022	9.62E-11	<i>TNFSF10</i>
cg10508317	17	76355146	-0.0101	1.36E-16	-0.0058	4.32E-06	<i>SOCS3</i>
cg26804423	7	8201134	0.0113	1.43E-16	0.0101	6.08E-06	<i>ICA1</i>
cg23570810	11	315102	-0.0206	1.57E-16	-0.018	6.43E-05	<i>IFITM2</i>
cg20566897	11	313527	-0.0164	2.88E-16	-0.017	2.05E-04	<i>IFITM2</i>
cg01101459	1	234871477	0.0136	9.14E-16	0.0137	3.52E-10	<i>LINC01132</i>
cg19821297	19	12890029	-0.0159	9.20E-16	-0.0206	1.25E-11	<i>HOOK2</i>
cg26470501	19	45252955	-0.0131	9.23E-16	-0.0154	2.40E-08	<i>BCL3</i>
cg06051311	6	30131001	-0.0158	5.34E-15	-0.008	2.08E-04	<i>TRIM15</i>
cg10472711	7	797592	-0.0135	1.62E-14	-0.0119	7.39E-05	<i>HEATR2</i>
cg13781414	9	138951648	-0.0099	5.28E-14	-0.0106	6.43E-08	<i>NACC2</i>
cg27209729	11	64428925	-0.0181	1.58E-13	-0.0218	1.17E-06	<i>NRXN2</i>
cg00159243	12	109023799	-0.013	1.42E-12	-0.0159	4.04E-13	<i>SELPLG</i>
cg14162417	1	226315857	-0.0121	1.71E-12	-0.0125	1.38E-05	<i>RP11-396C23.3</i>
cg20995564	2	145172035	-0.0165	3.19E-12	-0.0217	6.43E-07	<i>ZEB2</i>
cg19459791	15	65363022	0.0115	4.06E-12	0.0154	2.55E-10	<i>RASL12</i>
cg18860310	4	87752504	-0.0111	1.34E-11	-0.0138	1.41E-06	<i>SLC10A6</i>
cg05316065	8	130799007	-0.0105	2.25E-11	-0.0102	1.92E-04	<i>GSDMC</i>
cg04326337	20	36664969	0.0208	3.24E-11	0.0127	4.16E-05	<i>RPRD1B</i>

CpG site	Chr	Position	Effect size (450K)	P-value (450K)	Effect size (EPIC)	P-value (EPIC)	Gene
cg24859433	6	30720203	-0.0081	4.84E-11	-0.007	1.01E-06	<i>RN7SL353P</i>
cg11032810	4	141057042	-0.0074	6.97E-11	-0.0075	2.08E-05	<i>MAML3</i>
cg12728588	1	36025489	0.0059	8.89E-11	0.0064	1.42E-04	<i>NCDN</i>
cg02112168	14	45579561	0.014	9.75E-11	0.0121	6.55E-05	<i>PRPF39</i>
cg15342087	6	30720209	-0.0074	1.32E-10	-0.0051	2.46E-05	<i>RN7SL353P</i>
cg15310871	8	20077936	0.0088	1.85E-10	0.0124	2.13E-10	<i>ATP6V1B2</i>
cg16896911	19	33895690	0.0105	2.07E-10	0.0138	2.52E-06	<i>PEPD</i>
cg22959742	10	13913931	0.0133	2.64E-10	0.0132	1.08E-04	<i>FRMD4A</i>
cg03940776	6	158490013	-0.0079	3.47E-10	-0.0084	4.63E-06	<i>SYNJ2</i>
cg03998636	13	111210121	-0.0093	3.87E-10	-0.0113	1.98E-06	<i>RAB20</i>
cg18217136	20	36157651	0.007	4.91E-10	0.0078	1.22E-05	<i>PPIAP3</i>
cg23866916	19	1155738	-0.008	5.21E-10	-0.0077	1.25E-04	<i>SBN02</i>
cg26663590	16	28959310	0.0124	6.69E-10	0.0159	1.47E-06	<i>NFATC2IP</i>
cg06192883	15	52554171	0.0108	8.05E-10	0.0126	6.87E-06	<i>MYO5C</i>
cg04523589	3	48265146	0.0082	8.41E-10	0.0091	1.27E-05	<i>CAMP</i>
cg24678869	1	153919638	0.007	1.16E-09	0.0073	3.68E-05	<i>DENND4B</i>
cg10967866	10	134362164	0.012	1.34E-09	0.014	8.95E-06	<i>INPP5A</i>
cg02650017	17	47301614	-0.0039	1.71E-09	-0.0057	8.90E-15	<i>PHOSPHO1</i>
cg19769147	14	105860954	0.0075	2.52E-09	0.0083	3.84E-05	<i>PACS2</i>
cg07830855	6	56524142	0.009	3.21E-09	0.0098	8.91E-05	<i>DST</i>
cg14472551	11	33563060	0.0113	3.58E-09	0.0109	1.27E-04	<i>KIAA1549L</i>
cg17953136	2	20232577	0.0105	3.75E-09	0.0123	8.71E-05	<i>LAPTMA4</i>
cg19053223	7	105359070	0.0059	3.78E-09	0.0055	1.22E-04	<i>ATXN7L1</i>
cg01581098	7	105754353	0.0091	4.48E-09	0.0092	5.72E-05	<i>SYPL1</i>
cg05279866	8	37378355	0.0087	5.62E-09	0.0107	8.12E-06	<i>RP11-150O12.1</i>
cg00138407	3	47386505	0.0094	7.14E-09	0.0105	1.54E-04	<i>KLHL18</i>
cg20692268	1	25358981	-0.0115	8.60E-09	-0.0121	1.23E-04	<i>MIR4425</i>
cg02508743	8	56903623	0.0113	9.79E-09	0.0127	1.19E-05	<i>LYN</i>
cg15901722	5	175974973	-0.0105	1.06E-08	-0.0105	4.92E-06	<i>CDHR2</i>
cg17736772	1	155246828	0.007	1.41E-08	0.0081	1.22E-04	<i>CLK2</i>
cg04232816	2	96905390	0.0073	1.47E-08	0.0084	1.08E-04	<i>STARD7-AS1</i>
cg12269535	6	43142014	-0.0103	1.57E-08	-0.0132	6.91E-05	<i>SRF</i>
cg25653947	8	144443217	0.0071	1.68E-08	0.0106	1.74E-06	<i>TOP1MT</i>
cg25953130	10	63753550	-0.0167	2.25E-08	-0.0214	3.61E-05	<i>ARID5B</i>
cg27307975	15	58797839	-0.0066	2.34E-08	-0.0069	6.88E-07	<i>LIPC</i>
cg05304729	1	158800024	0.0117	3.86E-08	0.0118	5.51E-05	<i>MNDA</i>
cg23966214	17	48203188	-0.0066	5.19E-08	-0.0055	2.66E-05	<i>SAMD14</i>
cg05166473	16	88103629	0.0075	5.91E-08	0.0087	9.75E-06	<i>BANP</i>
cg13522406	6	30581820	0.0072	9.63E-08	0.0154	7.02E-07	<i>PPP1R10</i>



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cg19137806	10	134362170	0.0094	1.01E-07	0.0126	4.10E-06	<i>INPP5A</i>
cg00607627	16	28995994	-0.0067	1.01E-07	-0.0074	3.32E-05	<i>RPI1-264B17.3</i>
cg07719512	2	219246576	0.0059	4.97E-07	0.0107	7.04E-09	<i>SLC11A1</i>
cg25739715	22	30663881	-0.0038	1.95E-06	-0.0058	2.62E-10	<i>OSM</i>
cg25114611	6	35696870	-0.0075	7.10E-06	-0.0116	3.30E-08	<i>FKBP5</i>
cg22460173	19	6536042	0.0055	4.49E-05	0.0122	1.17E-08	<i>TNFSF9</i>
cg23842572	17	17030253	0.0061	4.50E-05	0.013	3.43E-09	<i>MPRIP</i>

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