Atherosclerosis Risk in Communities

Study participants

The Atherosclerosis Risk in Communities (ARIC) Study is a prospective cohort study of cardiovascular disease risk in 15,792 men and women. Participants aged 45-64 were enrolled from Forsyth County, NC; Jackson, MS (African Americans only); suburban Minneapolis, MN; and Washington County, MD and underwent a baseline clinical examination (Visit 1, 1987-89). Four subsequent follow-up exams (Visits 2-5, through 2013) have occurred. The present analysis is restricted to African Americans from Jackson and Forsyth County with stored blood samples available for methylation quantification from either Visit 2 (1990-92) or Visit 3 (1993-95). The study protocol was approved by the institutional review board of each participating university, and each participant provided written informed consent.

Hemostatic factors and covariates

Blood was drawn after an 8-hour fast and plasma samples were stored at -70°C until the time of analysis in a central laboratory. Fibrinogen, factor VIII, factor VII, von Willebrand factor (vWF), and activated partial thromboplastin time (aPTT) were each measured using stored plasma from Visit 1, and D-dimer was measured using stored plasma from Visit 3, as previously described. Only those with methylation quantification using Visit 3 blood samples were included in the D-dimer analyses.

Height and weight were measured by trained technicians with participants in scrub suits and without shoes. Body mass index (BMI) was calculated as weight (in kg) divided by height (in m) squared. Participants self-reported their smoking and physical activity behaviors. The BMI and smoking measures used in analysis were taken from the same exam from which stored blood was used for methylation quantification, while physical activity was from Visit 1.

DNA methylation quantification & epigenetic age calculation

Genomic DNA was extracted from peripheral blood leukocyte samples using Gentra Puregene Blood Kit (Qiagen; Valencia, CA) according to the manufacturer’s instructions (www.qiagen.com). Bisulfite conversion of 1 μg genomic DNA was performed using the EZ-96 DNA Methylation Kit (Deep Well Format) (Zymo Research; Irvine, CA) according to the manufacturer’s instructions (www.zymoresearch.com). Bisulfite conversion efficiency was determined by PCR amplification of the converted DNA before proceeding with methylation analyses on the Illumina platform using Zymo Research’s Universal Methylated Human DNA
Standard and Control Primers. The Illumina Infinium HumanMethylation450K Beadchip array (HM450K) was used to measure DNA methylation (Illumina, Inc.; San Diego, CA). Background subtraction was conducted with the GenomeStudio software using built-in negative control bead types on the array. Positive and negative controls and sample replicates were included on each 96-well plate assayed. After exclusion of controls, replicates, and samples with integrity issues or failed bisulfite conversion, a total of 2841 study participants had HM450K data available for further QC analyses. We removed poor-quality samples with pass rate of <99%, that is, if the sample had at least 1% of CpG sites with detection P-value > 0.01 or missing, indicative of lower DNA quality or incomplete bisulfite conversion, and samples with a possible gender mismatch based on evaluation of selected CpG sites on the Y chromosome. Additional details have been published elsewhere. 4,5

Average normalized methylation data was input into the Horvath online age calculator (http://labs.genetics.ucla.edu/horvath/dnamage/).6 Samples were excluded if there was a mismatch of self-reported sex and the methylation predicted sex by the Horvath calculator or if a participant had no hemostatic measures available. The Horvath calculator did not predict any tissue type other than peripheral blood, and no samples had low correlation with “gold standard” probes used for epigenetic age calculation. After exclusions, there were a maximum of 2,742 ARIC participants available for analysis.

**Advanced Study of Aortic Pathology**

**Study Participants**

Tissue biopsies from aneurysmal ascending aorta were collected from the Advanced Study of Aortic Pathology (ASAP) biobank. The ASAP study includes 600 patients undergoing elective open-heart surgery for aortic valve and/or ascending aortic disease at the Karolinska University Hospital, Stockholm, Sweden. Patients with syndromic aortic pathologies and/or significant coronary artery disease (according to angiography) were excluded. A detailed description of the study population is presented elsewhere.7 Demographic variables, as well as information on current and past smoking behavior and physical activity was collected via questionnaires at the time of exam prior to the surgery. Peripheral blood was collected for biochemical measurements. The study was approved by the Human Research Ethics Committee at Karolinska Institutet (application number 2006/784-31/1), Stockholm, Sweden. Written informed consent was obtained
from all patients according to the declaration of Helsinki. Methods were carried out in accordance with relevant guidelines.

Biopsies were taken from the anterior part of the ascending aorta, at the site of aortotomy a few cm above the aortic valve. Biopsies used for DNA isolation were snap frozen on dry ice at the site of surgery and stored at -80°C pending extraction; biopsies used for RNA isolation was stored in RNAlater RNA Stabilization Reagent (QIAGEN) prior to extraction. RNA and DNA was extracted from the intima-media portion of aneurysmal aorta (n=22).

**DNA methylation quantification & epigenetic age calculation**

Genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN), according to manufacturer’s instructions, and quantified using NanoDrop ND-1000 (NanoDrop Technologies). For each sample, 500 ng of DNA was bisulfite converted using EZ-96 DNA Methylation™ Kit (ZYMO Research, Orange, CA) according to manufacturer’s recommendations. Genome-wide DNA methylation was measured using Infinium HumanMethylation450 BeadChip Array (Illumina Inc.). All samples were converted, fragmented, hybridized to array and further processed at the core facility for Bioinformatics and Expression Analysis at the Department of Biosciences and Nutrition, Novum, Karolinska Institutet Huddinge, Stockholm, Sweden, according to manufacturer’s recommendations. To avoid sampling bias, samples were randomized prior to conversion and subsequent analysis. Raw methylation levels (beta values) were calculated and extracted by Illumina GenomeStudio® software. Preprocessing was performed using R (2.13.0) scripts including packages lumi8 and methylumi9. For the quality control purpose, only CpG sites that are located on autosomes are selected; probes with low detection p-value, probes measuring SNPs and non-CpG probes were excluded from the analyses.

**Gene Expression**

RNA was isolated using RNeasy Mini Kit (QIAGEN), according to manufacturer’s instructions, and quantified using NanoDrop ND-1000 (NanoDrop Technologies). RNA quality was assessed by 2100 Bioanalyzer using the RNA 6000 Nano Lab Chip (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiling was performed using Affymetrix GeneChip® Human Exon 1.0 ST arrays and protocols, as previously described. All expression measurements were RMA normalized and log2 transformed.

**Acknowledgements**
The work was supported by the Swedish Research Council [12660]; the Swedish Heart-Lung Foundation [201202729]; the Leducq Foundation [MIBAVA, 12CVD03]; Fundació La Marató de TV3 [20151332]; donation by Fredrik Lundberg.

**Cardiovascular Health Study**

**Study Participants**

The Cardiovascular Health Study is a population-based cohort study of risk factors for coronary heart disease and stroke in adults ≥65 years conducted across four field centers. The original predominantly European ancestry cohort of 5,201 persons was recruited in 1989-1990 from random samples of the Medicare eligibility list. In 1992-1993, an additional predominantly African-American cohort of 687 persons was enrolled. This analysis includes subjects from a randomly selected subset of 329 African-American participants and 336 participants of European ancestry who participated in the 3rd annual follow-up visit (study year 5) and had DNA available from that visit. European-ancestry participants had no baseline history of coronary vascular disease (defined as coronary heart disease, heart failure, peripheral vascular disease, valvular heart disease, stroke, or transient ischemic attack).

CHS was approved by institutional review committees at each field center and individuals in the present analysis had available DNA and gave informed consent including consent to use of genetic information for the study of cardiovascular disease.

**DNA methylation quantification & epigenetic age calculation**

Methylation was assessed for all selected participants using DNA collected at study year 5. Methylation measurements were performed at the Institute for Translational Genomics and Population Sciences at the Harbor-UCLA Medical Center Institute for Translational Genomics and Population Sciences using the Infinium HumanMethylation450 BeadChip (Illumina Inc, San Diego, CA). Quality control was performed in the minfi R package (version 1.12.0, http://www.bioconductor.org/packages/release/bioc/html/minfi.html). Samples with low median intensities of below 10.5 (log2) across the methylated and unmethylated channels, samples with a proportion of probes failing detection of greater than 0.5%, samples with QC probes falling greater than 3 standard deviation from the mean, sex-check mismatches, failed concordance with prior genotyping, or > 0.5% of probes with a detection p-value > 0.01 were
removed. In total, 11 samples were removed for sample QC resulting in a sample of 323 European-ancestry and 326 African-American samples. Methylation values were normalized using the SWAN quantile normalization method. Since white blood cell proportions were not directly measured in CHS they were estimated from the methylation data using the Houseman method. Epigenetic age measures were estimated using the Horvath online calculator.

**Hemostatic factors and covariates**

Blood was drawn after an 8 to 12 hour fast and processed according to protocol. Plasma fibrinogen levels were measured by the Clauss method with Dade fibrinogen calibration reference (Baxte-Dade, Bedford, MA) and bovine thrombin (Parke-Davis, Lititz, PA). Factor VII and factor VIII levels were measured on the Coag-A-Mate X2 (Organon-Teknika, Durham, NC). D-dimer was measured in SCAT-1 plasma by ELISA using 2 monoclonal antibodies directed against non-overlapping antigenic determinants. The assay detects D-dimer from cross-linked fibrin but not D-monomer.

Fibrinogen and factor VII levels were measured at the same visit as the methylation measures, as were D-dimer levels for many participants. Factor VIII levels were only available from baseline, and some participants only had D-dimer levels available from baseline. When hemostatic measures and methylation were determined at different time points, an additional term for difference in age at methylation and age at hemostatic measure was included in the models. All other covariates were assessed at the same visit as the methylation measures.

**Acknowledgements**

Infrastructure for the CHARGE Consortium is supported in part by the National Heart, Lung, and Blood Institute grant R01HL105756. The CHS research was supported by NHLBI contracts HHSN268201200036C, HHSN26820080007C, N01HC55222, N01HC85079, N01HC85080, N01HC85081, N01HC85082, N01HC85083, N01HC85086; and NHLBI grants U01HL080295, U01HL130114, K08HL116640, R01HL087652, R01HL092111, R01HL103612, R01HL105756, R01HL103612, R01HL111089, R01HL116747 and R01HL120393 with additional contribution from the National Institute of Neurological Disorders and Stroke (NINDS). Additional support was provided through R01AG023629 from the National Institute on Aging (NIA), Merck Foundation / Society of Epidemiologic Research as well as Laughlin Family, Alpha Phi
Foundation, and Locke Charitable Foundation. A full list of principal CHS investigators and institutions can be found at CHS-NHLBI.org. The provision of genotyping data was supported in part by the National Center for Advancing Translational Sciences, CTSI grant UL1TR000124, and the National Institute of Diabetes and Digestive and Kidney Disease Diabetes Research Center (DRC) grant DK063491 to the Southern California Diabetes Endocrinology Research Center.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**French-Canadian Family study on Factor V Leiden**

**Study Participants**

The French-Canadian Family study on Factor V Leiden (F5L) included 369 individuals from five multi-generational families. In total, 255 subjects consented to participate in the study. The study was originally designed to investigate genetic determinants of venous thromboembolism (VT) endophenotypes, and we recently expanded its scope to include the investigation of epigenetic determinants. The families were identified through the Thrombosis Clinic of the Ottawa Hospital between 2005 and 2006, and probands were ascertained on objectively diagnosed, idiopathic VT (i.e. VT in the absence of cancer, myeloproliferative disease, pregnancy, puerperium, prolonged immobilization, trauma, surgery, antiphospholipid syndrome, and inherited thrombophilia) and heterozygote for the F5L mutation. All probands reported to be of French-Canadian origin and were free of rare genetic risk factors, including anti-thrombin, protein C, and protein S deficiencies, and homozygosity for the Factor V Leiden and Factor II G20210A mutations. Participants completed an interviewer-administered questionnaire on their personal and medical information. Subjects self-reported their height and weight, and BMI as calculated as body mass divided by the square of body height. Ethics approval was obtained from the Ottawa Hospital and the University of Toronto ethics boards.

**DNA methylation quantification & epigenetic age calculation**

DNA was extracted from peripheral blood using a salting out procedure adapted from. Bisulphite conversion and DNA methylation measurements were performed at The Center for Applied Genomics, Toronto, Canada in 227 subjects from F5L family study and in 350 randomly selected subjects from the MARTHA study as described elsewhere. Eleven duplicated samples
from both studies were included for quality control. The DNA samples from the F5L and MARTHA studies were randomly mixed by plate and batch to minimize potential technical biases between the study samples. Bisulphite conversion was performed on 1 µg genomic DNA for each sample using the Qiagen EpiTect 96 Bisulfite Kit and 200 ng of bisulfite-converted DNA at 50 ng/µl was independently amplified, labeled and hybridized to Infinium HumanMethylation450 BeadChip microarrays. For each sample, the intensities of the methylated and unmethylated signals were measured at 485,577 CpG sites using the Illumina iScan (with default settings). The methylation level at individual CpG sites was reported as the β-value, i.e. the ratio of the methylated probe intensity to the overall intensity (sum of methylated and unmethylated probe intensities).

Methylation results from the F5L family and MARTHA studies were merged for quality control and data normalization as previously described. The data were normalized using SWAN, Noob and a dye bias adjustment method proposed by Illumina. Individual outliers were detected based on a principal component analysis, and four individuals from the F5L family study were excluded. Probe quality was assessed by the detection P-value as defined in the minfi R-package and low detection P-values indicated that the probe signal differed from the background signal. We excluded 6033 probes with detection P-value > 0.05 in more than 5% of the samples, as well as 66,877 polymorphic and 30,969 cross-reactive probes identified by Chen et al. The final dataset included 378,594 autosomal probes were included.

Epigenetic age measures were estimated using the Horvath online calculator. Raw methylation values were provided to the website. One sample was discarded due to the low correlation with “gold standard”. We observed an agreement with the estimated tissue type for all the samples. Since we observed from the principal component analysis that samples were clearly clustered by sex, the data was not filtered based on the estimated sex. In total, 217 individuals were included in the final dataset.

**Hemostatic factors**

Partial thromboplastin time (aPTT, in seconds) and plasma levels of FVII (in U/mL) and FVIII (in U/mL) activity were measured by a clotting assay on the BCS instrument (Siemens Diagnostics, Marburg Germany) and vWF antigen (in U/mL) was measured with a commercially available ELISA kit from Diagnostica Stago. PAI-1 levels (in IU/mL) were measured with the
Spectrolyse (pL) PAI kit (Biopool distributed by ESBE, Toronto, ON). Fibrinogen levels (in g/L) were determined on a STA compact analyzer using STA Fibrinogen 5.

**Framingham Heart Study**

*Study Participants*

The Framingham Heart Study is a population-based, prospective study that began in 1948 with the recruitment of an original cohort of 5,209 men and women from Framingham, MA. The FHS Offspring cohort was recruited in 1971, including 5,124 offspring and spouses of offspring of the FHS Original cohort. Participants underwent examinations every four years (except eight years between the first and the second examinations) to collect demographic and clinical measures and medical history. The Generation 3 cohort (Gen3) was recruited starting in 2002 and includes 4,095 adults having at least one parent in the Offspring Cohort. Gen3 participants have undergone two examination cycles with the third currently underway. DNA methylation was measured in 2,846 Offspring cohort participants who attended the eighth examination cycle from 2005-2008 and 1,522 Gen3 participants who attended the second examination cycle from 2009-2011. All participants included in this study provided written consent for genetic research.

**DNA methylation quantification & epigenetic age calculation**

DNA methylation was measured on 2,846 Offspring and 1,522 Gen3 participants who gave consent for genetic studies. Peripheral whole blood samples were collected from these participants at the eighth examination. Buffy coat fractions were obtained and genomic DNA was extracted using the Gentra Puregene DNA extraction kit (Qiagen, Venlo, Netherlands). Bisulfite conversion of genomic DNA was performed with the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). After whole genome amplification, fragmentation, array hybridization, and single-base pair extension, DNA methylation was quantified in two laboratories for the Offspring cohort and a third laboratory for Gen3. The first laboratory analyzed 576 samples that were previously selected for a cardiovascular disease (CVD) case-control study. The second laboratory analyzed 2,270 samples from the remainder of the Offspring cohort.

Due to possible laboratory effects on methylation arrays, data was processed within each laboratory using the DASEN methodology implemented in the `wateRmelon` R package (version 3.0.2, http://www.bioconductor.org/packages/release/bioc/html/wateRmelon.html). The first step of data processing was to adjust for methylated and unmethylated fluorescent intensities.
(M and U) and technical variations. The second step was to perform quantile normalization of the M and U values with consideration of two types of probe technologies. Beta values were then derived as the ratio of methylated probe intensity to the overall intensity. For quality control purposes, we retrieved overlapped SNPs (Number of SNPs=65) from previous genotyping effort. We excluded samples with a probe missing rate >1% (n=45), poor SNP matching to the 65 SNP control probe locations (n=79), and outliers by multi-dimensional scaling techniques (n=73). At the probe level, we excluded those with missing rate >20% at p<0.01 (n=466 from S1 1 and n=366 from S2), as well as probes previously identified to map to multiple locations or have an underlying SNP (minor allele frequency >5% in European ancestry (EUR) 1000 genomes project data) at the CpG site or <=10 bp of the single base extension (n=42,251).

Epigenetic age measures were calculated using the Horvath online calculator (https://dnamage.genetics.ucla.edu/) 6. As the online calculator performs an internal normalization the raw methylation values for Gen3 were used. As the online calculator provides back several quality control statistics we used these to filter potentially lower quality samples. In particular, we filtered any samples with sex mismatches, low correlation with “gold standard” probes used for the epigenetic age calculation development, and any samples whose estimated tissue type was not peripheral blood. This filtering left 2,654 samples for analysis in FHS Offspring and 1,522 samples in FHS Gen3 for analysis.

**Hemostatic factors**

Plasma fibrinogen levels were measured using the Clauss method 16. ELISA was used to assess plasma PAI-1 antigen (TintEliza PAI-1, Biopool AB), von Willebrand factor (Diagnostica Stago) and D-dimer (BioPool AB).

**Acknowledgements**

The Framingham Heart Study is funded by National Institutes of Health contract N01-HC-25195. The laboratory work for this investigation was funded by the Division of Intramural Research, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD. The analytical component of this project was funded by the Division of Intramural Research, National Heart, Lung, and Blood Institute, and the Center for Information Technology, National Institutes of Health, Bethesda, MD. A portion of this research utilized the Linux Cluster for Genetic Analysis (LinGA-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center.
The views expressed in the manuscript are those of the authors and do not necessarily represent the views of the National Heart, Lung, and Blood Institute; the National Institutes of Health; or the U.S. Department of Health and Human Services.

**Genetic Epidemiology Network of Arteriopathy**

**Study Participants**

The Genetic Epidemiology Network of Arteriopathy (GENOA) study is a community-based study of hypertensive sibships that was designed to investigate the genetics of hypertension and target organ damage in African Americans from Jackson, Mississippi and non-Hispanic whites from Rochester, Minnesota\(^3\). In the initial phase of the GENOA study (Phase I: 1996-2001), all members of sibships containing \(\geq 2\) individuals with essential hypertension clinically diagnosed before age 60 were invited to participate, including both hypertensive and normotensive siblings. Exclusion criteria of the GENOA study were secondary hypertension, alcoholism or drug abuse, pregnancy, insulin-dependent diabetes mellitus, or active malignancy. Eighty percent of African Americans (1,482 subjects) and 75% of non-Hispanic whites (1,213 subjects) from the initial study population returned for the second examination (Phase II: 2001-2005). Study visits were made in the morning after an overnight fast of at least eight hours. Demographic information, medical history, clinical characteristics, lifestyle factors, and blood samples were collected in each phase. Written informed consent was obtained from all subjects and approval was granted by participating institutional review boards. DNA methylation levels were measured only in African Americans participants, so only African Americans were included in the current analysis.

**GENOA Methylation Data 450K**

DNA methylation was measured on the peripheral blood leukocytes of 422 African American participants using stored blood samples collected during the **Phase I** examination. Peripheral blood leukocytes were isolated from blood samples and used to measure DNA methylation levels. The EZ DNA Methylation Gold Kit (Zymo Research, Orange CA) was used for bisulfite conversion. The methylation assay was performed at the Mayo Clinic Advanced Genomics Technology Center using Illumina® Infinium HumanMethylation450 BeadChip and Illumina
BeadXpress reader. The Minfi R package \(^{11}\) was used to preprocess, subset quantile within-array normalization (SWAN), and calculate beta values. Principle component analysis was performed on beta values and two outliers were identified (out of mean ± 6 SD range from any of the top 10 PCs) and excluded from analysis, leaving a total of 420 samples. Detection p value was calculated for each site. In all samples, >95% of probes had a detection P-value<0.01.

**Phenotype Measures**

Inflammatory markers were measured from fasting blood samples obtained during Phase II. Fibrinogen level (g/L) was measured from citrated plasma using the clotting time based Clauss method.\(^{35}\) Fibrinogen levels were converted to g/L (divided by 100).

**Acknowledgements**

Support for the Genetic Epidemiology Network of Arteriopathy (GENOA) was provided by the National Heart, Lung and Blood Institute (HL054464, HL054457, HL054481, HL100185, HL119443, and HL133221). We would also like to thank the families that participated in the GENOA study.

**Cooperative health research in the Region of Augsburg**

**Study Participants**

The KORA study (Cooperative health research in the Region of Augsburg) is a population-based cohort from the region of Augsburg, Southern Germany. Whole blood samples of the KORA S4 survey and KORA F3 survey were used. The KORA S4 survey is a baseline survey of 4,261 individuals living in Augsburg, Germany sampled in 1999 – 2001.\(^{36}\) The KORA F3 survey is the follow-up survey to the MONICA/KORA S3 survey which involved 4,856 participants\(^{37,38}\) examined in 1994 – 1995 and of these 3,006 subjects participated in the follow-up conducted in 2005-2005.\(^{38}\) Demographic and clinical variables were collected via standardized questionnaires at each exam and peripheral blood was collected for later analysis. Body mass index (kg/m\(^2\)) was estimated based on the weight and height at exam with participants wearing underwear or light clothing. Information on current and past smoking behavior and physical activity was also collected during the examination. All participants gave a written informed consent form and the study has been approved by the local ethics committee (Bayerische Landesärztekammer).

**DNA methylation quantification & epigenetic age calculation**
Processing of the methylation data was done similarly for both KORA S4 and KORA F3. Briefly, whole Blood was drawn into serum gel tubes. 1 µg genomic DNA was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer’s procedure, with the alternative incubation conditions recommended when using the Illumina Infinium Methylation Assay. Genome-wide DNA methylation was analyzed in 500 participants from KORA F3 and 1,526 participants from KORA S4 using the Infinium HumanMethylation450 BeadChip Array (Illumina). Raw methylation data were extracted using the Illumina Genome Studio (version 2011.1) with the methylation module (version 1.9.0). Preprocessing was performed with R (version 3.0.1). Probes with signals from less than three functional beads, and probes with a detection p-value > 0.01 were defined as low-confidence probes. Probes that covered SNPs (MAF in Europeans > 5%) were excluded from the data set. A color bias adjustment was performed with the R package lumi (version 2.12.0) by smooth quantile normalization and background correction based on negative control probes present on the Infinium HumanMethylation BeadChip. This was performed separately for the two color channels and chips. β-values corresponding to low-confidence probes were set to missing. A 95% call rate threshold was applied on samples and CpG sites. Beta-mixture quantile normalization (BMIQ) was applied by using the R package wateRmelon, version 1.0.3.

**Epigenetic age assessment**

Epigenetic age measures were calculated using the Horvath online calculator (https://dnamage.genetics.ucla.edu/). As the online calculator performs an internal normalization the raw methylation values for KORA S4 and KORA F3 were used. As the online calculator provides back several quality control statistics we used these to filter potentially lower quality samples. In particular we filtered any samples with sex mismatches, low correlation with “gold standard” probes used for the epigenetic age calculation development, and any samples whose estimated tissue type was not peripheral blood. This filtering left 450 samples for analysis in KORA F3 and 1,495 samples in KORA S4 for analysis.

**Hemostatic factors**

Fibrinogen was assayed in KORA S4 via an immunonephelometric method (Dade Behring Marburg GmbH, Marburg, Germany) with a Behring Nephelometer II analyzer, and is reported in g/L. Plasminogen Activator Inhibitor 1 (PAI-1) and D-dimer were assessed in KORA F3. D-dimer was assessed using two monoclonal antibodies that are directed against nonoverlapping
epitopes.\textsuperscript{41} PAI-1 was measured in EDTA plasma via an ELISA assay (Technozym PAI-1 Actibind ELISA; Technoclone, Vienna, Austria), and standardized according to the WHO PAI-1 International Standard.\textsuperscript{42}

\textbf{Gene Expression}

Gene expression was assessed in KORA S4 cohort according to previously published methods for the follow-up KORA F4 study.\textsuperscript{43} After collection, RNA was stored in PAXgene tubes (BD, Heidelberg, Germany) using the PAXgene Blood miRNA kit provided by Qiagen (Hilden, Germany). RNA was isolated according to manufacturer’s (Qiagen) instructions using a QIAcube, and a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Scientific, Hennigsdorf, Germany). All samples were also analyzed on a 2100 Bioanalyzer and on RNA 6000 Nano Lab Chips from Agilent Technologies (Santa Clara, CA, USA) in order to insure a consistent and high RNA quality. Samples with a RNA integrity number (RIN) less than seven were removed. 500 ng of quality checked RNA was reverse transcribed and biotin-UTP labeled. Illumina HumanHT-12 v3 Expression BeadChip arrays were used to hybridize the cRNA (3000 ng) and washing steps were performed according to manufacturer’s protocols. Gene expression was analyzed using mixed linear effects models while adjusting for amplification plate and RIN.

\textbf{Acknowledgements}

The KORA study was initiated and financed by the Helmholtz Zentrum München – German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (BMBF) and by the State of Bavaria. Furthermore, KORA research was supported within the Munich Center of Health Sciences (MC-Health), Ludwig-Maximilians-Universität, as part of LMUinnovativ.

\textbf{Lothian Birth Cohort of 1921 and 1936}

\textbf{Lothian Birth Cohort of 1921 (LBC1921)}

The LBC1921 consists of 550 participants (316 female) who were born in 1921 and at age 11 took part in the Scottish Mental survey of 1932. Individuals who took part in the Scottish Mental Survey were identified through examining the records of those registered with a general practitioner in the area. At age 79 these individuals were followed up and those living in
Edinburgh and the surrounding regions were recruited into the LBC1921 cohort. They are healthy older age individuals living independently within the community. Venous whole blood was extracted following informed consent and ethical approval was given from The Lothian Research Ethics Committee.

Lothian Birth Cohort of 1936 (LBC1936)
The LBC1936 has a total of 1091 participants (543 female) who took part in the Scottish Mental Survey of 1947. These individuals were recruited in a similar fashion to the participants of the LBC1921 cohort. At age 70 these individuals were recruited into the LBC1936. These participants were healthy individuals who were able to live independently within the community. Venous whole blood was extracted following informed consent and ethical approval was granted by Scotland’s Multi-centre Research Ethics Committee and the Lothian Research Ethics Committee.

DNA methylation quantification & epigenetic age calculation
From LBC1921, DNA methylation was measured in 514 individuals at age 79 and in LBC1936 DNA methylation was measured in 1004 individuals at age 70. DNA methylation data was processed as described previously. In brief, DNA methylation data from each of the LBC cohorts was background corrected to methylation β-values using the R minfi package. Probes with a low detection rate (<95% detection at P <0.01) were removed from the analysis. Following this manual inspection of the array control probe signals was used to identify and remove samples of low quality.

Epigenetic age measures were derived using the Horvath online calculator (https://dnamage.genetics.ucla.edu/). Lower quality samples were filtered out by removing those with a sex mismatch, samples whose estimated tissue type was not peripheral blood, and
those with a low correlation with the gold standard probes used for epigenetic age calculation. In the LBC1921 cohort this resulted in a total of 435 samples for analysis whereas in the LBC1936, 919 samples were available.

**Hemostatic factors**

Fibrinogen was assayed in LBC1921 and LBC1936 using the Clauss method. Prothrombin time was assayed using the Clauss method and aPTT using the Synthasil method. All assays were measured using the Werfen ACLTOP700.

**Acknowledgements**

This work was undertaken in The University of Edinburgh Centre for Cognitive Ageing and Cognitive Epidemiology (CCACE), supported by the cross-council Lifelong Health and Wellbeing initiative (MR/K026992/1). Funding from the Biotechnology and Biological Sciences Research Council (BBSRC), the Medical Research Council (MRC), and the University of Edinburgh is gratefully acknowledged. CCACE funding supports I.J.D. W.D.H. is supported by a grant from Age UK (Disconnected Mind Project).

**MARseille Thrombosis Association**

**Study Participants**

The MARseille Thrombosis Association (MARTHA) study included 1592 unrelated venous thromboembolism patients of French origin, recruited between January 1994 and October 2005 from the Thrombophilia Center of La Timone Hospital, Marseille, France. The study was designed to investigate venous thromboembolism and quantitative traits related – or potentially related – to venous thromboembolism. Recruitment occurred at least 3 months after the venous thromboembolism event, which was objectively diagnosed by venography, Doppler ultrasound, angiography and/or ventilation/perfusion lung scan. Study subjects were free of chronic conditions, as well as any well-characterized strong genetic risk factors for venous thromboembolism as described above for the F5L family study. Medical and personal histories
were obtained from physician interviews. Informed consent was obtained from the participants. Ethics approval was obtained from the "Ministère de la Recherche et de l’Innovation".

**DNA methylation quantification & epigenetic age calculation**

DNA was extracted from peripheral blood using a salting out procedure. Bisulfite conversion and DNA methylation measurements were performed at The Center for Applied Genomics, Toronto, Canada in 350 subjects from the MARTHA study randomly selected from those with whole blood DNA available.\textsuperscript{21,22,45} Bisulfite conversion was performed on 1 µg genomic DNA for each sample using the Qiagen EpiTect 96 Bisulfite Kit and 200 ng of bisulfite-converted DNA at 50 ng/µl was independently amplified, labeled and hybridized to Infinium HumanMethylation450 BeadChip microarrays. For each sample, the intensities of the methylated and unmethylated signals were measured at 485,577 CpG sites using the Illumina iScan (with default settings). Probes that were measuring single nucleotide polymorphisms or were either cross-reactive or polymorphic at the targeted CpG site were excluded. DNA methylation data were expressed as a β-value, a continuous variable over the [0–1] interval, representing the percentage of methylation of a given CpG site.\textsuperscript{24} Methylation values were corrected for background by use of the Noob method implemented in the "methylumi" package\textsuperscript{24} for dye bias following the manufacturer's recommendation (http://support.illumina.com/downloads/genomestudio_m_module_v18_ug_%2811319130_b%29_ilmn) and normalized for design type bias according to the SWAN method\textsuperscript{12} implemented in the minfi R package.\textsuperscript{11} Probes (n=4,010) with a detection p-value (as described in the "minfi" package) greater than 0.05 in more than 5% of the total processed samples were then excluded from further analyses.

Epigenetic age measures were calculated using the Horvath online calculator (https://dnamage.genetics.ucla.edu/)\textsuperscript{6} on the β-values. A total of 345 individuals with phenotype data were used for analysis.

**Hemostatic factors**

Blood samples were collected by antecubital venipuncture into Vacutainer® tubes 0.105 M trisodium citrate (ratio 9:1, Becton Dickinson) for the coagulation test and the thrombin generation assay. Platelet-poor plasma (PPP) was obtained after double centrifugation of citrated blood (3000 g for 10 min at 25°C) and kept frozen at -80°C until analysis. Fibrinogen levels were measured using the Clauss method on STAR automatic coagulomater. PAI-1 were
measured using the Chromolize PAI-1 kit from Biopool and D-Dimers using the STA-Liatest D-Di from Stago.

Acknowledgements
The MARTHA project was supported by a grant from the Program Hospitalier de Recherche Clinique and the ICAN Institute for Cardiometabolism and Nutrition (ANR-10-IAHU-05). The Human450Meethylation epityping was funded by the Canadian Institutes of Health Research (grant MOP 86466) and by the Heart and Stroke Foundation of Canada (grant T6484).

Netherlands Twin Register
Study Participants
The Netherlands Twin Register (NTR)(Boomsma et al. 2002;Willemsen et al. 2013) was established in 1987 to study genetic and environmental influences on phenotypic differences between individuals. To this end, data from twins and their families (currently more than 200,000 participants) from all over the Netherlands are collected, with a focus on health, lifestyle, personality, brain development, cognition, mental health, and aging. In NTR Biobank, samples for DNA, RNA, cell lines and for biomarker projects have been collected (Willemsen et al. 2013). Body mass index (kg/m2) was computed based on weight and height obtained at the moment of blood sampling. Information on current and past smoking behavior was also collected as part of the NTR biobank project at the moment of blood draw. Information on voluntary exercise activity was obtained in longitudinal NTR surveys. For the current project, information on voluntary exercise activity obtained closest to the moment of blood draw was selected. A dichotomous variable was used that indicates whether a person participates in voluntary exercise activities at least 60 minutes per week with an intensity of at least 4 METS(Stubbe et al. 2006).

All subjects provided written informed consent. The study protocols were approved by the Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Centre, Amsterdam, an Institutional Review Board certified by the US Office of Human Research Protections (IRB number IRB-2991 under Federal-wide Assurance-3703; IRB/institute codes, NTR 03-180).

DNA methylation quantification & epigenetic age calculation
The blood sampling procedure(Willemsen et al. 2013) and DNA methylation data(van Dongen J. et al. 2016) have been previously described. Briefly, the Infinium HumanMethylation450
BeadChip (Illumina Inc, San Diego, CA, USA) was used to measure DNA methylation following
the manufacturer’s protocol in 500ng of genomic DNA obtained from whole blood. A number of
sample- and probe-level quality checks and sample identity checks were performed (described in
detail here(van Dongen J. et al. 2016)). In short, sample-level QC was performed using
MethylAid(van Iterson M. et al. 2014). Probes were set to missing in a sample if they had an
intensity value of exactly zero, or a detection p>.01, or a bead count of<3. After these steps,
probes that failed based on the above criteria in >5% of the samples were excluded from all
samples (only probes with a success rate≥ 0.95 were retained). Probes were also excluded from
all samples if they mapped to multiple locations in the genome(Chen et al. 2013).
Epigenetic age measures were calculated using the Horvath online calculator
(https://dnamage.genetics.ucla.edu/). As the online calculator performs an internal normalization,
the unnormalized methylation beta values were used as input. As the online calculator provides
back several quality control statistics we used these to filter potentially lower quality samples. In
particular we filtered any samples with sex mismatches, low correlation with “gold standard”
probes used for the epigenetic age calculation development, and any samples whose estimated
tissue type was not peripheral blood. This filtering left 3067 NTR samples for analysis.

**Hemostatic factors**

Fibrinogen levels were determined on the STA Compact Analyzer (Diagnostica Stago, France),
using STA Fibrinogen (Diagnostica Stago, France)(Willemsen et al. 2010), and are reported in
g/L.

**Acknowledgements**

The NTR received funding from: the BBRMI-NL-financed BIOS Consortium (NWO
184.021.007), the Netherlands Organization for Scientific Research (NWO):
Genotype/phenotype database for behavior genetic and genetic epidemiological studies
(ZonMwMiddelgroot 911–09–032). JvD is supported by ACTION. ACTION receives funding
from the European Union Seventh Framework Program (FP7/2007-2013) under grant agreement
no 602768.

**Rotterdam Study**

**Study Participants**
The Rotterdam Study (RS) is a population-based cohort study in Rotterdam, the Netherlands. The design of the RS has been previously described in detail elsewhere. In brief, The RS includes three sub-cohorts. In 1990, all residents of Ommoord, a district in Rotterdam, aged 45 years and older were invited to participate (RSI). In 2000, the cohort was extended with 3,011 participants who reached the age 55 years or who were 55 years and over and had moved into the research area (RSII). In 2006, a third cohort of 3,934 participant aged 45 years and older was initiated (RSIII). We performed the analyses on a random subset of 731 subjects from the third cohort (RSIII-1). Demographic and clinical variables were collected via standardized questionnaires at each visit and peripheral blood was collected for later analysis. Body mass index (kg/m²) was estimated based on the weight and height at exam with participants wearing underwear or light clothing. Information on current and past smoking behavior and physical activity was also collected during the visit.

The study was approved by the medical ethics committee at Erasmus University Rotterdam, Rotterdam, the Netherlands, and all examined participants gave written informed consent.

**DNA methylation quantification & epigenetic age calculation**

DNA was extracted from whole blood (stored in EDTA tubes) by standardized salting out methods. Genome-wide methylation levels were measured using the Illumina Infinium Human Methylation450 Beadchip (Illumina Inc., San Diego, CA). Briefly, samples (500 ng of DNA per sample) were bisulfite-treated with use of the Zymo EZ-96 DNA methylation kit (Zymo Research, Irvine, CA, USA). Thereafter, the samples were hybridized to the arrays according to the protocol of the manufacturer. During quality control in RSIII-1, samples showing incomplete bisulfite treatment were excluded (n=5) as were samples with a low detection rate (<99%) (n=7), and gender swaps (n=4). Probes with a detection p-value >0.01 in >1% of the samples, were filtered out. Per individual probe, participants with methylation levels higher than three times the inter-quartiles range (IQR) were excluded. The methylation proportion of a CpG site was reported as a beta-value ranging from 0 (no methylation) to 1 (full methylation). Epigenetic age measures were calculated using the Horvath online calculator (https://dnamage.genetics.ucla.edu/). As the online calculator provides back several quality control statistics we used these to filter potentially lower quality samples. We used 731 samples for analysis.

**Gene Expression**
Total RNA was isolated (PAXGene Blood RNA kits - Qiagen) from whole blood (PAXGene Tubes – Becton Dickinson). All RNA samples were analyzed using the Labchip GX (Calliper) according to manufacturer’s instructions, to ensure a constant high quality of RNA preparations. Samples with an RNA quality score >7 were amplified and labeled (Ambion TotalPrep RNA) and hybridized to the Illumina HumanHT12v4 Expression Beadchips, as described by the manufacturers protocol. RNA samples were processed at the Genetic Laboratory of Internal Medicine, Erasmus University Medical Centre Rotterdam. The dataset including 881 expression samples from RSIII-1 is available at GEO (Gene Expression Omnibus) public repository under the accession GSE338828. Gene expression data was quantile-normalized to the median distribution and log2-transformed. Probe and sample means were centered to zero. Genes were considered significantly expressed when detection p-values calculated by Genome Studio were less than 0.05 in more than 10% of all discovery samples, which added to a total number of 21,238 probes. The eQTL-mapping pipeline was used to perform quality control.7 Gene expression was analyzed using mixed linear effects models while adjusting for amplification plate and RIN.

Acknowledgements
The Rotterdam Study is supported by Erasmus MC (Erasmus Medical Center Rotterdam), the Erasmus University Rotterdam, the Netherlands Organization for Scientific Research (NWO), the Netherlands Organization for Health Research and Development (ZonMW), the Research Institute for Diseases in the Elderly (RIDE), the Ministry of Education, Culture and Science, and the Ministry of Health, Welfare and Sports. The authors are grateful to the Rotterdam Study participants, the staff involved with the Rotterdam Study and the participating general practitioners and pharmacists. The generation and management of the Illumina 450K methylation array data for the Rotterdam Study was executed by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, the Netherlands. The generation and management of RNA-expression array data for the Rotterdam Study was executed and funded by the Human Genotyping Facility of the Genetic Laboratory of the Department of Internal Medicine, Erasmus MC, the Netherlands. We thank Mr. Michael Verbiest, Ms. Mila Jhamai, Ms. Sarah Higgins, Mr. Marijn Verkerk, and Lisette Stolk PhD for their help in creating the methylation database. We thank Ms. Mila Jhamai, Ms. Sarah Higgins,
Ms. Marjolein Peters, Mr. Marijn Verkerk and Jeroen van Rooij for their help in creating the RNA array expression database.

**Other Disclosures/Acknowledgements**
This study was reviewed by the US Environmental Protection Agency. The results in this study do not necessarily represent the policy of the US Environmental Protection Agency. Use of products or trade names does not constitute endorsement by the US Environmental Protection Agency.

**References**


Supplemental Figure 1. Association between accelerated epigenetic aging and hemostatic factors measured at different time points.

Shown is the association between accelerated epigenetic aging and both fibrinogen (left column) and PAI-1 (right column) for longitudinal data from the Framingham Heart Study (FHS) Offspring cohort collected at examination 5 (FHS5, 1991-1995), 6 (FHS6, 1995-1998), and 7
(FHS7, 1998-2001). Epigenetic aging for all samples was assessed based on samples collected at examination 8 (2005-2008). The blue dashed line represents the line of null association while the red dashed line represents the estimated effect based on FHS5. AAD = epigenetic age acceleration difference; EEAD = extrinsic epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; PAI-1 = plasminogen activator inhibitor-1
Supplemental Figure 2.
Associations between epigenetic aging and fibrinogen amongst cohorts with fibrinogen and methylation measured at the same time point.
Restriction of the meta-analysis for the fibrinogen – epigenetic aging associations to just those cohorts with fibrinogen and DNA methylation measured at the same time point, i.e. on the same sample. This removed all of the African-ancestry cohorts from the analysis. The associations are somewhat attenuated as compared to the analysis on the complete cohort and the confidence interval has...
widened (likely due to the reduced number of cohorts). EEAD = epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; RE = random effects
Supplemental Figure 3

Associations between epigenetic aging and PAI-1 amongst cohorts with PAI-1 and methylation measured at same time point.

Restriction of the meta-analysis for the PAI-1 – epigenetic aging associations to just those cohorts with fibrinogen and DNA methylation measured at the same time point, i.e. on the same sample. The associations remain nearly identical as compared to the
analysis on the complete cohort although the confidence interval did widen (likely due to the reduced number of cohorts). EEAD = epigenetic age acceleration difference; IEAD = intrinsic epigenetic age acceleration difference; RE = random effects
Supplemental Figure 4

Tissue specific expression from data from the genotype tissue expression consortium\textsuperscript{48,49} is compiled and presented by the Functional Mapping and Annotation of Genome-Wide Associations Studies GENE2FUNC tool (http://fuma.ctglab.nl/gene2func).\textsuperscript{50} The heat map colors correspond to the average (log\textsubscript{2} transformed) reads per kilobase of transcript per million mapped reads. All of the tissue types assayed by the genotype tissue expression consortium are given.