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Blood DNA Methylation Patterns Across Carotid, Coronary, and Peripheral Atherosclerosis

A Comparative Analysis in 2 Prospective Cohorts

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ABSTRACT

BACKGROUND Epigenetic modifications have been linked to atherosclerotic cardiovascular disease and could constitute therapeutic targets.

OBJECTIVES In this study, we sought to identify differentially methylated CpG sites related to atherosclerosis across vascular beds, and to investigate to what extent these epigenetic signatures reflect cardiovascular risk factors (CVRFs).

METHODS Blood DNA methylation at 767,735 CpG sites was investigated in 3,688 individuals from 2 prospective cohort studies to create a comprehensive atlas of epigenetic modifications in carotid, coronary, and peripheral atherosclerosis. Atherosclerosis was objectively assessed by medical diagnoses, ultrasonography-assessed carotid plaque presence, and ankle-brachial index. Epigenome-wide association studies (EWAS) with 5% false discovery rate correction were performed to explore the relationship between DNA methylation patterns and atherosclerosis, as well as with CVRFs. Methylation scores were trained with the use of ridge regression for each atherosclerosis phenotype in one cohort, and evaluated for discriminatory and prognostic ability in a separate cohort. The influence of CVRFs on atherosclerosis-associated methylation was investigated by: 1) quantification of overlapping CpG sites associated with CVRFs in EWAS; and 2) observing the effect of CVRF trait adjustment on estimates and *P* values.

RESULTS Totals of 1,687, 3,131, and 5,852 CpG sites were significantly associated with, respectively, carotid, coronary, and peripheral atherosclerosis; 2,155 sites were significantly associated in 2 or more settings. The most strongly associated CpG sites across phenotypes mapped to 4 loci—an intergenic region on chromosome 2 (near *ALPP*/*ALPG*), *AHRR*, *PRSS23*, and *F2RL3*—with additional strong signals in *ABCG1* and *DHCR24* in coronary atherosclerosis. Epigenetic scores were predictive of 3-point major adverse cerebrovascular and cardiovascular events (HRs ranging from 1.23 to 1.39; all $P < 0.001$). Overall, >90% of atherosclerosis-associated CpG sites intersected with CVRF-associated sites, particularly smoking (up to 90%), followed by inflammation (60%) and metabolic traits (44%). Atherosclerosis EWAS adjustment for smoking pack-years alone reduced the median absolute estimate of significant CpG sites by 19.6% to 29.0%, and joint adjustment for all CVRF markers by 25.5% to 32.8%.

CONCLUSIONS We identified novel and validated previously known associations of DNA methylation with 3 subtypes of atherosclerosis. Results suggest that in blood, the epigenetic signature of atherosclerosis largely reflects cumulative exposure, particularly smoking and cardiometabolic dysregulation, rather than strongly distinguishing vasculature-specific biological processes. (JACC. 2026;■:■–■) © 2026 by the American College of Cardiology Foundation.

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ABBREVIATIONS AND ACRONYMS

ABI = ankle-brachial index

CpG = cytosine-phosphate-guanine

CRP = C-reactive protein

CVD = cardiovascular disease

CVRF = cardiovascular risk factor

EWAS = epigenome-wide association study

FDR = false discovery rate

FEV_1 = forced expiratory volume

FVC = forced vital capacity

HDL = high-density lipoprotein

HOMA-IR = homeostasis model assessment of insulin resistance

LDL = low-density lipoprotein

MACCE = major adverse cerebrovascular and cardiovascular events

MI = myocardial infarction

PAD = peripheral artery disease

Ischemic cardiovascular diseases (CVD), including cardiac disease such as coronary artery disease and myocardial infarction (MI), cerebrovascular disease such as stroke, and diseases of the peripheral arteries such as peripheral artery disease (PAD), are the leading causes of death and disability worldwide.[1] A common underlying mechanism for these diseases is atherosclerosis, the development of atheromatous lesions due to the accumulation of lipids, cholesterol, and other material. Established risk factors for atherogenesis include immutable factors such as ageing, male sex, and genetic predisposition, as well as modifiable risk factors such as dyslipidemia, obesity, diabetes mellitus, arterial hypertension, smoking, physical inactivity, and chronic inflammation.[1,2] CVD is therefore highly driven by exposures in the natural, social, and personal environment (the "exposome").[3] Biological changes caused by the exposome are in part mediated by epigenetic modifications, as was previously shown for exposure to various chemicals, nutrition, and smoking.[4]

In particular, DNA methylation at CpG sites (cytosine-guanine dinucleotides where methyl groups regulate gene expression) have been suggested to provide a molecular record of cumulative environmental[5] and metabolic exposures,[6] and several previous studies have shown a link between DNA cytosine methylation and CVD.[7] As such, blood-based methylation patterns may serve as integrated markers of exposure to risk factors such as smoking, obesity, and metabolic dysregulation, offering a potential link between the exposome and atherosclerotic disease. It remains unclear whether blood-based epigenetic signals of atherosclerosis reflect vasculature-specific mechanisms, capture systemic exposure biology, or even act as a mediator between the environment and disease. In light of recent work on in vivo epigenome editing, which has illustrated how methyl groups at CpG sites now represent promising and specific targets of direct therapeutic relevance,[8,9] answering this question is crucial.

We aimed to determine the extent to which blood DNA methylation distinguishes atherosclerosis across vascular beds and to what degree these signals reflect established cardiovascular risk factors (CVRFs). To do so, we used large and deeply phenotyped cohorts (combined n >3,600) with comprehensive DNA methylation quantification and systematic screening of atherosclerosis in carotid, coronary, and peripheral (leg) arteries. In addition, we systemically mapped methylation at the identified CpG sites to the exposome, to determine to which degree these factors contribute to the identified differences in methylation states.

METHODS

STUDY PARTICIPANTS. The study sample comprised individuals from the population-based prospective Gutenberg Health Study (GHS) (first wave n = 15,010) and the prospective MyoVasc (MyoVasc Study on the Development and Progression of Heart Failure; NCT04064450) study (n = 3,289). Participants were predominantly of European descent. Design and methods have been reported previously.[10,11] Briefly,

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the Author Center.

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participants in both were examined during a highly standardized 5.5-hour visit, including questionnaires, medical-technical examinations, and biomaterial collection after standardized laboratory analyses and storage. Both studies were approved by local ethics committees, and every participant provided written informed consent before enrollment into the studies. Both studies adhere to the Declaration of Helsinki and the principles of Good Clinical and Epidemiological Practice. Whole-blood DNA methylation was available in 3,688 individuals (1,648 in GHS; 2,040 in MyoVasc). A detailed description of available participants is provided in the Supplemental Methods.

DNA METHYLATION MEASUREMENT AND DATA PROCESSING. DNA extraction from peripheral blood was performed within 2 hours after sample collection with the use of salting and dissolution in TE buffer. Samples were stored at −80 °C. DNA methylation measurements were conducted using the Illumina Infinium MethylationEPIC BeadChip platform. Data processing is detailed in the Supplemental Methods. After processing, methylation measurements at a total of 767,735 CpG sites were available as beta values. All beta values were standardized to a mean of 0 and SD of 1 before analysis.

DEFINITION OF ATHEROSCLEROSIS PHENOTYPES. Carotid atherosclerosis was determined via the presence of plaques in ultrasound imaging of the common and internal carotid arteries. Coronary atherosclerosis was determined with a prior diagnosis of coronary artery disease or history of MI. Peripheral atherosclerosis was determined with a prior diagnosis of PAD or ankle-brachial index (ABI) <0.9, fulfilling diagnostic criteria of PAD.[12] Atherosclerosis-free control subjects had no known atherosclerosis as defined above and in addition no history of stroke and an ABI in the physiologic range, 0.9 to 1.4, following guideline recommendations.[12]

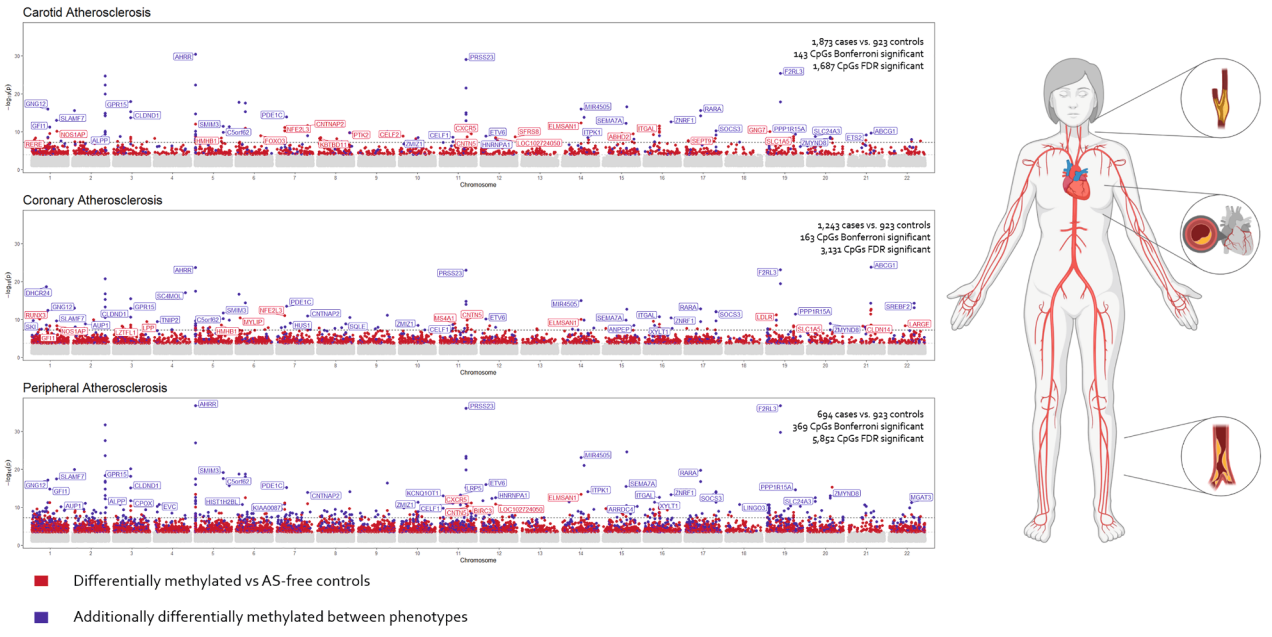
DEFINITION OF CVRF-RELATED TRAITS. In this study, continuous markers representing CVRFs were used where possible. Diabetes mellitus was represented by the glycated hemoglobin (HbA_{1c}) level. Insulin resistance was defined using the homeostasis-model assessment of insulin resistance (HOMA-IR) index, based on fasting plasma insulin and glucose. Dyslipidemia was operationalized with low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol levels, apolipoprotein (Apo) A and B, as well as triglyceride counts. Smoking was classified as never, former, or current smoking, and pack-years defined as number of packs smoked daily multiplied by number of years smoked. Obesity was substituted by the body mass index, and the waist-to-height ratio was used to capture central adiposity. Arterial hypertension was defined by systolic and diastolic blood pressure. The family history of MI or stroke was self-reported. Systemic inflammation was operationalized by continuous C-reactive protein (CRP) levels.

MEASUREMENTS OF SUBCLINICAL ORGAN DAMAGE AND DYSREGULATION ACROSS SYSTEMS. For vascular function and structure, the ABI, flow-mediated dilation, and intima-media thickness were determined with the use of ultrasonography. Vascular stiffness was quantified according to the stiffness index and augmentation index. Cardiac function and structure, ie, left ventricular ejection fraction, left ventricular filling pressure (E/E′), relative wall thickness, and left ventricular mass, were assessed with the use of echocardiography. Cardiomyocyte damage was assessed according to troponin I concentrations. Renal function was assessed by serum cystatin-C and the serum creatinine-based estimated glomerular filtration rate, using the 2012 Chronic Kidney Disease-Epidemiology Collaboration equation. Liver function was assessed based on alkaline phosphatase, gamma-glutamyl transferase, glutamic oxaloacetic transaminase, the Fib4 score, and fatty liver index. Lactate dehydrogenase was used to represent systemic cell death. Thyroid-stimulating hormone concentrations represented thyroid function. Creatine kinase was a surrogate for skeletal muscle damage. Forced vital capacity (FVC) and forced expiratory volume (FEV_1), measured with the use of spirometry, represented pulmonary function.

DEFINITION OF ENDPOINT OF INTEREST. The clinical endpoint of interest was 3-point major adverse cardio- and cerebrovascular events (MACCE), defined as the composite of incident MI, cardiac death, stroke, or transient ischemic attack over 4 years of follow-up, assessed by means of a combination of computer-assisted telephone interviews and hospital records. In addition, all-cause death was examined over 10 years of follow-up, based on continuous vital status information retrieval from the local registry offices.

EPIGENOME-WIDE ASSOCIATION STUDIES. Each atherosclerosis subtype was contrasted with the common reference group of the atherosclerosis-free control subjects, adjusted for age, sex, blood cell composition, and study cohort, in logistic regression models. Additional covariate adjustment was performed to investigate the influence of CVRFs. First, models were additionally adjusted for each CVRF

FIGURE 1 Epigenome-Wide Associations of Atherosclerosis by Vascular Bed



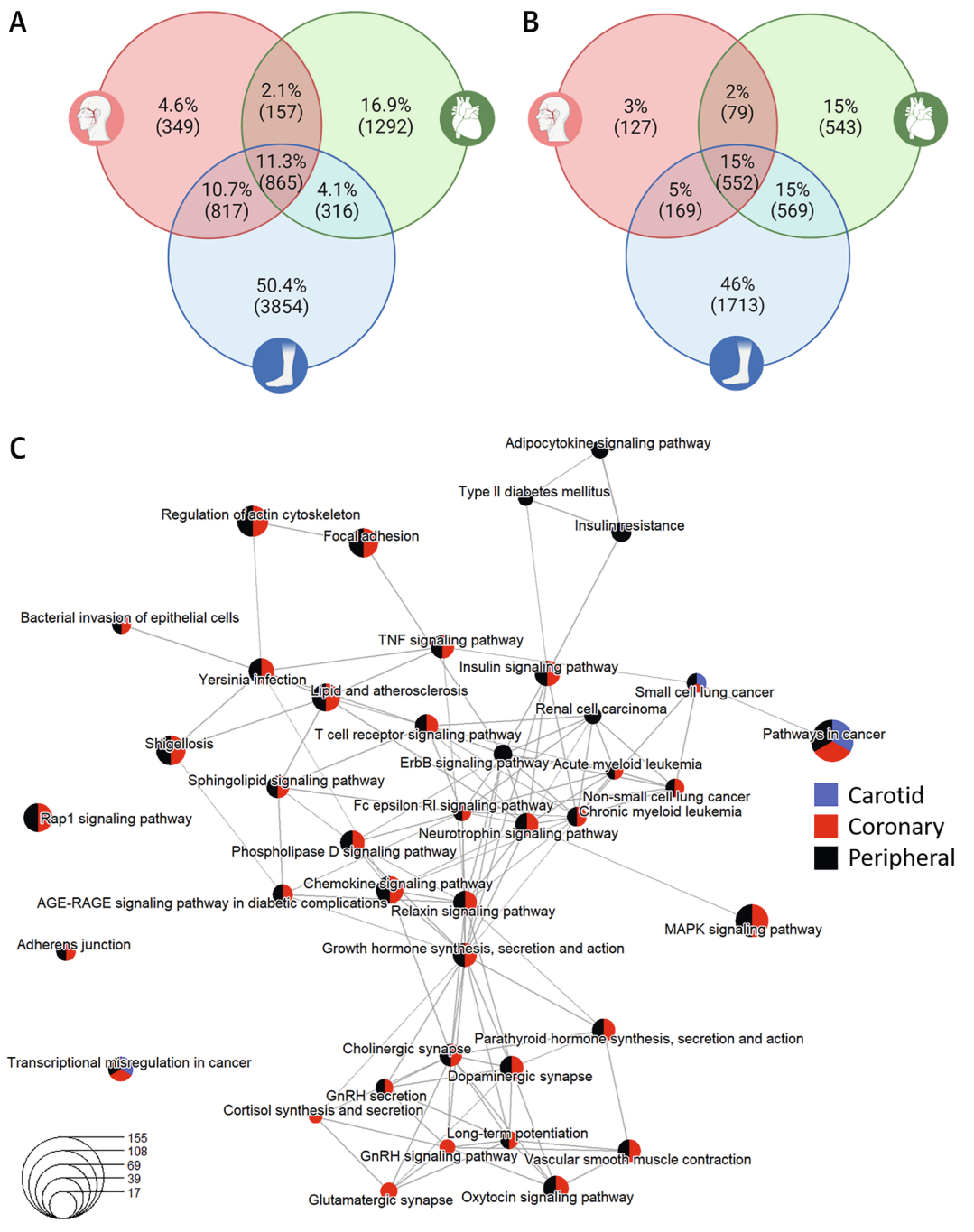
False discovery rate–significant CpG sites are colored according to whether there were or were not significant differences (blue or red, respectively) between the atherosclerosis subgroups in an analysis of covariance adjusted for age, sex, and cohort. CpG sites colored in gray were not significantly associated. The horizontal dotted line denotes the Bonferroni threshold. The 100 strongest sentinel sites per locus were annotated with their corresponding genes.

marker individually. Then, models with joint adjustment for all CVRF markers were created. In the models incorporating traits representing dyslipidemia, diabetes mellitus, and arterial hypertension, adjustment for the indicated medication intake was performed simultaneously. Sensitivity analyses included additional adjustments for the presence of atherosclerosis in the other arterial beds, and for cg05575921 (*AHRR*) methylation as proxy for smoking behavior.[13] To identify CpG sites associated with CVRF markers, additional epigenome-wide association studies (EWAS) were performed, applying logistic, linear, or Poisson regression models as appropriate. Individuals with a missing observation were omitted on a per-analysis basis. Quantile-quantile (Q-Q) plots of EWAS results were generated with the use of the *CMplot* v4.5.1 R package.[14] To further investigate differences in CpG methylation between vascular beds, analysis of covariance adjusted for age, sex, and study cohort was performed for each CpG site. Significance was declared with the use of a 2-sided α of 0.05. *P* values were corrected with the use of the Benjamini and Hochberg false discovery rate (FDR) procedure, as well as the Bonferroni family-wise error controlling procedure, to provide inclusive and more stringent selections of relevant CpG sites, respectively. All analyses were conducted using the R statistical software version 4.2.2.[15]

DEVELOPMENT AND EVALUATION OF EPIGENETIC PHENOTYPE SCORES. Epigenetic atherosclerosis scores were created using ridge regression using the *glmnet* version 4.1-8 R package.[16] The penalty λ was tuned by maximizing the 10-fold cross-validated AUC. In GHS, which was used as the training cohort, all FDR-significant CpG sites alongside age and sex were used as input variables for these models. Phenotype-specific epigenetic scores were then created in the independent validation cohort (MyoVasc) by a matrix multiplication of the CpG data with the vector of model coefficients for the CpG sites. Before analyses, each score was standardized to a mean of 0 and SD of 1.

EVALUATION OF ASSOCIATION OF EPIGENETIC SCORES WITH OUTCOME, RISK FACTORS, AND ORGAN MARKERS. Evaluation of epigenetic scores was performed in the validation cohort only. Kaplan-Meier plots were used to assess the ability of quartiles of the atherosclerosis methylation scores to

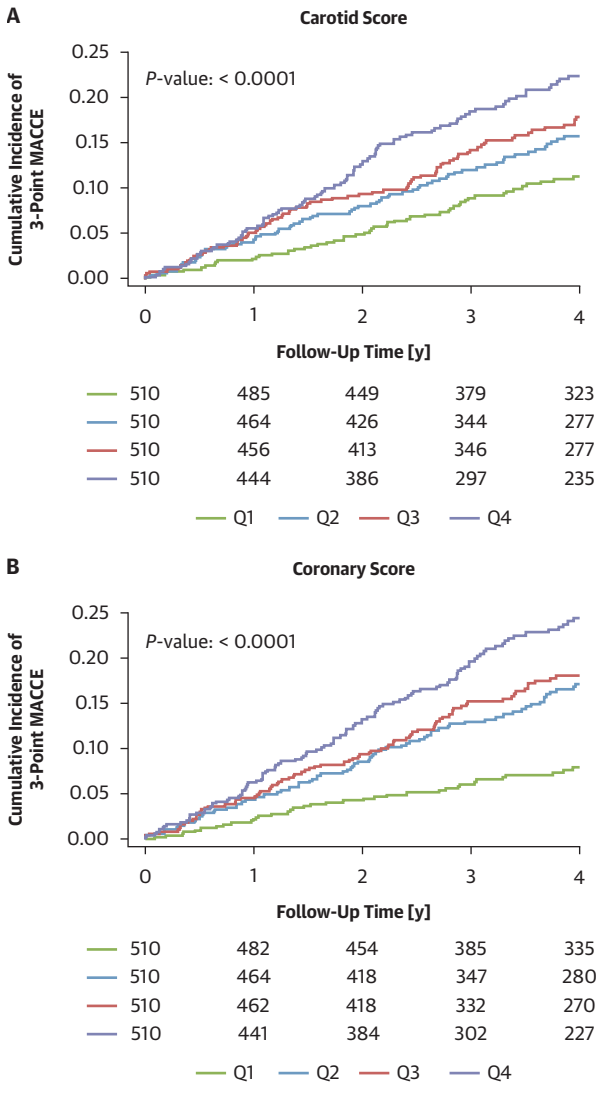
FIGURE 2 **Shared and Distinct Epigenetically Regulated Mechanisms for Atherosclerosis by Vascular Bed**



Overlap of false discovery rate significant (A) CpG sites and (B) genes in subgroup epigenome-wide association studies. (C) Arterial bed-wise similarity matrix enrichment of Kyoto Encyclopedia of Genes and Genomes pathway-associated genes. Node size denotes the number of genes detected in the pathway, and edges (ie, connections between nodes) denote a Jaccard similarity coefficient between pathways above 0.2.

stratify the risk of 3-point MACCE. In addition, Cox regression adjusted for age and sex was applied to each of the clinical endpoints separately. Age- and sex-adjusted linear regression was applied to risk factor and organ damage markers, with methylation scores as the outcome. Regression results were visualized with forest plots, with the use of the *forplo* version 0.2.5 R package.[17] The *P* values of

FIGURE 3 Prognostic Ability of Atherosclerosis Methylation Scores for Clinical Outcome



Cumulative incidence of 3-point major adverse cerebrovascular and cardiovascular events (MACCE) in individuals stratified by (A) carotid, (B) coronary, and (C) peripheral atherosclerosis methylation scores. (D) Forest plot of Cox regression results on several endpoints, adjusted for age and sex only. MACCE = major adverse cerebrovascular or cardiovascular events; HR per SD = hazard ratio per standard deviation of methylation score.

Continued on the next page

associations with organ damage markers were additionally visualized with the use of the *PheWAS* version 0.99.6-1 R package.[18] Cell deconvolution and gene enrichment analyses are described in the Supplemental Methods.

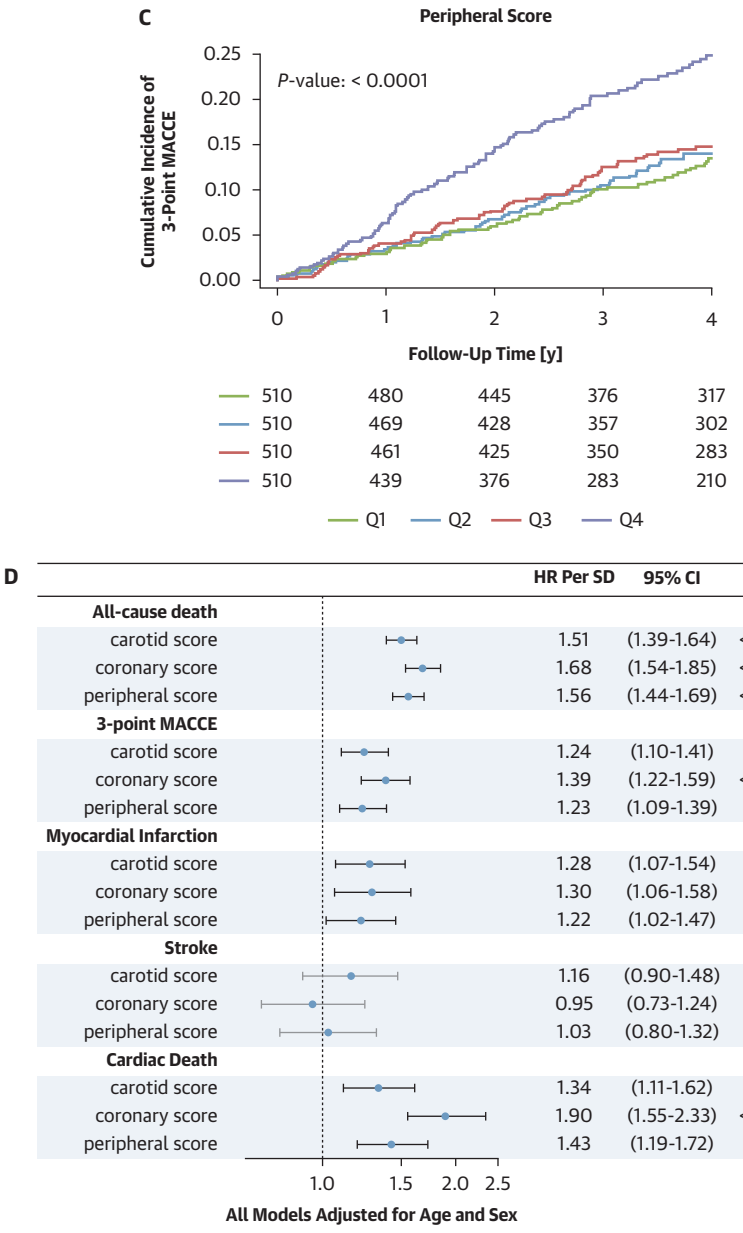
RESULTS

COHORT CHARACTERISTICS. The combined cohort used in this work included 3,688 individuals. Of these, 1,873 individuals had carotid atherosclerosis, 1,243 coronary atherosclerosis, and 694 peripheral atherosclerosis, and 923 were atherosclerosis-free control subjects, with per-setting sample sizes from 1,617 to 2,796. Overlaps between case groups are visualized in Supplemental Figure 1. Characteristics of the study participants stratified by atherosclerosis phenotype are listed in Supplemental Table 1. Control subjects were on average younger, had fewer comorbidities, were less often smokers, and were less likely to be on chronic medication. The coronary atherosclerosis group had a high prevalence of dyslipidemia (>90%), and individuals with peripheral atherosclerosis were often smokers (prevalence >70%). The carotid atherosclerosis group had relatively fewer risk factors and comorbidities.

EWAS OF ATHEROSCLEROSIS PHENOTYPES. In carotid, coronary, and peripheral atherosclerosis, respectively, 1,687, 3,131, and 5,852 CpGs reached FDR significance at a 5% threshold (143, 163, and 369 CpG sites, respectively, survived Bonferroni correction). These sites mapped to 926, 1,742, and 3,002 unique genes, and 29%, 27%, and 24% of CpG sites mapped to intergenic regions (compared with 29% across all investigated sites). The most strongly associated CpG sites across phenotypes were located in 4 peaks—in an intergenic region on chromosome 2 (near *ALPP*/*ALPG*), *AHRR*, *PRSS23*, and *F2RL3*—with additional strong signals in *ABCG1* and *DHCR24* in the coronary atherosclerosis EWAS (**Figure 1**). In total, 7,650 CpG sites were associated with at least 1 atherosclerosis phenotype. Of these, only 28% were significantly associated in at least 2 atherosclerosis phenotypes (**Figure 2A**). On the gene level, 37% of mapped genes were shared between at least 2 vascular beds. In a pooled analysis of all atherosclerosis case vs control subjects, respectively, 1,837 and 141 CpG sites reached 5% FDR and Bonferroni significance. Of the former, only 111 (6%) were not detected in vascular bed-specific EWAS (Supplemental Figure 2).

To investigate potential vascular bed-specific signals, the EWAS were repeated while additionally adjusting for the presence of atherosclerosis in the other 2 vascular beds. This identified the *ABCG1* and *DHCR24* loci as more prominently associated with atherosclerosis in the coronary artery (Supplemental Figure 3).

FIGURE 3 Continued



COMPARISON WITH GENOME-WIDE ASSOCIATION STUDIES AND ENRICHED PATHWAYS. Comparison with the NHGRI-EBI GWAS Catalog[19] revealed that, respectively, 157, 302, and 467 of the genes mapped to carotid, coronary, and peripheral atherosclerosis (or 17.0%, 17.3%, and 15.6%) were previously implicated in atherosclerosis phenotypes (peripheral artery disease [EFO_0004265], atherosclerosis [EFO_0003914], carotid atherosclerosis [EFO_0009783], coronary atherosclerosis [MONDO_0021661], and coronary artery disease [EFO_0001645]).

Kyoto Encyclopedia of Genes and Genomes terms of established relevance, eg, "Insulin Signalling," "TNF Signalling," "Lipid and Atherosclerosis," or "AGE-RAGE signalling," were enriched in coronary and peripheral atherosclerosis (**Figure 2C**). Pathway analysis according to the Reactome database further showed enrichment for "platelet activation, signalling, and aggregation" in all phenotypes (Supplemental Figure 4). By visualizing pathway diagrams such as "Lipid and Atherosclerosis," it became clear that large parts of the pathway were

TABLE 1 Prognostic Ability of Atherosclerosis Methylation Scores for Clinical Outcomes in the Validation Cohort

Outcome	Score	HR	95% CI	*P* Value	C-Index
Cox proportional hazard models adjusted for age and sex					
All-cause death	Carotid	1.51	1.39-1.64	8.93E-23	0.68
	Coronary	1.68	1.54-1.85	5.21E-29	0.69
	Peripheral	1.56	1.44-1.69	3.96E-27	0.68
3-point MACCE	Carotid	1.24	1.10-1.41	4.94E-04	0.61
	Coronary	1.39	1.22-1.59	7.77E-07	0.62
	Peripheral	1.23	1.09-1.39	7.11E-04	0.61
MI	Carotid	1.28	1.07-1.54	8.02E-03	0.64
	Coronary	1.30	1.06-1.58	1.02E-02	0.64
	Peripheral	1.22	1.02-1.47	2.90E-02	0.63
Stroke	Carotid	1.16	0.90-1.48	2.51E-01	0.54
	Coronary	0.95	0.73-1.24	7.00E-01	0.52
	Peripheral	1.03	0.80-1.32	8.24E-01	0.52
Cardiac death	Carotid	1.34	1.11-1.62	2.04E-03	0.66
	Coronary	1.90	1.55-2.33	6.51E-10	0.70
	Peripheral	1.43	1.19-1.72	1.18E-04	0.67
Cox proportional hazard models with CV risk factors[a]					
All-cause death	Carotid	1.42	1.28-1.57	5.03E-12	0.71
	Coronary	1.54	1.38-1.71	7.60E-15	0.72
	Peripheral	1.42	1.29-1.57	5.34E-13	0.71
3-point MACCE	Carotid	1.14	0.98-1.32	9.35E-02	0.65
	Coronary	1.32	1.13-1.54	5.26E-04	0.66
	Peripheral	1.15	1.00-1.33	5.56E-02	0.65
MI	Carotid	1.23	0.98-1.53	7.35E-02	0.69
	Coronary	1.24	0.98-1.56	7.57E-02	0.69
	Peripheral	1.17	0.94-1.46	1.47E-01	0.69
Stroke	Carotid	1.14	0.85-1.55	3.80E-01	0.62
	Coronary	1.00	0.73-1.38	9.86E-01	0.6
	Peripheral	1.11	0.82-1.49	4.98E-01	0.61
Cardiac death	Carotid	1.15	0.92-1.44	2.27E-01	0.75
	Coronary	1.66	1.31-2.11	3.25E-05	0.76
	Peripheral	1.23	0.99-1.53	5.97E-02	0.75

Prognostic ability of atherosclerosis methylation scores for clinical outcome as determined by Cox proportional hazards regression with and without adjustment for cardiovascular risk factors. [a]Adjusted for age, sex, smoking status, pack-years, HbA_{1c}, HOMA-IR, ApoA, HDL-C, AboB, LDL-C, triglycerides, C-reactive protein, body mass index, waist/height ratio, systolic blood pressure, diastolic blood pressure, and family history of MI.

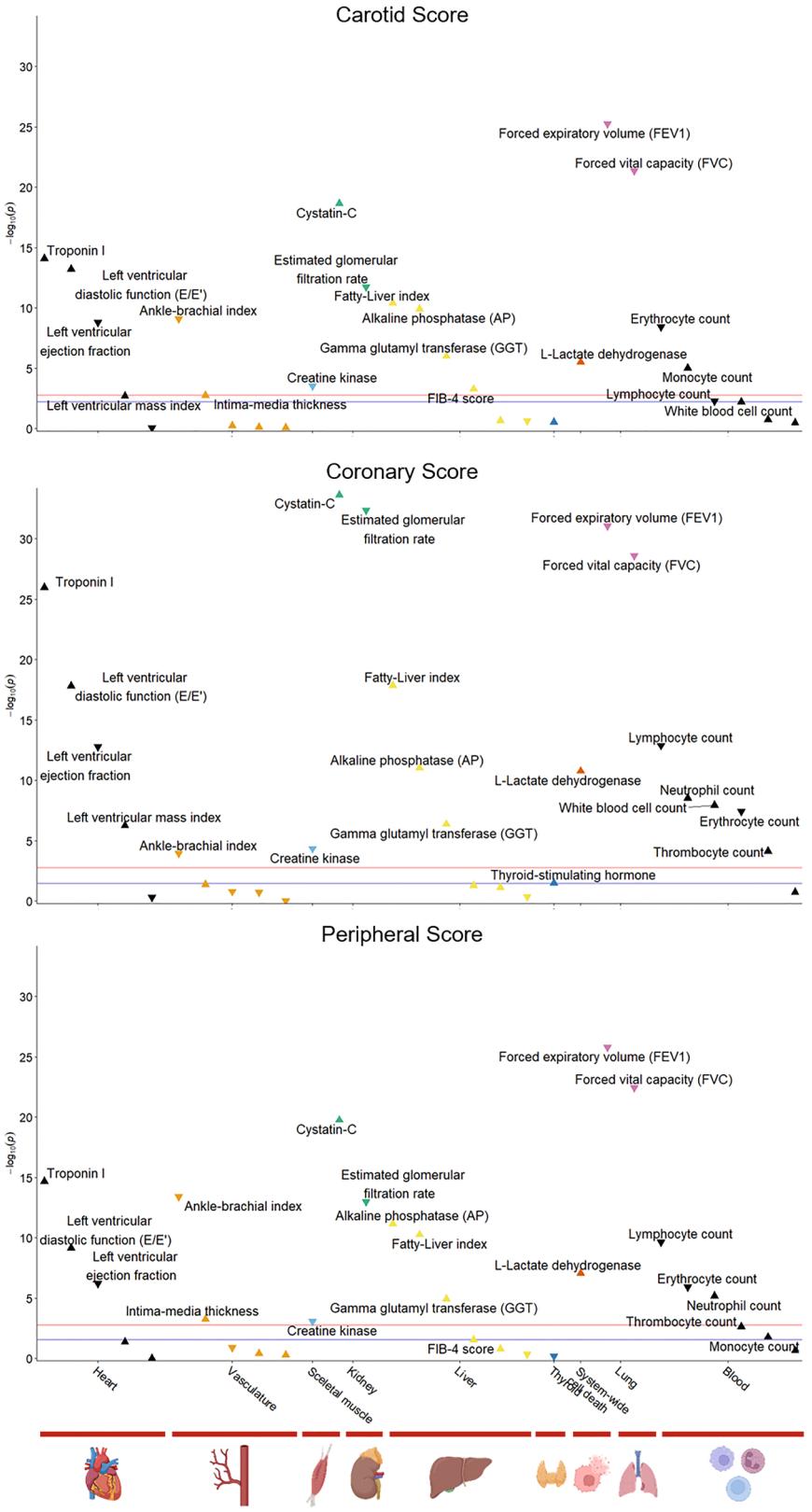
ApoA = apolipoprotein A; ApoB = apolipoprotein B; CV = cardiovascular; HbA_{1c} = glycated hemoglobin; HDL-C = high-density lipoprotein cholesterol; HOMA-IR = Homeostatic Model Assessment of Insulin Resistance; LDL-C = low-density lipoprotein cholesterol; MACCE = major adverse cerebrovascular and cardiovascular events; MI = myocardial infarction.

significantly differentially methylated, with only a few genes not covered by available CpG data (Supplemental Figures 5-8). Both hypo- and hypermethylation were observed, with the former more prevalent. In some cases, effect sizes for CpG sites from the same genomic regions were of opposite sign, eg, in *AHRR*, as shown in a Miami plot in Supplemental Figure 9.

CREATION OF EPIGENETIC ATHEROSCLEROSIS SCORES. Methylation scores of each atherosclerosis phenotype were trained in the GHS cohort with the use of ridge regression. Applying the resulting scores in the validation cohort (MyoVasc), the AUCs for classification of carotid, coronary, and peripheral case subjects vs atherosclerosis-free control subjects were 0.87, 0.87, and 0.92, respectively (Supplemental Figure 10). The scores were moderately strongly correlated with one another (all correlation coefficients >0.70) (Supplemental Figure 11).

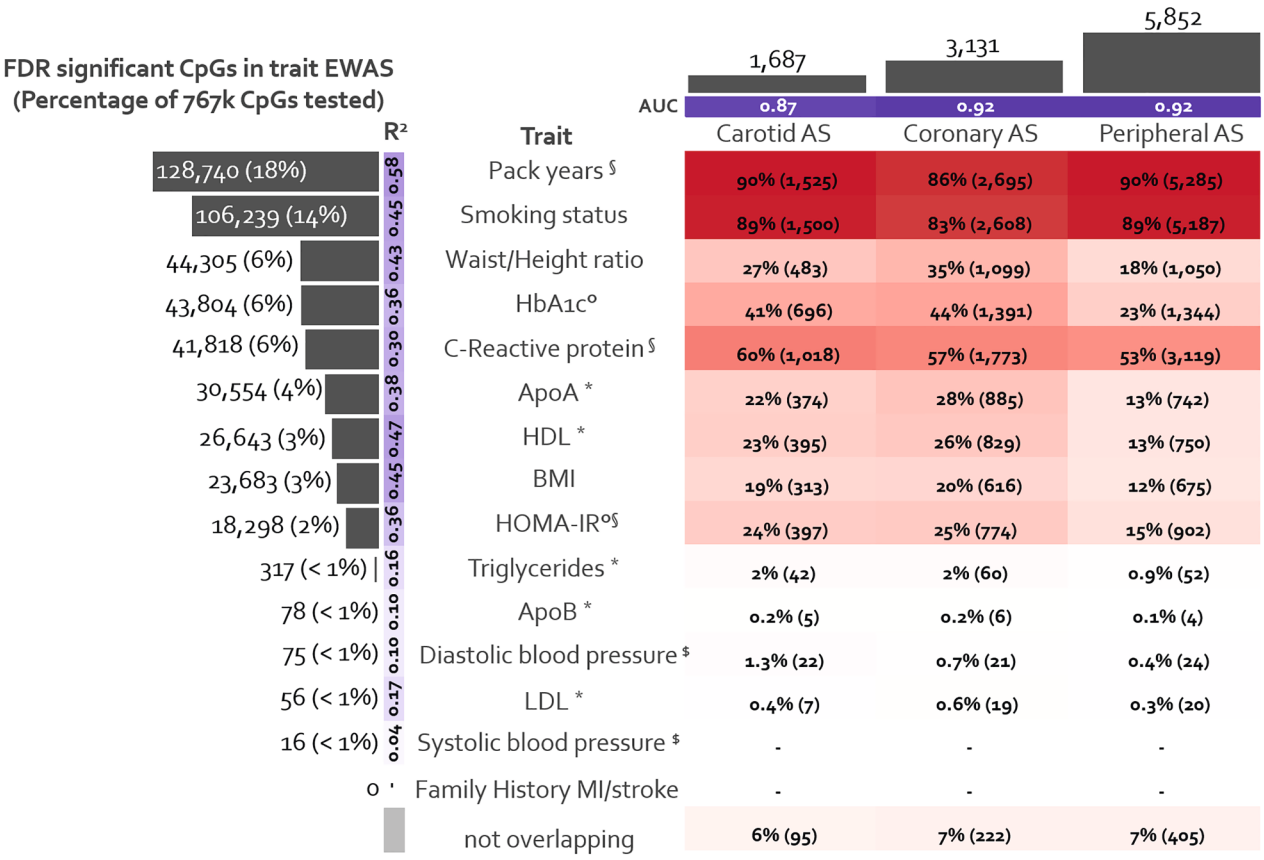
PROGNOSTIC ABILITY OF EPIGENETIC SCORES FOR CARDIOVASCULAR OUTCOME. In the validation cohort, methylation scores of each atherosclerosis phenotype predicted the incidence of 3-point MACCE and all-cause death (**Figure 3**), even after adjustment for CVRF markers (**Table 1**). For only the incidence of stroke, none of the scores were predictive.

FIGURE 4 **Association of Atherosclerosis Methylation Scores With Organ Damage and Blood Composition**



Phenome-wide association study–like plots of association between the carotid, coronary, and peripheral atherosclerosis epigenetic scores and organ damage markers and blood cell composition, derived with the use of linear regression models adjusted for age and sex. Variables were grouped by represented organ system. The horizontal red and blue lines denote the Bonferroni and false discovery rate (FDR) significance thresholds, respectively. Only variables reaching FDR significance were annotated.

FIGURE 5 Mapping Atherosclerosis-Associated Methylation Changes to Cardiovascular Risk Factors



CpG sites associated with each atherosclerosis subtype and degree of overlap with epigenome-wide association studies (EWAS) of cardiovascular risk factors. R^2 signifies the variance explained in each score by each trait. The AUC indicates the discriminative ability of each score for its corresponding atherosclerotic phenotype. *Adjusted for statin intake; §log-transformed; $adjusted for blood pressure medication intake. ApoA = apolipoprotein A; ApoB = apolipoprotein B; BMI = body mass index; HbA_{1c} = glycated hemoglobin; HDL = high-density lipoprotein cholesterol; HOMA-IR = Homeostatic Model Assessment of Insulin Resistance; LDL = low-density lipoprotein cholesterol; MI = myocardial infarction.

ASSOCIATION WITH SUBCLINICAL ORGAN DAMAGE AND BLOOD COMPOSITION. Significant associations were consistently observed across a range of organs, namely, the heart, vasculature, kidneys, liver, lungs, and blood (Figure 4). For all traits except the estimated glomerular filtration rate, which showed a more pronounced negative relationship with the coronary score, coefficient estimates were similar across vascular beds, with overlapping confidence intervals (Supplemental Figure 12). The most pronounced associations across atherosclerotic phenotypes were observed for spirometric measures of lung function (FEV_1, FVC). Troponin I measurements were consistently the third-strongest association.

ASSOCIATION BETWEEN ATHEROSCLEROSIS-RELATED DNA METHYLATION SCORES AND CARDIOVASCULAR RISK FACTORS. Next, we quantified the relationship between the atherosclerosis methylation scores and CVRFs in the MyoVasc cohort. The strongest associations were observed with smoking (15%-30% SD increases in atherosclerosis-associated differential methylation in smokers). The carotid and peripheral scores were more strongly associated with smoking traits, whereas the coronary score was more strongly associated with cardiometabolic traits and with blood pressure (Supplemental Figure 13).

INTERSECTION WITH EWAS OF CARDIOVASCULAR RISK FACTORS. More than 100,000 differentially methylated CpG sites were associated with

pack-years and smoking status, followed by HbA_{1c}, waist-to-height ratio, and CRP, with >40,000 associated sites each (**Figure 5**). Notably, blood pressure had only a few associations with DNA methylation, and none could be identified for family history of MI or stroke. Up to 90% of CpG sites differentially methylated in atherosclerosis phenotypes overlapped with those identified in relation to smoking or number of pack-years. The risk factor with the second-largest degree of overlap (up to 60%) in CpG sites with atherosclerosis phenotypes was systemic inflammation, as represented by CRP. Metabolic traits, including HbA_{1c}, HOMA-IR, body mass index, and lipids, were the next most prominently intersecting traits. Although a large number of CpGs were identified in relation to HDL-cholesterol and ApoA, few were associated with LDL-cholesterol and ApoB. Nevertheless, these few CpG sites still explained 17% of the variance in LDL and 10% of that in ApoB. Across atherosclerotic beds, 7%-8% of atherosclerosis-associated CpGs could not be traced back to a cardiovascular risk factor. Nevertheless, genes associated with these sites showed significant enrichment in genes found in GWAS on smoking status measurements, body mass index, and triglycerides, among others (Supplemental Figure 14).

THE EFFECT OF CVRF ADJUSTMENT ON ATHEROSCLEROSIS EWAS RESULTS. Adjustment for specific CVRFs showed a reduction in significant associations that was largest in smoking traits across settings (**Figure 6A, Central Illustration**). This was followed by metabolic traits (HbA_{1c} and HOMA-IR) and CRP in the carotid and peripheral settings; adjustment for lipid traits had a particularly large impact in the coronary atherosclerosis setting. Concordantly, genomic inflation measures of 1.31, 1.42, and 1.41, in carotid, coronary, and peripheral EWAS, respectively, were reduced to 1.05, 1.15, and 1.10 on pack-year adjustment alone. After smoking, metabolic marker adjustment led to the largest reduction in inflation. (**Figure 6B**, Supplemental Figures 15, 16A, and 16C). This was mirrored by a large attenuation of effect sizes (**Figure 6C**, Supplemental Figures 15, 16B, and 16D). A core set of CpG sites remained significant after Bonferroni correction in each adjustment setting across phenotypes (Supplemental Figure 17).

THE OUTSIZED INFLUENCE OF SMOKING STATUS ON THE ATHEROSCLEROSIS-ASSOCIATED METHYLATION RESULTS. On adjustment for smoking, the majority of previously significant CpG sites fell below the FDR threshold, and sites annotated to *AHRR*, *PRSS23*, *F2RL3*, and *ABCG1* remained significant across arterial beds. On adjustment for cg05575921 (annotated to *AHRR*), which has previously been investigated as a proxy for cumulative cigarette smoke exposure,[13] only *ABCG1* and *MYLIP* remained significant in all 3 settings (**Figure 7**, Supplemental Figures 18 and 19A-19C). When all previous smokers were omitted from the analysis, no CpG sites reached significance for carotid and peripheral atherosclerosis, and only *ABCG1* and *MYLIP* remained significant in the coronary setting (Supplemental Figure 20), although the large reduction in sample size should be factored in. All methylation scores were strongly associated with the number of pack-years smoked in a linear dose-dependent fashion. In addition, the methylation scores were linearly inversely related to years since smoking cessation, approaching the never-smokers' median methylation levels after several decades (**Figure 7**, Supplemental Figures 18, 19D, and 19E).

DISCUSSION

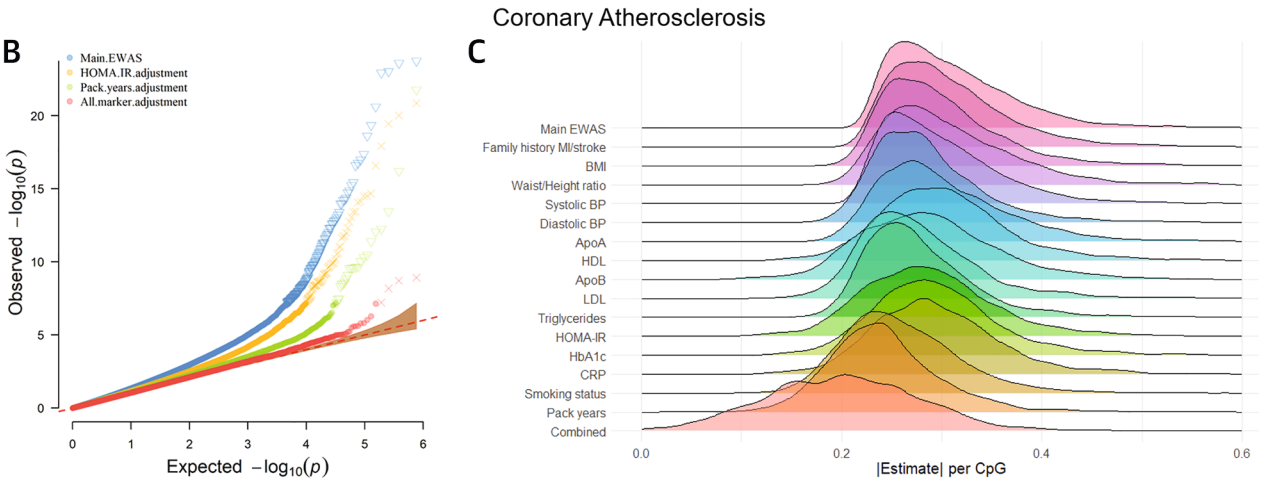
We have presented the largest combined EWAS on atherosclerosis phenotypes in blood conducted to date, establishing an atlas of DNA methylation changes across vascular beds. Previous studies had investigated methylation patterns in whole blood in relation to atherosclerosis in the carotid artery[20,21] and the coronary artery,[22] but did not have comparative data on both. One small study (n = 391) investigated markers representing atherosclerotic subtypes (ABI, intima-media thickness, carotid plaque presence, and coronary calcification) but only verified CpG sites found in a previous study.[23] To our knowledge, no EWAS on peripheral artery atherosclerosis had yet been conducted.

The deep phenotyping performed in the studies used in this investigation enabled us to objectively and systematically assess atherosclerosis in 3 vascular beds. Because phenotyping was performed prospectively and universally in all participants, independently from medical indication, atherosclerosis along the full clinical and subclinical spectrum was investigated. Long-term follow-up monitoring enabled the assessment of the prognostic ability of the scores and provided proof of the concept that DNA methylation contains prognostically relevant information years in advance of a first atherothrombotic event. Given that the methylation scores were trained and validated in separate cohorts, representing distinct settings, results were robust to variations in covariate distributions, underscoring that these scores captured robustly atherosclerosis-associated patterns.

FIGURE 6 The Effects of Cardiovascular Risk Factor Adjustment on Atherosclerosis EWAS Results

A

	Coronary			Carotid			Peripheral		
	Bonferroni	FDR	Inflation factor	Bonferroni	FDR	Inflation factor	Bonferroni	FDR	Inflation factor
Adjusted for									
All CVRF markers	4	5	1.06	2	3	1.05	9	16	1.1
Smoking pack years	23	62	1.15	5	8	1.05	12	27	1.1
Smoking status	34	157	1.22	13	54	1.1	42	259	1.17
LDL cholesterol	62	798	1.34	117	1456	1.31	264	5238	1.36
Triglycerides	68	934	1.3	113	1098	1.26	267	5161	1.35
Apolipoprotein A	72	1117	1.28	118	1122	1.24	279	5149	1.31
HbA1c	76	624	1.23	93	685	1.2	246	3710	1.29
HOMA-IR	76	624	1.21	114	845	1.2	275	4401	1.31
HDL cholesterol	77	1410	1.29	117	1177	1.24	298	5655	1.31
Apolipoprotein B	89	1426	1.36	124	1565	1.3	280	5396	1.37
Diastolic blood pressure	103	1408	1.35	120	1071	1.25	295	4402	1.38
Waist/height ratio	106	1064	1.29	134	1116	1.22	344	5087	1.35
C-reactive protein levels	120	1683	1.35	110	915	1.25	222	3192	1.3
Systolic blood pressure	123	2061	1.36	140	1532	1.27	352	5662	1.4
Body mass index	131	1844	1.38	142	1518	1.28	378	6087	1.4
Family history of MI/stroke	145	2406	1.42	128	1362	1.3	363	5890	1.41
Main EWAS	163	3131	1.42	143	1687	1.31	369	5852	1.41



(A) Table summarizing the consequences of single and joint adjustment for CVRF markers, compared with the main EWAS depicted in **Figure 1**, by reporting the reduction in Bonferroni and false discovery rate (FDR)-significant CpG sites and the inflation factor for carotid, coronary, and peripheral atherosclerosis. (B) Q-Q plot depicting expected vs observed *P* values for the main analysis (blue) and after adjustment for HOMA-IR (yellow), pack-years (green), and all CVRF markers jointly (red) in coronary atherosclerosis EWAS. (C) Density plots depicting absolute estimates of all CpG sites reaching 5% FDR in the main EWAS after single and joint CVRF-variable adjustments in the coronary atherosclerosis EWAS. The same results for the other vascular beds are presented in Supplemental Figure 15.
CVRF = cardiovascular risk factor; EWAS = epigenome-wide association study

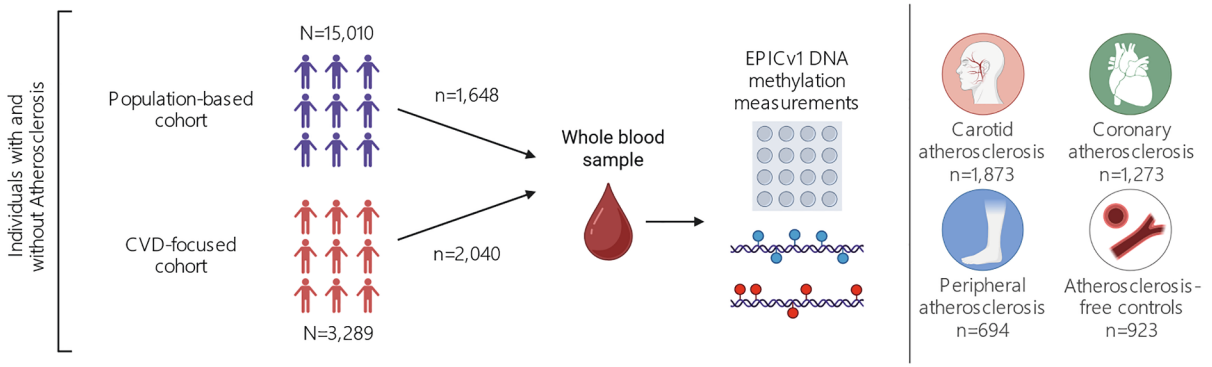
Gene enrichment analysis on the complete set of identified genes yielded terms expected in an analysis on atherosclerosis, confirming that methylation acts on well-established pathways involved in the disease. Differential methylation sites were further enriched in genes associated with cardiovascular measurements and CVRFs in previous GWAS, and this study replicated 70% to 100% of CpGs identified in 4 other studies on the presence of plaques or coronary artery disease (excluding sites unavailable in the present study).[20-22,24]

We report a large overlap of differentially methylated sites between atherosclerosis settings, especially in the most strongly associated CpG sites, located in 4 major loci: *AHRR, F2RL3, PRSS23,* and an intergenic locus on chromosome 2 near *ALPP/ALPG*.

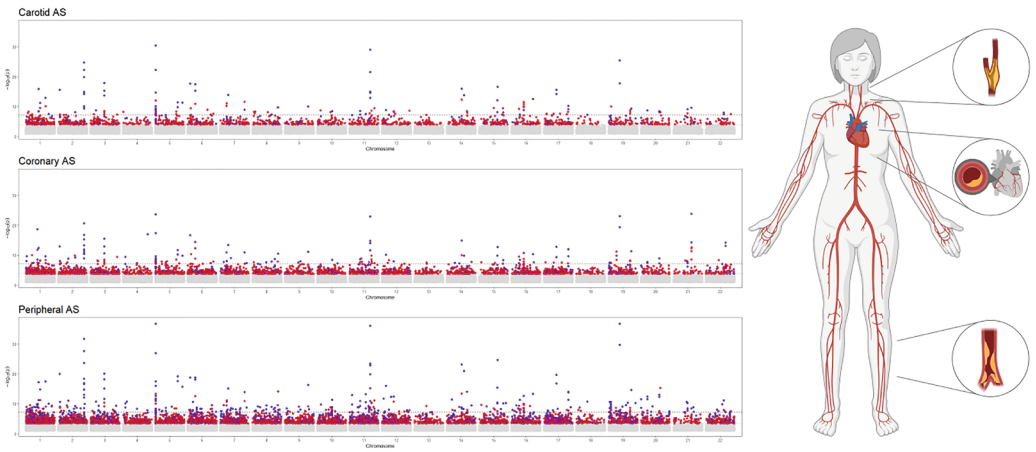
AHRR (aryl hydrocarbon receptor repressor) represses the transcription factor AHR by competing

CENTRAL ILLUSTRATION Blood DNA Methylation Patterns Across Atherosclerosis Subtypes

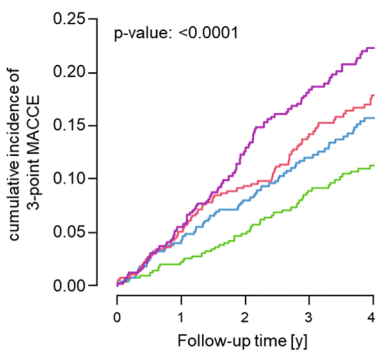
Study design and methods



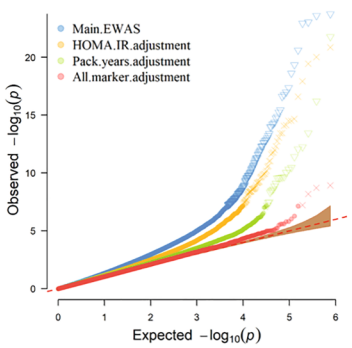
Epigenome-wide association studies detect signals across atherosclerosis subtypes

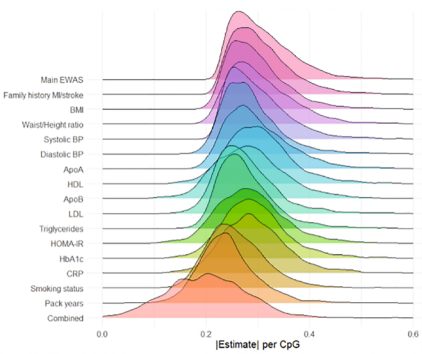


Polyepigenetic atherosclerosis scores predict 3-point MACCE incidence



Upon adjustment of EWAS for smoking pack years alone and CVRF markers combined, p-value inflation and estimates of signficant CpG sites are starkly reduced





Collectively, results suggest that the blood DNA methylation signature associated with atherosclerosis reflects the systemic exposure to cigarette smoke, cardiometabolic risk factors and inflammatory burden

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CVRF = cardiovascular risk factor; EWAS = epigenome-wide association study; MACCE = major adverse cerebrovascular and cardiovascular events.

FIGURE 7 The Association of Atherosclerosis Methylation Patterns With Smoking

Manhattan plots of (A) coronary EWAS for reference, (B) adjustment for pack-years and smoking status, and (C) adjustment for cg05575921 (*AHRR*) as a proxy for smoking. Scatter plots depict the association of the coronary methylation score with (D) pack-years and (E) time since smoking cessation. The median methylation score level in never-smokers is depicted as a dotted green line, and the linear regression fits as solid red line s.

for its binding sites.[25] AHR is a well-known target of dioxins and dioxin-like compounds, mediating their toxicity by changing its DNA-binding properties.[26] AHRR represses these adverse effects, thus playing a protective role against these toxins often found in cigarette smoke.[27] Indeed, *AHRR* methylation has previously emerged as a top association in EWAS of smoking behavior, not only in blood cells,[28] but also in lymphoblasts and lung alveolar macrophages.[29] Compared with self-reported smoking behavior, methylation at the locus has previously been shown to be a superior predictor of chronic obstructive pulmonary disease and lung cancer, suggesting it to be a superior biomarker of long-term smoking exposure.[30]

F2RL3 (F2R-like thrombin/trypsin receptor 3) encodes PAR4 (proteinase-activated receptor 4), which is involved in platelet activation through thrombin and therefore plays a key role in thrombosis.[31] In preclinical experimental studies, PAR4 has been directly implicated in the development of atherosclerosis.[32] Methylation in *F2RL3* also was shown to be linked to smoking, with dose-response relationships between CpG sites and pack years as well as the current number of cigarettes per day.[33] Interestingly, recovery of CpG methylation to nonsmoker levels has been shown gradually take place over the course of >20 years after smoking cessation.[33] Further epidemiologic and in vitro research showed that smoking-induced *F2RL3* methylation changes are likely to causally increase the risk of MI by enhancing platelet reactivity.[34]

Knowledge about PRSS23 (serine protease 23) in the cardiovascular context is more limited. It was shown to play a role in endothelial-to-mesenchymal transition during cardiac valve development in zebrafish[35] and is up-regulated during myocardial infarction in mice,[36] but little is known in the context of atherosclerosis. According to the HIPPIE database,[37] 2 large scale interactome studies report its interaction with CRP, which could be its target.

The intergenic CpG sites on chromosome 2 lie in close proximity to the cluster of germ-line, placental, and intestinal alkaline phosphatases (*ALPG*, formerly called *ALPPL2, ALPP*, and *ALPI*), not to be confused with the tissue-nonspecific alkaline phosphatase *ALPL*, which constitutes >90% of alkaline phosphatase in blood.[38] Serum levels of alkaline phosphatases in general and ALPP in particular are increased in smokers.[39,40] ALPI is known to play a role in innate immunity through the regulation of gut microbial communities[41] and neutralization of proinflammatory bacterial molecules such as lipopolysaccharide,[42] among others.[43] Genetic deficiency of *ALPI* causes inflammatory bowel disease.[44] Crucially, overexpression of human ALPI in Ldlr-deficient mice on Western diet reduced body weight and blood cholesterol and triglyceride levels, and attenuated the development of atherosclerotic lesions compared with control animals.[45]

Two other loci, *ABCG1* and *DHCR24*, were particularly strongly associated with coronary atherosclerosis in this analysis. ABCG1 is a cholesterol and phospholipid efflux carrier, overexpression of which has been shown to attenuate atherosclerosis in rabbits.[46] A possible mode of action is the suppression of cholesterol accumulation in macrophages and therefore differentiation into foam cells.[47] DHCR24 (24-dehydrocholesterol reductase) is an enzyme catalyzing the conversion of desmosterol to cholesterol. In mice, overexpression of *DHCR24* reduced the expression of *ABCG1* and increased plaque and necrotic core size.[48] Mendelian randomization using *DHCR24* expression quantitative trait loci (eQTLs) showed a significant harmful effect of higher expression on atherosclerosis.[48] Counterintuitively, the same study showed that down-regulation of *DHCR24* led to significantly heightened blood LDL levels, suggesting a locally beneficial mode of action within plaques to mediate the reduction in atherosclerosis.[48] However, the gene is located only ~300 kb from *PCSK9*; it is therefore possible that genetic effects detected by means of magnetic resonance and differential methylation in this region are rather connected to this strong effector of blood cholesterol levels.[49]

After adjusting all EWAS for cg05575921 (*AHRR*) as a proxy for smoking exposure, CpGs annotated to the *MYLIP* gene, which encodes myosin regulatory light chain-interacting protein, stood out in all atherosclerotic phenotypes. Its role in the cholesterol metabolism pathway is the degradation of the LDL receptor (LDLR).[50] Differentially methylated CpG sites mapping to *LDLR* itself were also detected in coronary atherosclerosis, with obvious connection to atherosclerosis.[50]

A unique aspect of this investigation is that the epigenetic signatures of atherosclerosis were mapped to the presence of CVRFs, to assess to what extent these might account for the observed differential methylation. Smoking emerged as the largest driver of differential methylation in all atherosclerosis phenotypes, with up to 90% of CpG sites associated with atherosclerosis overlapping with the methylation signature of smoking pack-years. Several analyses pointed to an overwhelming contribution of smoking to the observed methylation patterns: Adjustment for smoking exposure or its proxies

markedly attenuated observed effect sizes, leaving only a few CpG sites significantly associated with atherosclerosis. In addition, the epigenetic signatures of atherosclerosis correlated with pack-years, and were inversely related to years since smoking cessation, with similar levels to never-smokers observed after several decades. Beyond this, atherosclerosis methylation signatures were strongly associated with the pulmonary function markers FEV_1 and FVC. Both markers have extensive evidence of accelerated worsening over time in (former) smokers vs nonsmokers.[51,52]

Systemic inflammation, as captured through CRP levels, was the CVRF most strongly linked to the atherosclerosis methylation patterns after smoking, with an up to 60% overlap in differentially methylated sites with the atherosclerotic methylation signatures. Measures of obesity, diabetes mellitus, and HDL-cholesterol were likewise influential, overlapping with 12% to 44% of the atherosclerosis signatures. Surprisingly, LDL-cholesterol and blood pressure were associated with few methylation sites. The findings on cholesterol are in line with a previous study, where HDL-cholesterol–CpG associations were found in both larger number and with more pronounced effect sizes compared with LDL-cholesterol.[53]

The blood-based differential DNA methylation patterns we detected in atherosclerosis did not strongly differ by vascular bed, rather appearing to represent a potent and stable long-term readout of the systemic exposure to established behavioral, inflammatory, and cardiometabolic risk factors. This observation has several important implications.

First, these findings suggest that, if blood DNA methylation mediates part of the causal pathway from risk factor exposure to atherothrombosis, it is likely that exposures such as smoking, inflammation, and cardiometabolic risk factors exert much larger damage via this pathway than others, particularly hypertension. Although no inference on causality can be made based on this study, previous experimental work has shown that exposure-triggered DNA methylation changes can induce pathologic changes in this context, such as smoking exposure-induced *F2RL3* methylation changes causing elevated platelet reactivity.[34] Rather than merely representing confounding factors to adjust for, this tells us that CVRF-associated maladaptive methylation changes can represent real, pathomechanistically relevant treatment targets. Indeed, a number of companies are moving toward the application of epigenetic editing techniques to a wide range of indications, including dyslipidemias, cancers, and neurodegenerative diseases.[54] More research, including in vitro and in vivo experiments and mendelian randomization studies, are needed to prioritize robust candidate therapeutic targets.

Second, blood-based DNA methylation can serve as an accessible and high-quality surrogate of exposomal factors in studies where this information is not, or not reliably, available. A prime example is smoking, for which methylation-based scores are already available, discriminating current from never-smokers with AUCs of up to 0.98.[13] Methylation scores capturing difficult exposures, such as cannabis use and alcohol consumption,[5] have also been proposed and validated in independent cohorts. This is particularly valuable for epidemiologic studies with biobanking, as these exposures are prone to social desirability bias and difficult to quantify, even when self-reported in good faith.

And third, DNA methylation changes appear to be long-lived, eg, requiring decades to normalize after smoking cessation.[34] Such findings may serve as partial explanation for the epidemiologically reported persistently increased cardiovascular mortality risk in former vs never-smokers detectable up to 19 years after cessation.[55] Implied by this is that behavioral and pharmacologic interventions aimed at acute modification of CVRFs, eg, stopping smoking or initiating antidiabetic medication, may not be sufficient for atherothrombosis prevention. Epigenetic editing could represent a promising adjunct approach to correct these persistent risks.

STUDY LIMITATIONS. Comparisons of results between vascular beds should be treated with caution. Carotid atherosclerosis likely had the lowest detection threshold, because it was phenotyped objectively according to ultrasound-assessed presence of plaques. For the other vascular beds, no ultrasound was performed to detect subclinical lesions not yet obstructing blood flow. Instead, peripheral atherosclerosis was established by self-reporting of PAD or assessment of ABI.[12] Coronary atherosclerosis relied on self-reporting previous diagnoses of coronary artery disease or a history of MI. Therefore, atherosclerosis in each vascular bed may have been recognized at a different stage in its progression. Although training and testing of the atherosclerosis methylation scores was performed in 2 independent cohorts, the initial selection of CpG sites was based on EWAS analyses including both cohorts, potentially resulting in slightly optimistic estimates.

The CVRFs included in this work did not encompass the full spectrum of risk factors. For example, information on diet and physical activity could not be

evaluated in a harmonized manner between cohorts, and was omitted. Regarding alcohol intake, only information on current intake at the time of examination was available; to avoid counterintuitive associations due to selection bias, an equivalent to smoking pack-years would have been needed. For this reason, this risk factor was omitted as well. More generally, the possibility of residual confounding due to factors not considered in the analyses cannot be excluded.

Furthermore, DNA methylation is known to differ between ethnic groups,[56] which could not be investigated in the present cohort comprising almost exclusively white Europeans. Although results from MESA (Multi-Ethnic Study of Atherosclerosis) showed consistent effect sizes of atherosclerosis-specific CpG sites (the majority of which were replicated in this study) among Caucasians, African-Americans, and Hispanics,[20] more research in diverse populations is needed. This is particularly interesting in light of the locus *F2RL3*, polymorphisms in which have shown substantial differences in minor allele frequency between black and white Americans, but also among Sub-Saharan ancestries,[57] with clinically relevant implications on platelet function and atherothrombosis incidence.[58,59] In addition, although CpG methylation patterns highly correlate between tissues in their base state,[60] changes in the methylation state are often tissue specific.[61] Methylation states detected in whole blood may not necessarily generalize to tissues beyond blood cells, instead serving as biomarkers of systemic risk.

CONCLUSIONS

We have identified a large number of novel associations of DNA methylation with atherosclerosis across vascular beds. Of the identified differentially methylated sites, 90% were traceable to epigenetic signatures of CVRFs, and adjustment for these starkly attenuated atherosclerosis effect sizes. We conclude that in blood, the epigenetic signature of atherosclerosis largely reflects cumulative exposure, particularly smoking and cardiometabolic dysregulation, rather than strongly distinguishing vasculature-specific biological processes.

DATA AVAILABILITY. The raw data used in this study constitute protected health information and are under controlled access, in accordance with the consent provided by study participants. Request for data access can be made to the steering committees of the respective studies. EWAS summary statistics will be made available on request to the authors.

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APPENDIX For supplemental material, please see the online version of this paper.