Circulation

ORIGINAL RESEARCH ARTICLE

Blood Leukocyte DNA Methylation Predicts Risk of Future Myocardial Infarction and Coronary Heart Disease

A Longitudinal Study of 11 461 Participants From Population-Based Cohorts

BACKGROUND: DNA methylation is implicated in coronary heart disease (CHD), but current evidence is based on small, cross-sectional studies. We examined blood DNA methylation in relation to incident CHD across multiple prospective cohorts.

METHODS: Nine population-based cohorts from the United States and Europe profiled epigenome-wide blood leukocyte DNA methylation using the Illumina Infinium 450k microarray, and prospectively ascertained CHD events including coronary insufficiency/unstable angina, recognized myocardial infarction, coronary revascularization, and coronary death. Cohorts conducted race-specific analyses adjusted for age, sex, smoking, education, body mass index, blood cell type proportions, and technical variables. We conducted fixed-effect meta-analyses across cohorts.

RESULTS: Among 11 461 individuals (mean age 64 years, 67% women, 35% African American) free of CHD at baseline, 1895 developed CHD during a mean follow-up of 11.2 years. Methylation levels at 52 CpG (cytosine-phosphate-guanine) sites were associated with incident CHD or myocardial infarction (false discovery rate<0.05). These CpGs map to genes with key roles in calcium regulation (ATP2B2, CASR, GUCA1B, HPCAL1), and genes identified in genome- and epigenome-wide studies of serum calcium (CASR), serum calcium-related risk of CHD (CASR), coronary artery calcified plaque (PTPRN2), and kidney function (CDH23, HPCAL1), among others. Mendelian randomization analyses supported a causal effect of DNA methylation on incident CHD; these CpGs map to active regulatory regions proximal to long non-coding RNA transcripts.

CONCLUSION: Methylation of blood-derived DNA is associated with risk of future CHD across diverse populations and may serve as an informative tool for gaining further insight on the development of CHD.

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Clinical Perspective

What Is New?

- Among 11 461 individuals across 9 population-based cohorts from the United States and Europe, differences in blood leukocyte DNA methylation at 52 CpG loci were robustly associated with incident coronary heart disease (CHD).
- Several of the differentially methylated loci map to genes related to calcium regulation and kidney function.
- Exploratory analyses with Mendelian randomization supported a causal effect of DNA methylation on incident CHD at loci in active regulatory regions, with links to noncoding RNAs and genes involved in cellular and tissue structural components.

What Are the Clinical Implications?

- Leukocyte genome regulatory mechanisms, via DNA methylation, are robustly linked to risk of developing CHD.
- Our findings provide the first evidence that genomic regulation via epigenetic modifications in kidney function- and calcium homeostasis-related pathways may be involved in the development of CHD.
- Our findings of epigenetic loci related to noncoding RNAs highlight pathways that have not emerged in genome-wide studies of CHD, and may represent novel therapeutic targets which are thus far unexplored.

Coronary heart disease (CHD) is a major contributor to global morbidity and mortality.[1] Despite substantial progress in CHD prevention, improved approaches are needed to further reduce CHD incidence. Methylation of DNA at CpG (cytosine-phosphate-guanine) dinucleotides is a stable yet environmentally responsive epigenetic regulatory mechanism. DNA methylation at a CpG site is dependent on both underlying genetic variation as well as exposures to environmental factors.[2] In vitro and animal-based studies provide evidence that DNA methylation changes are involved in the development of CHD,[3] and large-scale population-based studies have shown that risk factors for CHD including smoking,[4] obesity,[5] hypertension,[6,7] serum lipids,[8,9] and type-2 diabetes mellitus[10] are linked to persistent differences in leukocyte DNA methylation. Hence DNA methylation, as a molecular bio-archive integrating genetic predisposition and risk factor exposures, may provide further insight on CHD development and identify novel, modifiable, pathways related to CHD. Previous studies of DNA methylation and CHD in humans[11–15] have generally been small in sample size (eg, n < 300), focused on repetitive elements[11,13] or selective genomic regions,[15] or have been cross-sectional or case-control in design.[11,13–15] Whether blood leukocyte DNA methylation predicts future CHD has not been comprehensively investigated.

We conducted a longitudinal, large-scale, multicohort, epigenome-wide investigation of incident CHD events among 11 461 participants in the CHARGE (Cohorts for Heart and Aging Genetic Epidemiology) Consortium.[16] We first assessed whether leukocyte DNA methylation was associated with risk of CHD. We then combined information on the identified CHD-associated methylation sites with genetic sequence variation to provide an integrated genomic map reflecting CHD risk, and evaluated if there was evidence for causal effects of DNA methylation variation on incident CHD.

METHODS

Study Design and Population

We selected cohorts participating in the CHARGE Consortium in which genome-wide leukocyte DNA methylation was assessed using the Infinium 450k microarray, and CHD events were prospectively ascertained. Nine population-based cohorts comprising a total of 11 461 participants from the United States and Europe were included: the ARIC (Atherosclerosis Risk in Communities) study, the CHS (Cardiovascular Health Study), the EPICOR (Long-Term Follow-Up of Antithrombotic Management Patterns in Acute Coronary Syndrome Patients), the FHS (Framingham Heart Study), the InCHIANTI (Invecchiare in Chianti) study, the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) study, the NAS (Normative Aging Study), the WHI-EMPC (Women's Health Initiative "Epigenetic Mechanisms of Particulate Matter-Mediated CVD") ancillary study, and the WHI-BAA23 ("Integrative genomics and risk of CHD and related phenotypes in the Women's Health Initiative") ancillary study (Detailed information in Methods in the online-only Data Supplement). Each cohort study obtained informed consent from participants and protocol approval from its respective institutional review board and ethics committee. DNA methylation data was only collected for African Americans of the ARIC cohort, from the Jackson, MS, and Forsyth County, NC, study sites of the cohort. Cohorts comprising participants of both African American and European Ancestry were analyzed in a race-specific manner. Accordingly, we performed an epigenome-wide analysis within each of the 12 study samples, and then meta-analyzed the resulting summary statistics from the 12 analyses. We also examined the association between DNA methylation and cis-genetic variants (±500 KB) in a subset of the cohorts and conducted Mendelian randomization to evaluate potential causal relations between DNA methylation and incident CHD (Figure 1).

Data and Materials

The DNA methylation datasets from ARIC and CHS data can be requested from the corresponding author. EPICOR data are available upon request from the Human Genetics Foundation; requests should be sent to info@hugef-torino.org. The FHS DNA methylation datasets are available from the dbGAP repository: phs000724. The genotype datasets are available from

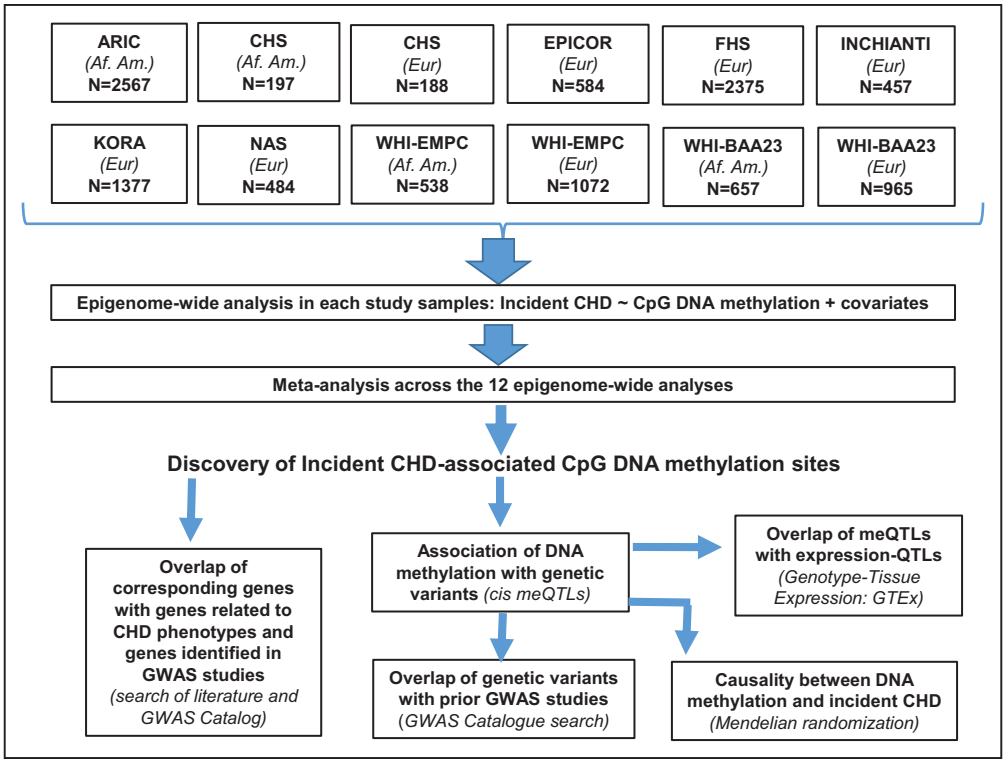

Figure 1. Overall analytic workflow.
Af. Am indicates individuals of African-American ancestry; ARIC, Atherosclerosis Risk in Communities; CHS, Cardiovascular Health Study; CHD, coronary heart disease; EPICOR, Long-Term Follow-Up of Antithrombotic Management Patterns in Acute Coronary Syndrome Patients; Eur, individuals of European ancestry; FHS, Framingham Heart Study; GWAS, genome-wide association study; INCHIANTI, Invecchiare in Chianti; KORA, Kooperative Gesundheitsforschung in der Region Augsburg; meQTLs, methylation quantitative trait loci; NAS, Normative Aging Study; WHI-BAA23, Integrative Genomics and Risk of CHD and related phenotypes in the Women's Health Initiative; and WHI-EMPC, Women's Health Initiative Epigenetic Mechanisms of Particulate Matter-Mediated CVD.

the dbGAP repository: phs000007. The InCHIANTI data are available on request from the corresponding author. The KORA data can be requested at KORA Project Application Self-Service Tool from the Helmholtz Zentrum München German Research Center for Environmental Health. The NAS DNA methylation datasets are available at the dbGAP repository: phs000853. The WHI-BAA23 DNA methylation dataset is available at dbGAP repository phs001335. WHI-EMPC data are available on request from the WHI website or the corresponding author.

Measurement of DNA Methylation

For all cohorts, DNA was extracted from whole blood samples and bisulfite-converted using a Zymo EZ DNA methylation kit. The Illumina Infinium Human Methylation450K BeadChip (Illumina Inc, San Diego, CA) was used to measure DNA methylation. Quality control, filtering, and normalization of the methylation data were independently conducted for each cohort according to established criteria[4,6] and other diagnostics unique to the cohort (details available in Methods in the online-only Data Supplement). For each CpG, methylation = M/(M+U+ε), where M and U are the average fluorescence intensity from the probe (ie, the oligonucleotide that hybridizes to the target CpG) corresponding to the methylated (M) and unmethylated (U) target CpG, respectively, and ε=100 to protect against division by zero. Therefore, the methylation at each CpG is contained in the interval 0–1, with 0 indicating no methylation and 1 indicating 100% methylation at the target CpG across DNA from cells in the sample.

Definition of CHD and Myocardial Infarction Events

Our primary outcome of interest was incident CHD, defined as any of the following: recognized nonfatal or fatal myocardial infarction (MI; hospitalization with diagnostic ECG changes or biomarkers of MI), coronary insufficiency/unstable angina, coronary revascularization, or coronary death. We also conducted a secondary meta-analysis restricted to incident MI-only (recognized nonfatal or fatal MI), to evaluate whether analysis with this more homogenous outcome measure supported robustness of the results.

Statistical Analyses

Individual Study Epigenome-Wide Analyses

Baseline was defined as the time of blood sampling for DNA methylation assays, and all cohorts excluded individuals with prevalent CHD at baseline. Seven cohorts conducted time-to-event analyses using Cox proportional hazard models, and 3 of these cohorts adapted Firth's penalized Cox regression[17] because of a low number of CHD events. Two prospective cohorts, EPICOR and WHI-BAA23, employed a nested case-control design with incident CHD events and performed logistic regression analyses, which, under specific assumptions, provide risk estimates that are unbiased in relation to the estimates derived from Cox regression.[18,19] All analyses were race-specific, and adjusted for age, sex, body

mass index (BMI; kg/m^2), smoking status (current, former, never), education (as years of education or categorical levels of school degrees completed), differential cell counts,[20] family structure (if present), and batch-related technical variables (for additional details see Methods in the online-only Data Supplement).

Meta-Analysis

We performed an inverse variance-weighted fixed-effects meta-analysis using the *metafor* package in R. The fixed-effects method is standard practice in genome-wide studies[21,22] and has been consistently used in previous large-scare epigenome-wide studies in CHARGE and other consortia.[4,6] It is well-documented that using the random-effects approach leads to substantially diminished power,[23] which has major implications for such genome-wide meta-analysis studies that are more exploratory than standard epidemiological studies and are aimed at uncovering new loci previously not possible. However, we also include, as sensitivity analyses, results with random-effects models (described further in the results section). We accounted for multiple-testing by controlling the false discovery rate at 5%. Of the CpGs that exceeded this a priori multiple-testing threshold, we excluded CpGs where the CpG probe sequence harbored a single nucleotide polymorphism (SNP) assayed in the 1000 Genomes Project with a minor allele frequency >0.01 (given that the frequency of underlying genetic variation differs between race/ancestry groups, we excluded CpGs with the potential for underlying SNPs specific to that cohorts race/ancestry), and CpGs that had high inter-study heterogeneity assessed using Cochran's Q test (Q<0.05).

Identification of Associated Genetic Variants and Mendelian Randomization Analyses

We investigated whether genetic variants within ±500 kb (cis) of the incident CHD- and MI-associated CpGs contributed to variation in methylation levels, ie, were methylation-quantitative trait loci (meQTLs). The discovery analysis was conducted on 3868 individuals from the FHS, followed by replication in 1731 individuals from KORA. Genotyping was conducted with the Affymetrix 500K and MIPs 50K platforms in FHS, and the Affymetrix Axiom array in KORA, and imputation was performed using the 1000 Genomes reference panel in both cohorts. meQTLs detected at $P<1x10^{-4}$ in the discovery stage, followed by *P*<Bonferroni threshold (ie, *P*<0.05/number of significant discovery stage meQTLs) at the replication stage were selected for conducting Mendelian randomizations using a 2-sample instrumental variable approach (implemented with MRbase[24]) to infer causal relations between DNA methylation and incident CHD. Single strongest cis-meQTLs were utilized: (1) to minimize potential of horizontal pleiotropy, and (2) because of lack of sufficient meQTLs from independent loci in low linkage disequilibrium. Genotype associations for CHD and MI were obtained from the CARDIoGRAMplusC4D 2015 genome-wide association study (GWAS; n=60 801 cases and n=123 504 controls).[25] We used the meQTLs for differential methylation at CpGs that were significant for a causal relationship between DNA methylation and incident CHD in Mendelian randomization analyses (*P*<0.05) for further inquiry on putative effects on gene expression, by overlapping meQTLs with expression-QTLs (eQTLs) from the Genotype-Tissue Expression (GTEx) resource (The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on 11/25/2018 and 02/10/2019).

RESULTS

Participant characteristics among the 11 461 participants, mean age at baseline was 64 years, 67% were female, and 35% were of African American ancestry (Table 1). During a mean follow-up of 11.2 years, a total of 1895 CHD events and 1183 MI events occurred.

Association of DNA Methylation With Risk of CHD

Among 442 192 CpGs analyzed, methylation levels at 30 CpGs were associated (false discovery rate <0.05) with incident CHD (Table 2; individual forest plots for each CpG in Figure I in the online-only Data Supplement), after excluding CpGs with underlying SNPs that could interfere with probe binding (n=7) and CpGs that demonstrated substantial heterogeneity (Q <0.05) in the meta-analyses (n=8). Methylation levels at 29 CpGs were associated with our secondary outcome of incident MI at a false discovery rate <0.05 (Table 3; individual forest plots for each CpG in Figure II in the online-only Data Supplement), after similarly excluding CpGs with underlying SNPs (n=4) and high heterogeneity (n=5). Additional genomic information on these CpGs are provided in Tables I and II in the online-only Data Supplement. Among these 30 and 29 CpGs identified in the incident CHD and incident MI-only meta-analyses, respectively, 7 CpGs met the false discovery rate <0.05 threshold in both analyses, resulting in 52 unique CpGs identified across the 2 meta-analyses. We found that the direction, magnitude, and precision of estimated effects for these 52 CpGs were highly concordant when comparing results from the 2 different meta-analyses (Figure 2). Manhattan plots indicated that significant associations were distributed across the genome (Figure III in the online-only Data Supplement). Neither meta-analysis was strongly influenced by inflation from technical or batch effects, and both had a uniform distribution of *P* values and symmetry in the coefficient direction of effect (Figure III in the online-only Data Supplement). As results obtained from the secondary, incident MI-only meta-analysis did not materially differ from the primary CHD meta-analysis, we henceforth combined the results from the 2 meta-analyses and simply refer to all 52 CpGs as CHD-associated CpGs.

Table 1. Demographic Characteristics of Participants Across the 9 Cohorts Included in the Study

	Total N	No. CHD Events	No. MI-Only Events	Mean Age (SD)	Female (%)	Smoking Status %former;%current	Mean BMI (SD)	Mean Years of Education or % >HS
ARIC-Af. Am.	2567	389	233	56.5 (5.8)	64%	30%;25%	30.1 (6.3)	13.3 (5.2)
CHS-Af. Am.	197	57	26	72.9 (5.4)	66%	33%;50%	28.8 (5.0)	11.4 (3.7)
CHS-White	188	54	26	76.1(5.1)	55%	41%;13%	27.1 (4.9)	12.5 (3.2)
EPICOR*†	584	292	292	52.9 (7.4)	36%	33%;31%	26.6 (3.8)	11.3 (5.0)
FHS	2375	116	70	65.8 (8.8)	57%	55%;8%	28.1 (5.3)	14.3 (2.6)
InChianti†	457	50	50	62.1 (15.8)	53%	24%;19%	27.1 (3.9)	12%
KORA†	1377	32	32	54.2 (8.8)	51%	37%;17%	27.7 (4.5)	11.4 (2.3)
NAS	484	102	50	72.0 (7.1)	0%	65%;4%	28.0 (4.1)	15.2 (2.9)
WHI (EMPC‡)-Af. Am.	538	38	–	62.7 (7.0)	100%	40%;10%	31.5 (6.0)	62%
WHI (EMPC‡)-Eur,	1072	88	19	64.7 (7.1)	100%	41%;8%	28.8 (5.9)	62%
WHI (BAA23*‡)-Af. Am.	657	254	112	62.9 (6.7)	100%	51%; 2%	31.8 (6.6)	28%
WHI (BAA23*‡)-Eur.	965	423	273	68.3 (6.3)	100%	45%;0%	28.8 (5.7)	30%

The dashed line (–) indicates that WHI (EMPC)-Af. Am. was not included in the MI meta-analyses because the number of MI-only cases was too low (n <10). Af. Am. indicates African American ancestry; BMI, body-mass index; CHD, coronary heart disease; Eur, European ancestry; HS, high school; MI, myocardial infarction; and SD, standard deviation.

*EPICOR and WHI (BAA23) had a case–control design nested within their respective prospective cohorts; all cases and controls were adjudicated during follow-up.

†EPICOR, InChianti, and KORA only contributed MI events to the meta-analyses; thus, all CHD events are MI, and the numbers of events in columns 2 and 3 are the same.

‡EMPC refers to the WHI ancillary study Epigenetic Mechanisms of Particulate Matter-Mediated CVD; BAA23 refers to another sample of WHI in the substudy Integrative Genomics and Risk of CHD and Related Pin the Women's Health Initiative.

Sensitivity-Analyses and Race-Specific Meta-Analyses

We observed highly consistent results when comparing associations for the 52 CHD-associated CpGs from all cohorts (n=11 461) to results from the subset meta-analysis of the 7 cohorts that performed Cox regression (n=9255; Figure IV in the online-only Data Supplement). Similarly, we performed 4 additional meta-analyses, each time excluding 1 of the 4 largest cohorts (FHS, NAS, ARIC, and KORA), and found similar results across these meta-analyses for a majority of the 52 CHD-associated CpGs (Figure V in the online-only Data Supplement).However associations appear to be notably driven by results from ARIC and FHS for 20 of the CpGs. In race-specific meta-analyses, the effect size and direction of effects for the majority of the CHD-associated CpGs were similar when comparing those of European versus African American ancestry (Figure VI in the online-only Data Supplement). However, 11 of the 52 CpGs showed race-specific differences in the association of DNA methylation with incident CHD (P<0.05 for difference in t-statistic; Table III in the online-only Data Supplement). Finally, we also conducted a random-effects sensitivity meta-analysis on the 30 CpGs that were associated with incident CHD (ie, those reported in Table 2). When comparing results from models run under a fixed-effects meta-analysis vs a random-effects meta-analysis, the majority of these 30 CpGs had either the same or very similar effect sizes, and many had the same P value as well. Exceptions to this include: cg22617878, cg02155262, cg06596307, and cg08853494 (Table IV in the online-only Data Supplement).

Associations of DNA Methylation With Genetic Variants

For 10 of the 52 CHD-associated CpGs, we were able to detect and replicate multiple meQTLs for each CpG, comprising 1634 unique SNPs total (full list available as an Excel spreadsheet in the online-only Data Supplement). Across these 1634 meQTLs, we observed overlap with SNPs identified in previous genome-wide association studies on diabetic kidney disease, age-related macular degeneration, prostate cancer, neutrophil count, multiple sclerosis, follicular lymphoma, diffuse large B cell lymphoma, multiple sclerosis, and kidney stones (Table V in the online-only Data Supplement).

Identifying Causal Associations Between DNA Methylation and Incident CHD Using Mendelian Randomization

For each of the 10 CpGs with replicated meQTLs, we proceeded to select the cis-meQTL(+/-500kb) with the lowest P value to utilize as an instrumental variable to model the causal exposure of differential methylation at the 10 CpGs on development of inci-

Table 2. DNA Methylation at 30 CpG Sites Associated With the Risk of CHD*

CpG Name	β Coefficient†	Nominal *P* Value	Hazard Ratio (95% CI)	Gene‡
cg22617878§	–0.3719§	1.99E-08§	0.69 (0.61, 0.79)§	ATP2B2§
cg13827209§	0.2680§	3.76E-08§	1.31 (1.19, 1.44)§	TGFBR1§
cg14185717	–0.2878	1.38E-07	0.75 (0.67, 0.83)	BNC2
cg10307345	–0.1480	1.86E-07	0.86 (0.82, 0.91)	PTPN5
cg13822123	0.4138	2.03E-07	1.51 (1.29, 1.77)	PSME4
cg23245316	–0.4674	2.17E-07	0.63 (0.53, 0.75)	TSSC1
cg24977276	–0.3256	2.54E-07	0.72 (0.64, 0.82)	GTF2I
cg24447788	–0.2679	4.33E-07	0.76 (0.69, 0.85)	(PTBP1‖)
cg08422803	0.1994	7.52E-07	1.22 (1.13, 1.32)	ITGB2
cg01751802	0.1473	9.35E-07	1.16 (1.09, 1.23)	KANK2
cg02449373	0.3715	9.98E-07	1.45 (1.25, 1.68)	FUT1
cg02683350	–0.5062	1.55E-06	0.60 (0.49, 0.74)	ADAMTS2
cg05820312	0.5031	1.60E-06	1.65 (1.35, 2.03)	TRAPPC9
cg06639874	–0.2506	1.83E-06	0.78 (0.7, 0.86)	MLPH
cg06582394	0.1657	1.90E-06	1.18 (1.1, 1.26)	CASR
cg02155262	0.4770	1.97E-06	1.61 (1.32, 1.96)	AGA
cg12766383	–0.6194	1.98E-06	0.54 (0.42, 0.69)	UBR4
cg05892484	–0.5020	2.01E-06	0.61 (0.49, 0.74)	MAD1L1
cg03031868	0.3461	2.29E-06	1.41 (1.22, 1.63)	ESD
cg25497530	–0.2225	2.62E-06	0.80 (0.73, 0.88)	PTPRN2
cg06596307	–0.4198	2.99E-06	0.66 (0.55, 0.78)	IGF1R
cg10702366	–0.1093	3.09E-06	0.90 (0.86, 0.94)	FGGY
cg26470101	0.3052	3.09E-06	1.36 (1.19, 1.54)	(DLX2‖)
cg26042024	–0.3109	3.13E-06	0.73 (0.64, 0.84)	ZFAT
cg00466121	0.4646	3.16E-06	1.59 (1.31, 1.93)	ZNHIT6
cg04987302	–0.3378	3.71E-06	0.71 (0.62, 0.82)	(OTX2-AS1‖)
cg08853494	0.2210	4.03E-06	1.25 (1.14, 1.37)	RCHY1;THAP6
cg26467725	–0.4225	4.22E-06	0.66 (0.55, 0.78)	SLCO3A1
cg06442192	–0.5241	4.89E-06	0.59 (0.47, 0.74)	ZNF541
cg00393373	–0.3156	4.91E-06	0.73 (0.64, 0.84)	ZNF518B

*The CpGs reported as significant do not include X, Y chromosome probes, cross-reactive probes, single nucleotide polymorphism (SNP)–associated probes, or probes that were significant for heterogeneity in the meta-analysis (ie, QEp <0.05).

†Effect estimates represent log hazard ratio per 5% increase in DNA methylation.

‡Gene information is based on Illumina annotation (February 2009 - GRCh37/hg19) assembly).

§CpGs with false discovery rate <0.05 in are shown (Bonferroni-significant sites).

‖For CpG sites annotated to inter-genic regions, information on nearest annotated gene is from R Bioconductor package FDb.InfiniumMethylation.hg19.

dent CHD (Table 4). For 2 of the 10 CpGs, Mendelian randomization analyses supported a causal effect of DNA methylation on incident CHD: cg26470101 (β [95% CI] for 1% increase in DNA methylation =0.042 [0.002, 0.08]; *P*=0.037) and cg07289306 (β [95% CI] for 1% increase in DNA methylation =-0.148 [-0.288, -0.009]; *P*=0.04) on CHD (Table 4). Both CpGs map to regulatory active intergenic regions within CpG islands, and cg07289306 is located proximal to 2 long non-coding RNAs (lncRNA) transcripts[26] (Figure 3).

Expression-QTLs Overlapping With Methylation-QTLs of CpGs Showing Causal Associations Between DNA Methylation and Incident CHD

For methylation at CpGs cg07289306 and cg26470101, which had evidence for causal effects on CHD development in Mendelian randomization analyses, as described above, we took all corresponding meQTLs (n=26 and 261, respectively) to identify whether these meQTLs overlapped with eQTLs, using

Table 3. DNA Methylation at 30 CpG Sites Associated With the Risk of MI*

CpG Name	β Coefficient†	Nominal *P* Value	Hazard Ratio (95% CI)	Gene‡
cg22871797§	–0.599§	5.29E-08§	0.55 (0.44, 0.68)§	CYFIP1§
cg24977276§	–0.366§	9.97E-08§	0.69 (0.61, 0.79)§	GTF2I§
cg18598861	–0.671	1.61E-07	0.51 (0.4, 0.66)	IRF9
cg09777776	0.287	2.25E-07	1.33 (1.19, 1.48)	ZNF254
cg20545941	–0.885	2.47E-07	0.41 (0.29, 0.58)	MPPED1
cg19935845	–0.336	4.65E-07	0.71 (0.63, 0.81)	TNXB
cg24423782	–0.398	5.37E-07	0.67 (0.58, 0.78)	MIR182
cg00393373	–0.401	7.68E-07	0.67 (0.57, 0.79)	ZNF518B
cg00466121	0.487	7.79E-07	1.63 (1.34, 1.97)	ZNHIT6
cg19227382	–0.504	8.12E-07	0.60 (0.49, 0.74)	CDH23
cg03467256	–0.408	8.33E-07	0.67 (0.57, 0.78)	HPCAL1
cg25196881	–0.269	1.05E-06	0.76 (0.69, 0.85)	(THBS1‖)
cg02321112	0.390	1.08E-06	1.48 (1.26, 1.73)	(MNX1-AS1‖)
cg00355799	–0.216	1.40E-06	0.81 (0.74, 0.88)	(LOC339529‖)
cg17556588	–0.154	1.45E-06	0.86 (0.8, 0.91)	PRRG4
cg04987302	–0.428	1.50E-06	0.65 (0.55, 0.78)	(OTX2-AS1‖)
cg07289306	0.616	1.71E-06	1.85 (1.44, 2.38)	(MIR138-1‖)
cg05892484	–0.551	1.84E-06	0.58 (0.46, 0.72)	MAD1L1
cg10702366	–0.150	2.11E-06	0.86 (0.81, 0.92)	FGGY
cg22618720	–0.424	2.37E-06	0.65 (0.55, 0.78)	(MIR5095‖)
cg14010194	–0.484	2.71E-06	0.62 (0.5, 0.75)	GUCA1B
cg13827209	0.285	2.71E-06	1.33 (1.18, 1.5)	TGFBR1
cg24318598	–0.254	2.79E-06	0.78 (0.7, 0.86)	ANO1
cg07015775	0.479	3.13E-06	1.61 (1.32, 1.97)	ZNHIT6
cg21018156	–0.135	3.17E-06	0.87 (0.83, 0.92)	(LINC01312‖)
cg07475527	–0.225	3.77E-06	0.80 (0.73, 0.88)	(RCAN3‖)
cg20000562	0.218	3.93E-06	1.24 (1.13, 1.36)	SFTA3
cg07436807	–0.779	4.10E-06	0.46 (0.33, 0.64)	STAMBPL1; ACTA2
cg14029912	–0.367	4.29E-06	0.69 (0.59, 0.81)	(BHLHE40‖)

*The CpGs reported as significant do not include X, Y chromosome probes, cross-reactive probes, single nucleotide polymorphism (SNP)–associated probes, or probes that were significant for heterogeneity in the meta-analysis (ie, QEp <0.05).

†Effect estimates represent log hazard ratio per 5% increase in DNA methylation.

‡Gene information is based on Illumina annotation (February 2009 – GRCh37/hg19) assembly.

§CpGs with false discovery rate <0.05 in are shown (Bonferroni-significant sites).

‖For CpG sites annotated to inter-genic regions, information on nearest annotated gene is from R Bioconductor package FDb.InfiniumMethylation.hg19.

the GTEx catalogue. For cg07289306, we found that 26 of 28 meQTLs for this CpG overlap with eQTLs for a lncRNA downstream of cg07289306: lncRNA RP4-555D20.2. Similarly, we found that for the 261 meQTLs detected and replicated for cg26470101, 84 overlapped with an eQTL for the ITGA6 (integrin subunit α 6) gene.

DISCUSSION

We conducted a large-scale analysis of DNA methylation in relation to incident CHD and MI among 11 461 adults across multiple cohort studies. Methylation at 52 CpGs across the genome were associated with future risk of CHD and MI. A 5% increase in methylation of identified CpGs was related to differences in CHD risk of a clinically relevant magnitude, ranging from a 46% decrease in the risk of CHD (cg12766383) to a 65% increase in risk (cg05820312), independent of age, sex, and other known CHD risk factors. In exploratory analyses to highlight candidates for functional experimentation, Mendelian randomization analyses revealed that methylation at 2 loci had a causal effect on incident CHD, potentially via non-coding RNA regulation and tissue structural elements.

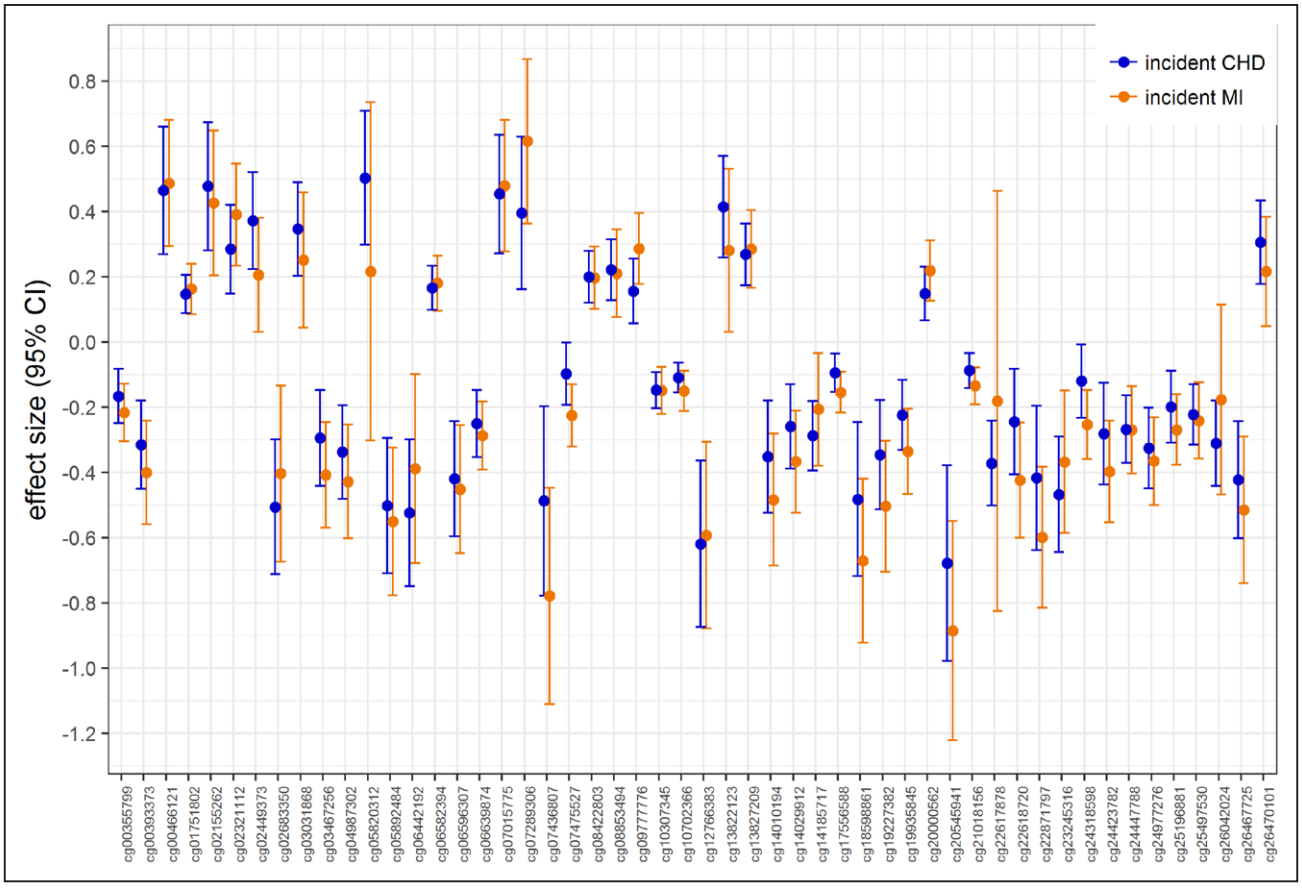

Figure 2. Plot of effect sizes (ie, log hazard ratios) and 95% CIs for the 52 coronary heart disease (CHD)–associated CpG DNA methylation sites, comparing results from the incident CHD meta-analysis (blue) vs the incident myocardial infarction (MI)–only meta-analysis (orange).

Biological Relevance and Clinical Implications

Several of the 52 CHD-associated CpGs in our study map to genes with roles in calcium regulation, as well as genes that have been identified in association with calcium levels and kidney function in previous GWAS and DNA methylation studies. CpG cg2261787 maps to the ATP2B2 (ATPase plasma membrane calcium transporter 2) gene from the plasma membrane calcium transporter family with critical roles in intracellular calcium homeostasis. Similarly, CpG cg06582394 maps to the CASR (calcium sensing receptor) gene, which has a key role in calcium homeostasis. SNPs in CASR have been consistently associated with serum calcium in populations from several different ancestries.[27–29] Furthermore, in a recent Mendelian randomization analysis of 184 305 individuals, Larsson et al[28] reported that a genetic variant at the CASR locus showed strong associations with coronary artery disease and MI. Similarly, CpGs cg14010194 and cg03467256 map to GUCA1B (guanylate cyclase activator 1B) and HPCAL1 (hippocalcin-like 1), respectively, both with roles in calcium-dependent regulation.[30,31]

We also identified CHD-associated CpG loci linked to renal function. CpGs cg19227382, cg03467256, and cg25497530 map to genes CDH23 (cadherin-related 23), HPCAL1, and PTPRN2 (protein tyrosine phosphatase receptor type N2), respectively. Both CDH23 and HPCAL1 were identified in a GWAS of kidney function in approximately 64 000 participants of European decent.[32] An epigenome-wide study of 400 individuals showed differential blood DNA methylation at the PTPRN2 locus in chronic kidney disease cases relative to controls.[33] However, genetic variants in PTPRN2 were also associated with coronary artery calcified atherosclerotic plaque in a meta-analysis GWAS among African Americans with type-2 diabetes mellitus.[34]

Observational studies and calcium supplementation randomized clinical trials provide evidence of associations between serum calcium levels and increased risk of CHD and MI.[35,36] Our results provide the first evidence that epigenetic regulation of calcium homeostasis may be involved in calcium-related CHD risk, an underdeveloped area of therapeutics. Similarly, kidney function is a well-recognized risk factor for CVD, with a recent American Heart Association report highlighting that individuals with an estimated glomerular filtration rate of 15 to 30 mL/min per 1.73 m^2 have the highest adjusted relative risk of CVD mortality.[37,38] Our results suggest that epigenetic regulation may be involved in pathways

Table 4. Mendelian Randomization Analysis for Assessing Causality Between DNA Methylation and Incident CHD, Using Identified meQTLs as Genetic Proxies

Exposure = DNA Methylation			Genetic Proxy as Instrumental Variable = SNP Associated With DNA Methylation (meQTL)			Outcome = CHD (CC4D 2015)			Outcome = MI (CC4D 2015)		
CpG	CpG Genomic Position	Gene or Nearest Gene*	Associated SNP (meQTL)†	SNP Genomic Position	SNP Fx Allele	Causal Estimates From Mendelian Randomization			Causal Estimates From Mendelian Randomization		
						β	SE	*P* Value	β	SE	*P* Value
cg01751802	19:11309639	KANK2	rs3745682	19:11313256	G	0.03	0.91	0.978	−0.45	1.02	0.660
cg06582394	3:121902622	CASR	rs9883099	3:121902945	A	0.94	0.78	0.228	0.37	0.87	0.667
cg06639874	2:238417703	MLPH	rs10187185	2:238416524	C	−0.40	1.27	0.751	0.29	1.37	0.832
cg07289306	3:44039357	(MIR138−1‡)	rs28731098	3:44019816	A	−14.84	7.13	0.037	−17.58	7.96	0.027
cg08422803	21:46341067	ITGB2	rs4050931	21:46346135	T	1.24	1.13	0.273	1.70	1.24	0.170
cg10307345	11:18771567	PTPN5	rs12575661	11:18771648	C	−0.41	0.63	0.518	−0.38	0.70	0.588
cg13827209	9:101912842	TGFBR1	rs1013186	9:101884337	C	−0.26	2.73	0.925	−3.64	3.01	0.226
cg14010194	6:42152817	GUCA1B	rs2395805	6:42163839	T	2.15	1.96	0.275	−1.08	2.16	0.618
cg25497530	7:158059944	PTPRN2	rs6953878	7:157943641	G	1.88	2.12	0.374	1.70	2.34	0.467
cg26470101	2:173099597	(DLX2‡)	rs2054832	2:173080117	C	4.24	2.04	0.037	4.13	2.16	0.056

Mendelian randomization analyses produce causal estimates for 1 unit increase in DNA methylation, which corresponds to 100% increase in DNA methylation. For a more appropriate interpretation, these estimates, shown here, are reported and discussed in the manuscript after converting them to correspond to a 1% increase in DNA methylation. CHR indicates chromosome; CHD, coronary heart disease; meQTL, methylation quantitative trait loci; MI, myocardial infarction; and SNP, single nucleotide polymorphism.

*Gene information are based on Illumina annotation (February 2009 – GRCh37/hg19) assembly). For CpG sites annotated to intergenic regions, information on nearest annotated gene (shown with ‡) is from R Bioconductor package FDb.InfiniumMethylation.hg19.

†For each CpG, we observed multiple meQTLs, but only selected the meQTL with the lowest *P* value / highest R^2-explained for reporting, and for conducting the Mendelian randomization analyses.

linking kidney function to CHD risk. We do note that since our analyses were adjusted for major risk factors such as smoking and BMI, we may not have identified BMI- and smoking-specific methylation signatures related to incident HD. Our goal was to identify methylation signatures related to CHD beyond major known risk factors such as smoking and BMI, and we direct readers to major epigenome-wide meta-analyses studies on BMI[39] and smoking[4] that have previously been published.

Other gene loci identified include the IGF1R (insulin growth factor 1 receptor), TGFBR1 (transforming growth factor β receptor 1), and ITGB2 (integrin subunit β 2). The roles of IGF1R and the TGF-β signaling in cardiac remodeling and function are well recognized,[40,41] and recently TGFBR1 gene expression levels in blood samples from acute MI patients strongly predicted left-ventricular dysfunction.[42] Furthermore, ITGB2 encodes a leukocyte cell-surface adhesion molecule that directly facilitates leukocyte transendothelial migration, a key step in formation of atherosclerosis.[43]

DNA methylation at CpGs cg26470101 and cg07289306 showed evidence of a causal effect on CHD. Both CpGs are located within CpG islands in intergenic regions, with cg07289306 located proximal to 2 lncRNAs. Furthermore, meQTLs for cg07289306 overlap with the eQTLs for a lncRNA downstream of cg07289306: lncRNA RP4-555D20.2. This suggests that methylation at cg07289306 may be part of regulatory pathways involving lncRNAs. Increasing evidence indicates that lncRNAs are key components of transcriptional regulatory pathways that govern cardiac development and cardiovascular pathophysiology.[44,45] Similarly, meQTLs we identified for CpG cg26470101 overlapped with eQTLs for ITGA6 (integrin subunit α 6) transcript expression. In a study of left ventricular myocardium tissue in mice, Lodder et al[46] assessed collagen levels combined with genome-wide genotyping and cardiac expression analyses and found that eQTLs for ITGA6 transcripts overlapped with QTLs related to cardiac collagen deposition. They report their findings to suggest that ITGA6 is an important part of the molecular network modulating collagen deposition in the heart. In another study of murine cardiac tissue,[47] ITGA6 was 1 of 6 identified (immune response) genes with decreased expression profiles in cardiac tissue macrophages from older mice compared to that of young mice. Our findings, in the context of the findings from these other studies, may suggest that DNA methylation plays a role in premature cardiovascular aging and risk of CHD via non-coding RNA as well as tissue cellular structural elements.

Findings in the Context of Previous Evidence

Our epigenome-wide study identifies numerous loci and related genes and pathways that have not been

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Figure 3. Adapted UCSC genome browser image for genomic location of CpGs cg07289306 and cg26470101.
The red zoom-in triangles are our addition to the UCSC image (http://genome.ucsc.edu), and represent a magnified region corresponding to the red marked region on each chromosome. The yellow highlights are our addition and highlight the exact genomic location of each CpG.

identified in incident CHD GWAS alone. Furthermore, our findings did not overlap with those of previous epigenome-wide studies of CHD, as previous studies were small and predominantly cross-sectional. Cross-sectional studies may identify CpGs and associated genes and pathways that are altered as a result of disease state, rather than the prospective design employed in our study which may be identifying loci involved in pathways preceding manifest disease. Previous studies were also often composed of select populations geographically and ethnically distinct from the populations in our meta-analysis. For example, Sharma et al identified differentially methylated regions near or within genes C1QL4, CCDC47, and TGFBR3 in a study of 36 men (18 CAD, 18 controls) from India.[15] Nakatochi et al[14] compared 192 MI cases with 192 controls in an epigenome-wide whole-blood analysis on elderly Japanese individuals, and reported DNA methylation at 2 CpGs, located in the ZFHX3 and SMARCA4 genes, to be associated with MI. In the prospective Italian EPICOR cohort, Guarrera et al[12] compared 292 MI cases with 292 matched controls ascertained prospectively during follow-up, and reported that a differentially-methylated region within the ZBTB12 (zinc finger and BTB domain containing 2) gene body was associated with MI.

Study Limitations

We used well-established statistical procedures to remove the effect of cell-type heterogeneity as a source of confounding,[20,48] however residual confounding is still possible. Further, while we used a stringent threshold to exclude any results for which there was between-study heterogeneity, some degree of heterogeneity is likely and may affect the results observed. However, some element of the heterogeneity likely reflects racial and environment specific sources of methylation differences. Additionally, our Mendelian randomization analyses provide evidence supporting a causal role of methylation at 2 CpGs but this does not prove causality, and thus, follow-up experimental work is needed. Currently, leukocyte-specific and trans-tissue meQTL datasets are limited with relatively small sample sizes, thus limiting our ability to use multiple independent meQTL loci for multi-SNP instrumental variables in Mendelian randomization analyses. Another limitation is the relatively large contribution from cohorts based in primarily Western countries in Europe and the United States because of the current limited availability of DNA methylation and incident CHD data in more ethnically diverse cohorts.

Study Strengths

Our study is by far the largest of its kind, with nearly 12 000 participants. We also made use of incident cases that were stringently adjudicated over a long-term follow-up. Furthermore, we used Mendelian randomization to build evidence regarding causal effects of DNA methylation on incident CHD.

Conclusions

We present novel and robust findings on associations of leukocyte DNA methylation with risk of CHD, with

effect sizes that are of a clinically relevant magnitude. In addition, our findings highlight known, as well as under-recognized, pathways to CHD, including calcium regulation, kidney function, and gene regulation mechanisms involving non-coding RNAs. Overall, the findings provide a deeper understanding of the molecular landscape of incident CHD and may present novel avenues for targeting disease pathways and development of therapeutic interventions.

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