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ORIGINAL ARTICLE

Epigenome-wide association study reveals differential DNA methylation in individuals with a history of myocardial infarction

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Abstract

Cardiovascular diseases (CVDs) are the leading causes of death worldwide and represent a substantial economic burden on public health care systems. Epigenetic markers have potential as diagnostic markers before clinical symptoms have emerged, and as prognostic markers to inform the choice of clinical intervention. In this study, we performed an epigenome-wide association study (EWAS) for CVDs, to identify disease-specific alterations in DNA methylation. CpG methylation in blood samples from the northern Sweden population health study (NSPHS) ($n = 729$) was assayed on the Illumina Infinium HumanMethylation450 BeadChip. Individuals with a history of a CVD were identified in the cohort. It included individuals with hypertension ($N = 147$), myocardial infarction (MI) ($N = 48$), stroke ($N = 27$), thrombosis ($N = 22$) and cardiac arrhythmia ($N = 5$). Differential DNA methylation was observed at 211 CpG-sites in individuals with a history of MI ($q < 0.05$). These sites represent 196 genes, of which 42 have been described in the scientific literature to be related to cardiac function, cardiovascular disease, cardiogenesis and recovery after ischemic injury. We have shown that individuals with a history of MI have a deviating pattern of DNA methylation at many genomic loci of which a large fraction has previously been linked to CVD. Our results highlight genes that might be important in the pathogenesis of MI or in recovery. In addition, the sites pointed out in this study can serve as candidates for further evaluation as potential biomarkers for MI.

Introduction

Cardiovascular disease (CVD) is among the leading causes of death worldwide and the number of deaths is predicted to increase even further (1,2). CVD is an umbrella term for several conditions affecting the heart, blood and vasculature of the body e.g. stroke, hypertension, thrombosis, myocardial infarction (MI), and cardiac arrhythmia. There are numerous known risk factors for developing CVDs, such as age, high blood pressure, tobacco smoking and obesity (2). Genome wide association studies have also identified more than fifty genomic loci associated with coronary artery disease (3–5). Despite known risk factors and the identification of genetic risk factors, much of the causality that underlies CVD remains undetermined. Data on epigenetic alterations, which represent genomic responses to environmental factors, also have the potential to provide insights into the genesis and progression of complex diseases such as CVDs (6).

Previous studies have reported increased global levels of DNA methylation in patients diagnosed with CVDs (2) as well as in patients with confirmed coronary artery disease (CAD) (7).

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A candidate gene-oriented studies have also reported associations between gene-specific methylation and atherosclerosis, CAD, hypertension, heart failure and stroke (8). One epigenome-wide association study (EWAS) for CAD has previously been performed utilizing the HCGI12K array, which contains probes for 12,192 CpG islands (9). Even though the sample size was limited (18 cases and 18 controls), as many as 72 differentially methylated regions (DMRs) were reported to be associated with CAD (9).

During the last years, genome-wide assays for high resolution mapping of DNA methylation at CpG sites has become available. In this study, we present the results from an EWAS for CVDs performed on blood samples from a cross-sectional study cohort; the Northern Sweden Population Health Study cohort (NSPHS). CpG methylation was assayed by utilizing the Ilumina Infinium HumanMethylation450 BeadChip, which interrogates approximately 485,000 individual sites across the genome.

Results

Clinical characteristics of the study cohort are presented in Table 1 and Table 2. After quality control of the DNA methylation, 729 participants (Table 1a) and 470,789 autosomal CpG sites remained for EWAS analyses. The NSPHS is a cross-sectional study and participants were not ascertained due to disease status. However, a total of 238 participants had experienced one or more CVDs or related risk factors, such as hypertension or type two diabetes (Table 2). Hypertension was reported by 147 participants; diabetes by 51; myocardial infarction by 48, stroke by 27, thrombosis by 22 and cardiac arrhythmia by five.

EWAS revealed that a history of MI was associated with altered methylation at 211 individual CpG sites (Fig. 1). These sites mapped to 196 individual genes (Supplementary Material, Table S1). Some degree of inflation in low *P*-values could be observed ($\lambda = 1.44 \pm 2.6\text{E-}5$) (Supplementary Material, Fig. 1), which is probably due to global alterations in CpG methylation following MI and consistent with previous reports on global hypermethylation in patients with a history of MI (2). A review of the published literature revealed biological function related to cardiovascular disease, cardiogenesis and recovery after ischemic injury for 42 of the MI-associated probe-linked genes (Table 3). For example, altered methylation at CpG-sites related to a number of cardioprotective genes, with known functions in recovery after an ischemic event, was observed, i.e. *DYSF*, *SFRP4*, *NRG1*, *BNIP3* and *GDF15* (Table 3). All significant associations were reanalysed with BMI excluded from the model to evaluate if BMI might influence the association between the disease phenotypes and DNA methylation. The re-analysis resulted in very similar P-values indicating that BMI did not influence the association between disease phenotypes and DNA methylation.

Enrichment analyses were performed on the results from the MI EWAS. A total of 19,746 gene names were entered into GOrilla (10) of which 19,351 were recognized. Twenty-four duplicated genes were removed, which left a total of 19,327 genes for the enrichment analyses. Of these, 16,826 genes were associated with GO terms. GOrilla identified 519 enriched biological

Table 1. Characteristics of the NSPHS cohort

N cases/control	*N* (men/women)	Age (years)	BMI	Weight (kg)	Height (cm)
238/491	341/388	47.4 ± 20.9	26.5 ± 4.9	71.6 ± 15.5	164.3 ± 9.6

Table 2. Prevalence and co-morbidity of CVD and diabetes in NSPHS participants (n)

Diagnosis	Hypertonia	Myocardial infarction	Stroke	Diabetes	Thrombosis	Cardiac arrhythmia
Hypertension	147	29	17	30	12	3
Myocardial infarction		48	11	11	9	1
Stroke			27	6	10	2
Diabetes				51	7	1
Thrombosis					22	4
Cardiac arrhythmia						5

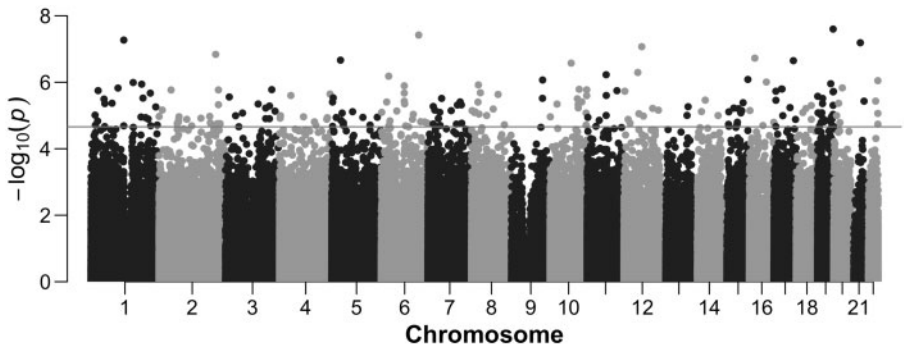

Figure 1. Manhattan plot showing chromosomal locations of –log10 (*P*-values) of all CpG sites in MI. The dashed line designates 5% FDR ($P \geq 2.19\text{E-}05$).

Table 3. Probe-linked genes with biological roles in cardiac function, cardiac development and CVD

ID	P	Closest gene	Description	Related biological role
cg01926051	2.13E-06	*ESRRG*	Aestrogen-related receptor gamma	Transcriptional coordinator of cardiac energy production (32)
cg06201642	4.28E-06	*ST6GALNAC5*	α-N-acetylgalactosaminide α-2.6-sialyltransferase 5	Mutations identified in family with CAD (33)
cg07914084	5.52E-06	*RYR2*	Ryanodine receptor 2	Regulation of calcium influx. Mutations linked to CAD (34)
cg23716800	1.11E-05	*NMNAT2*	Nicotinamide nucleotide adenylyltransferase 2	Regulator of cardiotrophic processes (35)
cg08137080	1.95E-05	*EPHA2*	EPH receptor A2	Regulates inflammation and cardiomyocyte death after ischemic events (36, 37)
cg27508144	2.04E-05	*TGFB2*	Transforming growth factor β 2	Mutations associated with cardiac arrest in CAD-patients (38)
cg18303215	1.71E-06	*ABCG5*	ATP-binding cassette sub-family G member 5	Associated with lower LDL-C and reduced risk for CAD outcomes (39)
cg26179400	1.21E-05	*FMNL2*	Formin-like 2	Involved in myofibrillogenesis (40)
cg00672622	1.35E-05	*DYSF*	Dysferlin	Cytoprotective following myocardial ischemia (41)
cg25721451	1.75E-05	*MEIS1*	Homeobox protein Meis1	Cardiogenesis (42)
cg10953508	1.67E-06	*MECOM*	MDS1 and EVI1 complex locus protein EVI1	Associated with BP through GWAS (43)
cg00730653	2.77E-06	*WNT7A*	Protein Wnt-7a	Differentiation of cardiac conduction cells (44)
cg25933341	7.42E-06	*SOX2*	Transcription factor SOX-2	Induction of induced pluripotent stem cells for cardiac regeneration (45)
cg02774439	1.17E-05	*HAND2*	Heart and neural crest derivatives expressed 2	Cardiac transcription factor (46)
cg24573501	7.68E-06	*F2RL1*	Proteinase-activated receptor 2	Genetic variants associated with blood pressure and obesity (47)
cg23615676	1.14E-05	*KCNN2*	Small conductance calcium-activated channel protein 2	Susceptibility locus for coronary artery aneurysms in Kawasaki disease (48)
cg10948359	1.28E-06	*ME1*	NADP(+)-dependent Malic enzyme	Possibly related to hypertension onset (49)
cg26936429	4.47E-06	*TBX18*	T-box transcription factor TBX18	Cardiogenesis (50)
cg01912921	8.03E-06	*FOXC1*	Forkhead box protein C1	Reported as mutated in a family with Axenfeld-Rieger syndrome with congenital heart diseases (51)
cg01578017	7.15E-06	*SEMA3D*	Semaphorin 3D	Mutated in a case of congenital heart defect (52)
cg08261094	1.23E-05	*SFRP4*	Secreted frizzled-related protein 4	Cardioprotective after ischemic injury (53)
cg02621087	1.71E-05	*LMOD2*	Leiomodin 2	Formation of actin filaments in cardiomyocytes (54)
cg14391419	1.80E-05	*TWIST1*	Twist basic helix-loop-helix transcription factor 1	Sequence variations associated with ventricular septal defects (55)
cg17457560	2.08E-06	*NRG1*	Neuregulin 1	Myocardial repair following infarction (56, 57)
cg03079395	3.95E-06	*NKX2-6*	Homeobox protein NKx-2.6	Mutated in cases of congenital heart disease (58)
cg10090985	7.74E-06	*DLC1*	Rho GTPase-activating protein 7	Mutations observed in cases of congenital heart disease (59)
cg14919250	8.16E-06	*MIR598*	MicroRNA 598	Implicated in 8p23.1 duplication syndrome (60)
cg09626193	1.00E-05	*SOX17*	Transcription factor SOX-17	Cardiogenesis (61)
cg22473973	2.54E-06	*BNIP3*	BCL2/adenovirus E1B 19kda protein-interacting protein 3	Cardioprotection (62)
cg23944251	4.54E-06	*GPR158*	Probable G protein-coupled receptor 158	Implicated in age-dependant cardiac collagen deposition (63)
cg21052682	5.93E-07	*FGF19*	Fibroblast growth factor 19	Cardiogenesis (64)
cg15269503	2.50E-06	*ANO1*	Anoctamin 1	Implicated in ischemia induced cardiac arrhythmias (65)
cg24325551	2.12E-05	*WT1*	Wilms tumor protein	Expressed in cardiac resident stem cells (46)
cg23855989	5.07E-07	*AQP5*	Aquaporin 5	Mutation associated with blood pressure (66)
cg02781660	1.59E-05	*ALDH1A2*	Retinal dehydrogenase 2	Marker for epicardial lineage (67)
cg17658822	1.81E-05	*CGNL1*	Cingulin-like protein 1	Endocardial marker (68)
cg09320690	1.11E-06	*EHD2*	EH-domain containing protein 2	Cardiomyocyte membrane targeting protein (69)
cg16008327	3.06E-06	*GDF15*	Growth differentiation factor 15	Cardioprotective in CVD (70, 71)
cg07857792	4.39E-06	*KCNN1*	Small conductance calcium-activated potassium channel protein 1	Implicated in atrial and ventricular fibrillation (72)
cg22736850	4.30E-06	*OVol02521*	Transcription factor Ovo-like 2	Vascular angiogenesis (73)
cg00051068	1.01E-05	*JAG1*	Protein jagged 1	Mutated in Alagille syndrome (74)
cg25127852	8.95E-07	*MLC1*	Membrane protein MLC1	Implicated in ischemia/reperfusion injury (75)

Table 4. Potential overlapping MI-associated CpG sites and CVD-associated genetic loci

Genetic Locus	Chr	Leading SNP	Position	CpG site	Position	Distance (bp)	**Juicebox*(Rao, Huntley et al. 2014)**	WashU†
IL6R	1	rs4845625	154421817	cg00818872	154540270	118453	+	–
ABCG5-ABCG8	2	rs6544713	44073631	cg18303215	44059002	14629	+	+
HDAC9	7	rs2023938	19036525	cg14391419	19158646	122121	+	–
BCAP29	7	rs10953541	107244295	cg09660227	107643924	399629	+	+
RAB3D	19	rs1122608	11163601	cg05896042	11450089	286488	–	+
JAG1	20	rs1327235	10969030	cg00051068	10655610	313420	+	+ (Figure 2).

*'+' Data indicate CpG site and SNP to be located within the same topologically associated domain. '–' Data were not informative. † '+' ChIA-PET data support long range intereactions across genomic region with potential for interaction between CVD-associated loci and MI-associated DMPs. '–' Data were not informative..

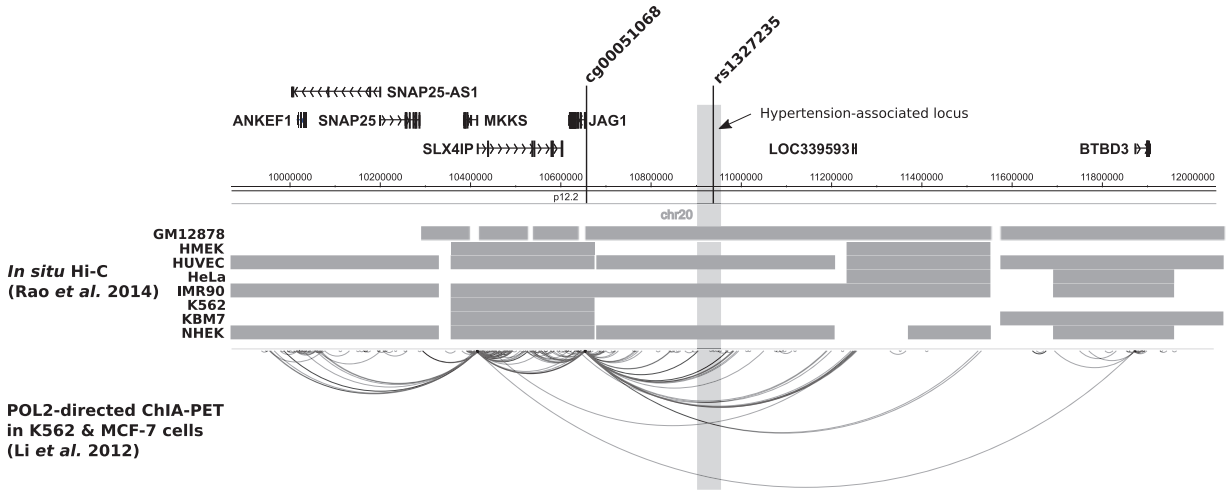

Figure 2. Long-range chromatin-chromatin interaction data from RNA polymerase 2 (POL2) -directed ChIA-PET (20), and *in situ* Hi-C experiments (23). Topologically associated domains predicted by Hi-C experiments on eight cell lines data from are visualized as bars. ChIA-PET data from experiments on two cell lines, MCF-7 breast cancer cells and K562 chronic myeloid leukemia cells, are represented as arcs which describe the two genomic regions that were co-precipitated with a POL2 antibody after formaldehyde-induced cross-linking. The hypertension-associated locus is highlighted in grey.

processes. At an allowed similarity of 0.5, REVIGO (11) reduced the list of enriched GO terms generated by GOrilla to 31 parent GO terms (Supplementary Material, Table S2) with strong enrichment for terms clustering under parent terms related to locomotory behavior, central nervous system neuron differentiation, calcium ion transport and regulation of synapse organization (Supplementary Material, Table S2). For the Enrichr-analysis (12), we utilized the list of 211 MI-associated CpG-sites. Sites where the methylation levels were significantly associated with a disease phenotype, and for which no gene annotation were available, were assigned to the nearest protein-coding transcript by examining sites manually using the UCSC genome browser (13). Comparison against the pathway databases KEGG (14,15) and Wikipathways (16) revealed enrichment of genes within the ERBB- and BDNF-signalling pathways, as well as genes related to neural crest differentiation. Comparison against the curated biological pathway database Reactome (17,18) revealed enrichment of genes related to neuronal systems and a number of categories relating to transmembrane ion transport and synaptic signalling (Supplementary Materials, Tables S3–S5).

Despite the large number of participants in NSPHS with hypertension, no associations of CpG methylation with hypertension were observed. Neither could any association be detected between CpG methylation and stroke, or thrombosis. Cardiac arrhythmia was excluded due to the low number of cases ($N = 5$).

Analysis of genomic function

A total of six of our MI-associated CpG-sites overlapped with previously identified CVD genome-wide association study (GWAS) loci (5,19) (Table 4). We performed additional analyses to test for association between leading SNPs at CVD-loci with methylation at proximal MI-associated CpG-sites as well as with MI. We were unable to detect associations between CVD-associated SNPs with MI or with methylation at proximal MI-associated CpG-sites (<0.05 for all tests). However, available data from chromatin-chromatin interaction studies reveal possible long-range interactions between MI-associated CpG-sites with previously reported CVD loci (Table 4). For example, long range chromatin-chromatin interaction data from the genome institute of Singapore (GIS) (20) were indicative of a series of long-range interactions between putative regulatory elements upstream of *JAG1*, which spans a hypertension-associated GWAS locus (19), with the *JAG1* promoter (Fig. 2) where one of our MI sites (cg00051068) is located. This points towards a possible link between our CpG site and the previously identified hypertension locus, despite their distance of about 320kb. Another potential overlap is between cg05896042 (chr19:11,450,089), which is located within the promoter region of *RAB3D*, and rs1122608, which is an intronic SNP within *SMARCA4*. Rs1122608 is located about 40kb upstream from *LDLR*, which has been associated with early-onset myocardial infarction (21) and aortic

calcification (22). GIS chromatin-chromatin interaction data are supportive of long-range interactions across this region (Table 4), despite the distance of about 280 kb, which separates our CpG site (cg05896042) and the SNP (rs1122608),

Discussion

In this study, we aimed to identify alterations in CpG methylation associated with common CVDs present in a population cohort from northern Sweden. We were able to find differential DNA methylation at 211 individual CpG sites, corresponding to 196 different genes, in participants with a history of MI.

A substantial number (N = 42) of the 211 MI associated CpG sites were mapped to genes with described biological functions that are highly relevant for cardiovascular function, myocardial development as well as response to responses ischemic injury. These include *RYR2* that encodes the ryanodine receptor 2, which regulates calcium influx from the sarcoplasmic reticulum, and *KCNN1* that encodes the small conductance Ca^{2+}-activated K-channel 1 (SK1). Other of our MI-associated genes are involved in cardiogenesis including nicotinamide mononucleotide adenylyltransferase 2 (*NMNAT2*), formin-like protein 2 (*FMNL2*), homeobox protein Meis1 (*MEIS1*), protein Wnt-7a (*WNT7A*), heart and neural crest derivatives-expressed protein 2 (*HAND2*), T-box transcription factor TBX18 (*TBX18*), leiomodin-2 (*LMOD2*), transcription factor SOX17 (*SOX17*), fibroblast growth factor 1 (*FGF1*) and putative transcription factor Ovo-like 1 (*OVol02521*) or have cardioprotective functions following ischemic events or reperfusion injury such as: ephrin type-A receptor 2 (*EPHA2*), dysferlin (*DYSF*), secreted frizzled-related protein 4 (*SFRP4*), pro-neuregulin-1 (*NRG1*), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (*BNIP3*), growth/differentiation factor 15 (*GDF15*) and membrane protein MLC1 (*MLC1*).

Two of the MI-associated CpG sites identified in our study were located within known genetic hypertension-associated loci identified in previous GWAS (5,19): cg10953508, which is located within the promoter region of *MECOM*; and cg18496965, which is located within the promoter region of *G6B* within the *BAT2-BAT3* locus. In addition, six MI-associated CpG-sites were located in the vicinity of GWAS-identified CVD-associated loci that have previously been associated with CVDs (Table 4). Long-range chromatin-chromatin interaction data were supportive of long-range chromatin interactions between the region of the CpG site and the previously known CVD loci (Table 4). E.g., the hypertension-associated *JAG1* locus, which is located in a probable regulatory region within an upstream gene desert: ChIA-PET data are supportive of chromatin–chromatin interactions between putative regulatory elements within the *JAG1* upstream region with the *JAG1* promoter, which contains one of the MI-associated CpG-sites identified in our analysis (Fig. 2). Even though many of the CpG sites map to cardiac-related genes, it is worth considering that variation at regulatory sites do not necessarily affect the most nearby gene. The study of genomic interaction utilizing chromatin configuration capture has shown that interactions between genomic elements can occur over a relatively large distance, even up to several million base-pairs (23,24).

Even though we adjusted for many potential technical and biological confounders in our analyses (e.g. batch effects, cell composition, age and sex) we still have a high inflation in our test statistics. This inflation is in agreement with previous studies of differential DNA methylation (25). In comparison to GWAS where the identified genetic variants are causal of the associated phenotypic variation, the differentially methylated CpG sites identified in an EWAS are less likely to be causal. Instead, MI alterations in DNA methylation can reflect underlying environmental/lifestyle factors (e.g. smoking or diet) that are risk factors for MI, or alterations that occur in association with the disease progression or the recovery after an episode of MI. It is well known that a MI causes a dramatic response in our body through the release of several signalling molecules and the activation of these can be reflected by alterations in DNA methylation. It is therefore important to consider that the inflation in low P-values in an EWAS is expected, and is not comparable to the low inflation commonly seen in GWA studies.

A limitation of our study is the relatively small sample size of the NSPHS cohort, and the limited number of participants that have experienced CVD. According to our power calculation, we expect to have an 80% chance to detect a difference in DNA methylation level of 3.0, 1.5, 1.3, 0.95, and 0.63 standard units for N = 5, 20, 30, 50, 150 cases, respectively. The MI-associated signals had an average difference between cases and controls of 0.70 standard units which is just below the 80% detection limit for 47 cases. It is therefore not surprising that the diseases with lower number of cases did not result in any significant findings due to the low sample size. Despite the limited cohort size, we were able to observe 211 altered CpG sites associated with a history of MI, which may reflect homogenous and specific biologic responses that occur after a myocardial ischemic injury. However, we could also observe a high degree of co-morbidity of different CVDs in participants from the NSPHS cohort (Table 2), which may bias our results leading to detection of DNA methylation patterns that represent a combination of co-morbidities, or individual co-morbidities themselves. Another limitation of this study is that we did not have an independent cohort for replication of our results, which would have improved the confidence of our results.

In this study, blood is utilized as a surrogate tissue for the study of more pathologically relevant tissues involved in cardiovascular disease, such as biopsies of the myocardium or of blood vessel walls. One of the challenges in epigenetic studies is how to utilize data on DNA methylation from accessible tissues, such as peripheral blood cells, in the prediction of pathological mechanisms in hard-to-reach tissues that are more relevant for the studied pathology. One previous study on peripheral blood leukocytes (PBL) and atrial biopsies collected from patients undergoing coronary bypass surgery was able to report a high correlation of DNA methylation between these tissues ($R^2 = 0.83$) (26), which supports the utility of DNA methylation in peripheral blood cells in predicting the function in cardiac cells. By utilizing machine learning based on paired DNA methylation datasets, Ma and colleagues were also able to predict the DNA methylation pattern in atrial biopsies from PBL DNA methylation data with very high precision ($R^2 = 0.98$–0.99)(26). The study by Ma *et al.* study shows that statistical utilities to accurately predict DNA methylation in target tissues based on data from surrogates are highly feasible. Development is however dependent on the collection of paired data sets to compare between tissues. For a more clinically relevant use as a biomarker, our results show that MI-associated alterations in DNA methylation take place in peripheral blood cells and can be studied for the development of biomarkers for diagnosis or disease prognosis. In addition, we also used information from when participants were interviewed for their history of CVD rather than having access to medical records is a limitation of our study. This also resulted in missing information on age of disease onset and time since the episode of the last MI, information that would have increased our power in finding disease associated differential

DNA methylation. However, it is worth considering that these limitations are mainly decreasing the power of our study, and there is no reason to believe that they would increase the risk of false positive findings.

Alterations in DNA methylation occur in concert with gene regulatory programs associated with specific biological responses. However, DNA methylation patterns are also determined by genetic variants. The interplay between genetic variation and the environment, and how DNA methylation factors into the emergence of a clinical phenotype are still unknown. DNA methylation is related to chromatin accessibility and the occupancy of transcription factor binding at gene regulatory sites and promoters. As such, the observed alterations in DNA methylation associated with myocardial infarction may reflect gene regulatory mechanisms that form part of the response to an ischemic event.

In summary, we have identified 211 CpG sites that are differentially methylated in blood samples from participants that have experienced an episode of MI. As many as 42 of the MI-associated probe-linked genes have previously been associated with CVDs or cardiac function, development and recovery after an ischemic event, which further points to the biological relevance of the observed changes in DNA methylation in blood samples. Even though we cannot pinpoint the role of these changes in the pathogenesis of MI, or in the recovery after an episode of MI, the list would be of great value for further investigation, particularly for identifying CpG-sites that can serve as diagnostic biomarkers or as prognostic biomarkers to identify at-risk patient groups.

Materials and Methods

Northern Sweden population health study

The NSPHS comprises data from a cross sectional cohort that was gathered during 2006 and 2009 from the population in the parishes of Karesuando and Soppero, Norrbotten County. All inhabitants aged 15 or above were invited to participate, which resulted in 1,068 participants. All participants provided written informed consent to the examination of genetic and environmental causes of disease. Peripheral blood samples were collected from all participants and immediately stored at -70°C. Data on disease history, medication and other physical traits were collected from all participants by interviews. The NSPHS study was approved by the local ethics committee at Uppsala University (Regionala Etikprövningsnämnden, Uppsala, permit number 2005:325) in compliance with the Declaration of Helsinki.

DNA methylation

Genomic DNA was extracted from blood samples of 743 participants and subjected to bisulfite-conversion using the EZ-DNA methylation kit (ZYMO research). DNA methylation was assayed on the HumanMethylation450K Beadchip (Illumina, San Diego, USA). Raw data were analysed using the minfi package in R. Normalization was performed using Subset-quantile Within Array Normalization (SWAN). A marker detection p-value $\leq$ 1.38e-10 was used to adjust for the number of individuals and the number of analysed CpG sites. The probe call rate was >0.98 and the individual call rate was >0.98. Control samples and duplicated samples were removed as described previously (27). In addition, ethnic outliers and participant with missing phenotypic data were also removed. White blood cell (WBC) fractions were inferred from methylation data (28) as described previously (27).

Statistical analysis

Statistical analyses were performed in R version 3.1.2. A rank-based inverse normal transformation was performed to adjust for non-normally distributed methylation data (29) using the 'rntransform' function included in the GenABEL package (30). Because the NSPHS is a population-based study that includes related individuals, all methylation values needed to be adjusted for relatedness among individuals. Methylation values were adjusted for relatedness, sex, age, estimated cell fractions, as well as array and slide information using the polygenetic function in the GenABEL package, which also adjusts for population stratification if present, using a genetic kinship matrix. The kinship matrix was estimated using the 'ibs' function in GenABEL including genotyped autosomal SNPs (MAF >0.05) from the cohort. Information about the SNP data has been published previously (31). The residuals from the polygenic models were exported for regression analyses in relation to CVDs. Linear regression analysis was performed using the generalized linear models-function, 'glm', in the stats package with adjusted methylation levels (residuals from the polygeneic model) as the response variable with the disease phenotype, age, sex, BMI and whether the individual was a smoker or not as explanatory variables. Individuals with a history of a specific disease were set as cases in the respective analysis and individuals with no prior history of cardiovascular disease or diabetes were set as controls. P-values were adjusted using the false discovery rate (FDR) and a q-value <0.05 was considered significant. Sensitivity analyses were performed for all significant sites in order to evaluate if BMI might influence the association between the disease phenotypes and DNA methylation. These analyses were performed as before but BMI was excluded in the regression analyses.

Enrichment analysis

Enrichment analyses were performed with the web-based gene ontology enrichment analysis and visualization tool Gorilla (10). We also used enrichment analyses to identify overrepresented biological pathways, using Enrichr (12) (accession date: Nov. 2015), for the MI-associated CpG sites. Enrichr is a one-stop online tool for enrichment analyses against a collection of gene-set libraries. We used REVIGO for summarizing and reducing lists of GO terms to make them more comprehensible. REVIGO relies on clustering by measures of semantic similarity to identify representative GO categories (11).

All genomic coordinates reported are in GRCh37 (hg19). For enrichment analyses, we used the annotation of DNA methylation sites provided by Illumina (HumanMethylation450_15017482_v.1.1.csv, accessed: September 1, 2012), to assign CpG-sites to a corresponding gene. When multiple CpG sites were mapped to the same gene, only the CpG site with the lowest P-value was kept, leaving one CpG site and one P-value per gene for the respective disease phenotype. Genes were ranked with regards to these P-values and the enrichment analyses in GOrilla were performed using the ranked list of genes.

Analysis of potential chromatin-chromatin interactions

MI associated CpG-sites that overlapped (within 1Mb) with previously reported CVD-associated loci were examined for potential functional interaction via open access datasets on chromatin-

chromatin interactions. *In situ* Hi-C data generated by the Aiden lab have demonstrated that the genome partitions into topologically associated domains (TADs) in which interactions take place (23). We examined TADs of all significant CpG sites that were located within 1 MB of previously known CVD loci via the standalone software Juicebox (http://www.aidenlab.org/juicebox/, accession date 2015/11/01). Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) allows analysis of genomic loci that bind a specific locus and has been performed for a number of transcription factors by the genome institute of Singapore (20). ChIA-PET data for DNA polymerase 2 was accessed to examine chromatin-chromatin interactions related to transcription, such as promoter-enhancer interactions. These data were accessed (accession date 2015/11/01) and visualized via the WashU epigenome browser at http://epigenomegateway.wustl.edu/.

Additional analyses were performed to test for associations between leading SNPs at CVD-loci with methylation at proximal MI-associated CpG-sites as well as with MI. Linear regression analyses were utilized as described in the statistical analysis section. In tests for association between SNPs and CpG-methylation, adjusted methylation levels (residuals from the polygeneic model) were set as the response variable with genotype, age, sex, BMI and whether the individual was a smoker or not as explanatory variables. In tests for association of SNPs with MI, the disease was set as the response variable with genotype, age, sex, BMI and smoking as explanatory variables.

Power calculation

To evaluate the power to detect differentially methylated regions depending on the number of cases we performed power calculations. In agreement with the DNA methylation values being rank transformed, we assume that DNA methylation levels in cases and controls are approximately normally distributed (SD = 1), with a difference in the mean value. Using the stats library in R, the power can then be calculated by: Power = pchisq(threshold, df = 1, lower.tail = FALSE, ncp = N * H2). Where the power depends on what fraction of variation in DNA methylation levels that can be explained by the difference between cases and controls. The threshold = qchisq (alpha, df = 1, lower.tail = FALSE) is the chi2 threshold for alpha = 0.05/470789 (Bonferroni adjustment for multiple testing) and N is the sample size, which is the number of cases (491) plus the number of controls in each analysis. H2 reflects the fraction in variance explained by the difference in DNA methylation levels between cases and controls and is determined by the difference in mean methylation level between the groups and the number of cases versus controls. Using the sample size of 491 controls and a threshold of significance of alpha = 0.05/470789, this gives that, to reach an 80% power to detect a difference between cases and controls, minimum H2 ranges from 0.077 to 0.055 when the number of cases increases from 5 to 200 (total samples size increase from 496 to 691). This is equal to a minimum difference in mean DNA methylation level of 3.0, 1.5, 1.3, 0.95, and 0.63 standard deviations for $N = 5$, 20, 30, 50, 150, respectively. Considering that the average standard deviation for the CpG sites in our data was 0.0316, this equals a difference in DNA methylation levels between cases and controls of approximately 0.093, 0.047, 0.040, 0.030, and 0.020 for the different number of cases respectively.

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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