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Exploring the causal inference of shear stress associated DNA methylation in carotid plaque on cardiovascular risk

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ABSTRACT

Background and aims: Atherosclerosis is a lipid-driven inflammatory disease presumably initiated by endothelial activation. Low vascular shear stress is known for its ability to activate endothelial cells. Differential DNA methylation (DNAm) is a relatively unexplored player in atherosclerotic disease development and endothelial dysfunction. Previous studies showed that the expression of 11 genes was associated with differential DNAm due to low shear stress in murine endothelial cells. We hypothesized a causal relationship between DNAm of shear stress associated genes in human carotid plaque and increased risk of cardiovascular disease.

Methods: Using Mendelian randomisation (MR) analysis, we explored the potential causal role of DNAm of shear stress associated genes on cardiovascular disease risk. We used data from the Athero-Expression Biobank Study for the discovery of methylation quantitative trait loci (mQTLs) in 442 advanced carotid plaques. Next, we performed MR analysis using these mQTLs and publicly available GWAS summary statistics of coronary artery disease (CAD) and ischemic stroke (IS).

Results: We discovered 9 mQTLs in plaque in the promoters of shear stress associated genes. We found no significant effect of shear stress gene promoter methylation and increased risk of CAD and IS.

Conclusions: Differential methylation of shear stress associated genes in advanced atherosclerotic plaques in unlikely to increase cardiovascular risk in human.

1. Introduction

Atherosclerosis is a lipid-driven inflammatory disease underlying many cardiovascular diseases, such as coronary artery disease (CAD) and ischemic stroke (IS). Low shear stress is likewise a key player in atherosclerosis and results in endothelial activation, ultimately leading to the initiation and progression of atherosclerotic plaque formation [1,2]. In mice, differential DNA methylation (DNAm) at the promoter region of 11 shear stress associated genes (*HOXA5*, *TMEM184B*, *ADAMTSL5*, *KLF4*, *KLF3*, *CMKLR1*, *PKP4*, *ACVRL1*, *DOK4*, *SPRY2* [3], and *ENOSF1* [4]), was shown to alter gene expression and influence endothelial dysfunction thus plaque progression [3,5].

To what extent differential DNAm at these genes would lead to increased risk in human is unclear. It is well established that DNAm, specifically at promoters, regulates gene transcription by modulating the interaction between DNA and chromatin binding proteins [6]. Given that common cardiovascular risk factors, such as smoking [7] and obesity [8–10], are known to associate with DNAm, these risk factors could give rise to aberrant DNAm, thereby impeding regulation of gene expression and negatively impacting atherosclerotic progression. Similarly, shear stress could also give rise to changes in the methylation of arterial DNA leading to differential expression and subsequently to more unstable plaques and increased risk of cardiovascular disease. However, differential arterial DNAm could also arise due to reverse causality or residual confounding, i.e. atherosclerotic disease manipulates DNAm or confounding factors, like smoking [7], manipulated DNAm. Also, genes identified in mouse models might not reflect the human condition under shear stress. These relations would be difficult to tease in traditional observation studies [11].

Much akin to randomized clinical trials, Mendelian randomisation (MR) studies make use of the intrinsic properties of the genome to assess causality. Alleles are randomly distributed from parents to offspring at conception, genetic variation is not influenced by disease (reverse causality), risk factors (residual confounding), and remains largely

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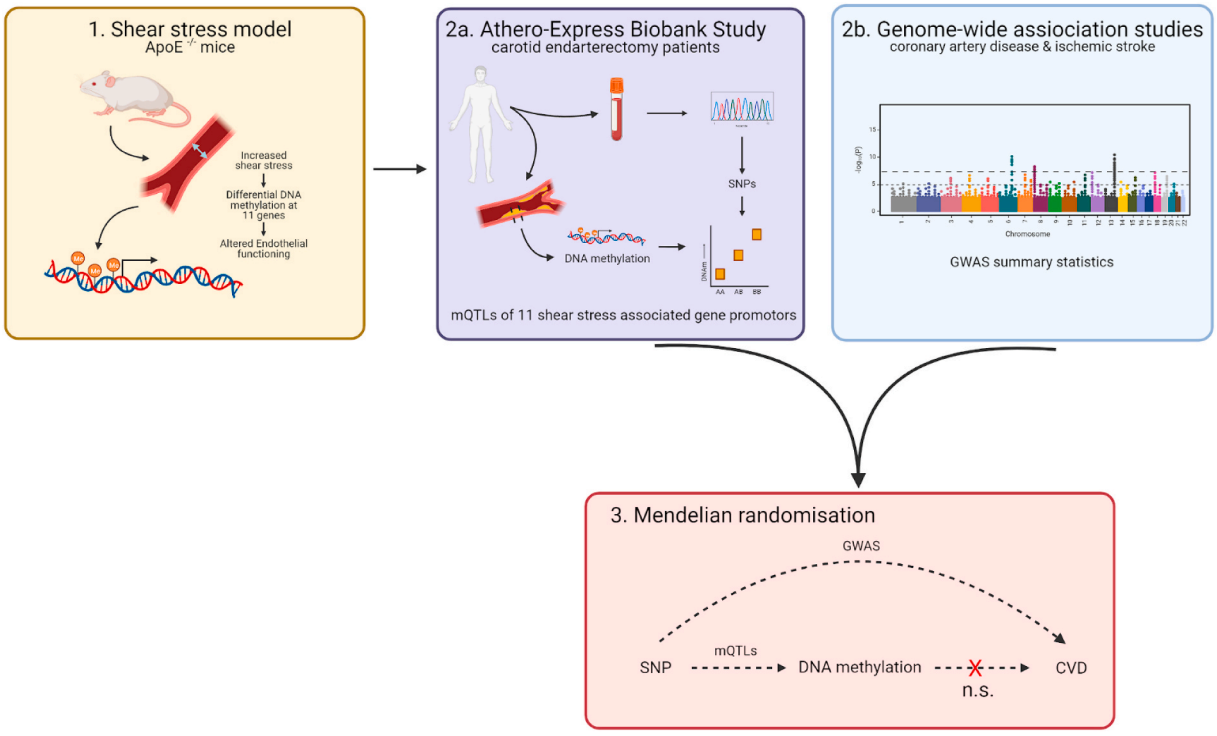

Fig. 1. Causal inference scheme of DNAm of shear stress associated genes on cardiovascular risk including origin of genes and risk factors. Yellow plane: Dunn et al. showed that low shear stress results in differential DNA methylation of 11 murine genes [3]. Blue plane: it has been shown that low(er) vascular shear stress is associated with an increased risk for cardiovascular disease in large genome-wide association studies. Purple plane: we set out to discover mQTLs of these shear stress associated genes in advanced human plaque as proxies (instrumental variables) for differential DNAm. Red plane: next, we assessed the effect of differential methylation on cardiovascular risk using Mendelian randomisation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

unchanged throughout life [11–13]. Therefore, genetic variation associated with DNAm at these 11 genes could be used, as instrumental variables, to assess the effect of differential arterial DNAm on cardiovascular risk, independent of risk factors.

To assess the impact of DNAm at these 11 genes on atherosclerotic plaque progression and cardiovascular disease, we performed an MR study in the Athero-Express Biobank Study comprising of patients undergoing carotid endarterectomy. We set out to identify common genetic variants associated with DNAm in advanced plaque and discovered 9 mQTLs in these 11 genes. We applied Two-Sample MR [14] with these mQTLs as instrumental variables to test the causal effect of differential DNAM on cardiovascular diseases (CAD [15] and IS [16]) using publicly available genome-wide association study (GWAS) summary statistics (Fig. 1).

2. Patients and methods

2.1. Athero-Express Biobank Study

The Athero-Express Biobank Study (AE) is a longitudinal biobank study including patients that undergo endarterectomy in two Dutch tertiary referral centres. The biobank study is ongoing and expanding since 2002; a detailed cohort description was published before [17]. In this study, genotype, methylation and phenotype data of carotid endarterectomy (CEA) patients were used [7]. The study was approved by the ethical commission of the participating medical centres. All participants provided informed consent. The study complies with the Declaration of Helsinki.

2.2. DNA isolation

Carotid plaque specimens were removed during CEA and processed following specific guidelines [17]. In short, specimens were cut into 5 mm segments and culprit lesions were identified to be fixed in 4% formaldehyde embedded in paraffin. Histological features were scored and remaining segmented were stored at −80 °C until further processing. DNA isolation was performed on these segments according to in-house protocols as described previously [18].

2.3. DNA methylation

Isolated DNA samples were randomly distributed on 96-well plates at equalized DNA concentrations of 600 ng. DNA was bisulfite converted using a cycling protocol and the EZ-96 DNA methylation kit (Zymo Research, Orange County, USA). The Infinium HumanMethylation450 Beadchip Array (HM450k, Illumina, San Diego, USA) was used to measure DNA methylation, processing according to manufacturer's protocol. The HM450K experiment was performed at the Erasmus Medical Center Human Genotyping Facility in Rotterdam, the Netherlands. In total, we collected data from 442 AE patients for the Athero-Express Methylation Study 1 (AEMS450K1) [7].

2.4. Genotyping and imputation

DNA was isolated from stored samples according to the above mentioned protocol and genotyped in two phases (Athero-Express Genomics Study 1 (AEGS1) and Athero-Express Genomics Study 2 (AEGS2)) [18]. Both AEGS1 and AEGS2 samples were genotyped using commercially available genotyping arrays, respectively the Affymetrix Genome-Wide Human SNP Array 5.0 and the Affymetrix Axiom® GW CEU 1 Array. Quality control was performed using community standards and assurance procedures [18,19]. Our reference panel consisted of a merge of phased haplotypes from the 1000 genomes project (phase 3, version 5) [20] and haplotypes from the Genome of the Netherlands

Table 1
Baseline characteristics of the Athero-Express Biobank cohort.

Characteristic	AEMS (n = 442)
Age, y (SE)	67.9 (9.01)
Males (%)	68.8
SBP, mm Hg (SE)	156.1 (25.88)
DBP, mm Hg (SE)	82.5 (13.24)
BMI, kg/m^2(SE)	26.7 (3.94)
Smoking (% (n))	40.3 (178)
Comorbidities (% (n))	
Diabetes mellitus	22.6 (100)
Hypertension	87.3 (386)
Medication use (% (n))	
Hypertensive drugs	77.4 (342)
Anticoagulants	12.4 (55)
LLDs	3.4 (15)
Symptoms (%)[a]	
TIA	41.4
Stroke	25.8
Asymptomatic	14.0
Ocular	13.1
Other	5.7

Patient characteristics at inclusion. AEMS: Athero-Express Methylation Study, SBP: systolic blood pressure, DBP: diastolic blood pressure, BMI: body-mass index, ocular: retinal infarction and amaurosis fugax; TIA: transient ischemic attack, smoking: self-reported current tobacco smoking status, comorbidities: diabetes and hypertension were defined as diagnosed by a medical doctor or medication use for the specific comorbidity, medication use: hypertensive drugs include all hypertensive drugs. Anticoagulant drug use includes use of aspirin, dipyridamole or any ADP-inhibitor. Lipid lowering drug (LLDs) use includes any lipid-lowering drug.

[a] Symptoms at presentation tertiary referral centre for carotid endarterectomy. When multiple cerebrovascular symptoms occurred in the six months prior to the operation, the most serious symptom counts in the following order: stroke > TIA > ocular.

(GoNL5) [21] and was imputed using IMPUTE2 [22].

2.5. Methylation quantitative trait loci analysis

We used the QTLToolKit workflow (swvanderlaan.github.io/QTLToolKit/) [23] which leverages QTLtools [24] to identify *cis*-acting mQTLs in carotid plaques of our genes of interest. The region of interest (ROI) was determined by flanking the outermost DNAm sites (CpGs) of the −2000 transcription start site (TSS) to the first exon by 250 kb upstream and downstream (Supplementary Table 1). We used these ROIs to test for phenotype-genotype pairs, i.e. associations between CpGs and variants. Two passes were performed, an initial pass to get nominal *p*-values on our dataset and a permutation pass to correct for multiple testing error (FDR < 5%) and get adjusted *p*-values. We filtered out potential false positives caused by variants affecting the binding of a probe on the array by removing CpG-variant pairs within the same probe and in linkage disequilibrium (LD) with the same probe. The top hit was visualised in a regional association plot using Regional Association ComparER (RACER) v0.1 (https://github.com/oliviasabik/RACER).

2.6. Two sample Mendelian randomisation

To determine causal effect of DNAm of shear stress associated genes on CAD and IS we applied the Two Sample Mendelian Randomisation (2SMR) design (using the R-package TwoSampleMR) [12]. The 2SMR design is able to infer causality between an exposure (DNAm) and an outcome (CAD or IS) using public genome wide association study (GWAS) summary statistics available through the MR-Base platform (http://www.mrbase.org). Variant proxies were used for outcome GWAS variants, if not available in that particular GWAS (linkage

Table 2
Shear stress related differential DNAm associated permutated *cis*-mQTLs in advanced plaques.

mQTL	CpG	CpG position	Chr	BP	Other allele	Coded allele	CAF	HWE	INFO	Gene Name	Beta	SE	Nominal *p*-value	Perm *p*-value
rs7235957	cg07100532	TSS1500	18	717,229	T	C	0.544	0.425285	0.9793	*ENOSF1*	0.794	0.054	1.12E-48	1.47E-38
rs7235957	cg26147554	TSS200	18	717,229	T	C	0.544	0.425285	0.9793	*ENOSF1*	0.752	0.058	2.95E-39	9.20E-33
rs7235957	cg16112050	TSS1500	18	717,229	T	C	0.544	0.425285	0.9793	*ENOSF1*	0.478	0.038	6.51E-36	6.91E-30
rs1061035	cg15158376	TSS200	18	722,118	A	G	0.121	0.535303	0.9902	*ENOSF1*	0.805	0.064	9.51E-37	1.69E-29
rs2741188	cg00955482	TSS200	18	708,299	T	C	0.554	0.630956	0.9893	*ENOSF1*	0.261	0.024	1.69E-27	1.62E-21
rs75588551	cg07283778	TSS200	18	725,330	A	G	0.122	0.901626	0.9703	*ENOSF1*	0.167	0.022	1.16E-14	7.39E-11
rs11113813	cg15448445	TSS1500	12	108,710,286	C	G	0.632	0.364196	0.9831	*CMKLR1*	−0.202	0.032	1.70E-10	5.78E-07
rs10861891	cg08110272	TSS1500	12	108,710,323	C	A	0.661	0.0392288	0.9903	*CMKLR1*	−0.307	0.052	2.31E-09	2.90E-06
rs4403843	cg03612522	TSS200	12	108,707,829	A	G	0.662	0.0389722	0.9685	*CMKLR1*	−0.102	0.018	4.62E-09	1.14E-05
rs11113813	cg03408433	TSS1500	12	108,710,286	C	G	0.632	0.364196	0.9831	*CMKLR1*	−0.174	0.038	2.15E-06	1.10E-03
rs11113813	cg25832824	TSS200	12	108,710,286	C	G	0.632	0.364196	0.9831	*CMKLR1*	−0.076	0.017	3.74E-06	2.70E-03
rs637718	cg08471037	TSS200	16	57,527,946	A	G	0.764	0.240815	0.9642	*DOK4*	0.104	0.017	1.27E-10	4.50E-07

Permuted advanced carotid plaque *cis*-mQTLs in 11 shear stress associated genes. *mQTL*: rsID of the SNP associated with plaque DNAm at a given the methylation site (*CpG*). CpG position: TSS1500, within 1500 base pairs (BP) of the transcription start site (TSS) of the gene; TSS200 within 200 BP of the TSS of the gene. Chromosome (*Chr*) and BP position relative to 1000 Genomes Project (Nov 2014, Hg19) is given. Other allele: the non-effect allele; Coded allele: the effect allele. CAF: coded allele frequency; HWE: Hardy-Weinberg-Equilibrium; INFO: imputation quality. Gene name: according to refSeq (GRCh37/hg19) canonical genes from UCSC. Beta: effect size relative to the cded allele; SE: standard error of the beta; Perm *p*-value: permutation *p*-value.

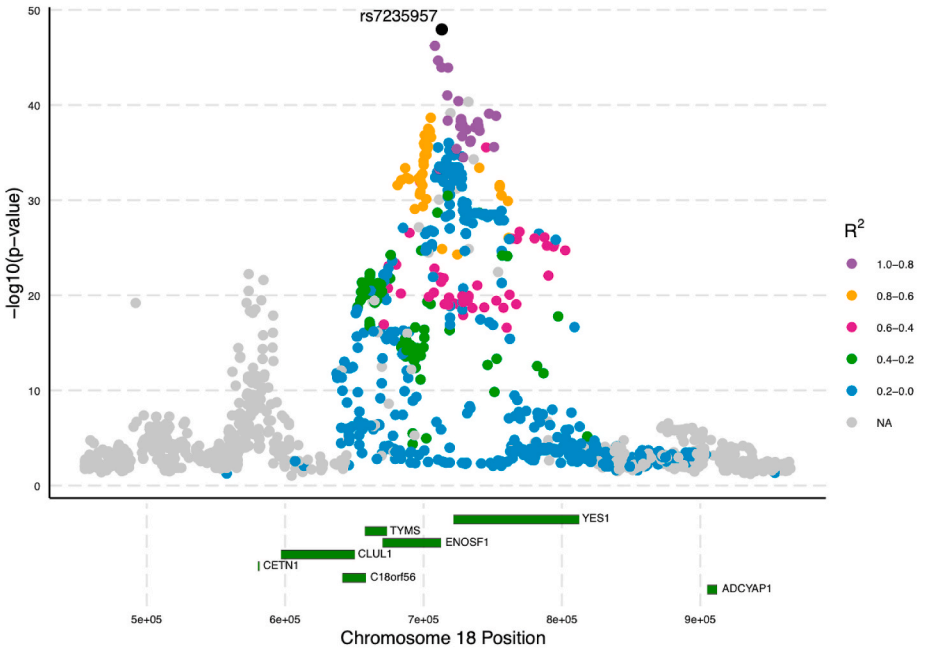

Fig. 2. Regional association plot rs7235957 *ENOSF1* on chromosome 18. Regional association of variants to DNA methylation in the *ENOSF1* promoter region. The strongest association is rs7235957 associated with multiple CpG sites in the *ENOSF1* promoter region in carotid plaques. Each dot represents an SNP and the lead SNP is indicated in black. The X-axis shows the chromosome location relative to 1000 Genomes Project (Nov 2014, Hg19) and refSeq canonical genes (green) from UCSC. The y-axis shows -$\log_{10}$(*p*-value) of the association with the CpG site in our region of interest. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

disequilibrium (LD) $r^2 < 0.8$). We used GWAS summary-statistics from the CARDIoGRAMplusC4D [15] for CAD and GWAS summary-statistics from the METASTROKE [16] for IS. We used the *cis*-acting mQTLs of plaque tissue as proxy of the exposure (DNAm). Three variants for CAD and one variant for IS passed LD clumping and harmonization to GWAS summary statistics and were used for 2SMR analysis.

2.7. Statistical analysis

Details on the study design and statistical analyses in CARDIoGRAMplusC4D, and METASTROKE were previously described [15,16]. For the discovery of *cis*-acting mQTLs in carotid plaques, we assumed an additive genetic model and corrected for sex, age, and genotyping array type. To declare a causal relationship between exposure and the significance, this was set at $p < 0.05$. We used simple mode, weighted mode, Inverse Variant Weighted (IVW), Weighted Median (WM), and MR-Egger (intercept) to determine causality. Simple mode, weighted mode, and IVW combine ratio estimates of individual genetic variants to a (weighted) mean, resulting in a consistent estimate of the causal effect, which converges to true values as sample size increases. Of these three methods, IVW is an efficient analysis method, but it will be biased if only a single genetic variant is invalid. WM provides a more consistent estimate if up to 50% of the genetic variants are invalid [13]. MR-Egger Regression performs a weighted linear regression and if there is no intercept term, it is equal to IVW. A non-zero of the intercept can be interpreted as an estimate of the horizontal pleiotropic effects (an effect not mediated via the exposure) of the genetic variants, indicating directional pleiotropy, and suggesting IVW is biased [25]. Furthermore, MR-Egger can provide a true causal effect if the genetic variant is not independent of the outcome, using the inSIDE (instrument strength independent of direct effect) assumption. F-statistic was used to determine weak instrument bias, a F-stat > 10 is generally considered a strong instrument [26]. mQTL power estimation showed a power of 85% and higher at minor allele frequencies (MAF) > 0.06 (Supplementary Fig. 1) [27].

2.8. Data availability

Scripts and full summary statistics available from: https://github.com/rubenmethorst/shear-stress-project. AE data for mQTL analysis is only available upon request due to GDPR restrictions and local rules and regulations.

3. Results

3.1. Common variants associated with methylation of shear stress genes in human carotid plaques

To facilitate Mendelian randomisation (MR) with DNAm, we first set out to identify common genetic variants associated with DNAm of shear stress associated genes in human carotid plaques. For this, we genotyped 1439 individuals from the AE and extracted DNA from 442 overlapping advanced atherosclerotic carotid plaque samples to assess methylation (Table 1) [7]. We defined regions of interest (ROIs) between transcription start site (TSS, −2000 base pairs (bp) from 5′-end of the gene) and the first exon for each of the 11 shear stress associated genes (Supplementary Table 1). We used the QTLToolKit [23] and QTLtools [24] to test for common *cis*-acting methylation quantitative trait loci (*cis*-mQTL) within ±250 kb of the ROIs and discovered 121,109 potential mQTLs near the 11 genes at nominal *p*-values (Supplemental Table 2). To correct for multiple testing, we applied permutation testing (1000 fold) and identified 12 significant *cis*-mQTLs-CpG pairs at 3 genes (Table 2). Regional association of the trongest associated variant-CpG pair corresponding to a shear stress associated gene, shows a significant relationship between rs7235957 and multiple CpG sites in the *ENOSF1* promoter (lowest *p*-value: $p = 1.47 \times 10^{-38}$) (Fig. 2 and Table 2).

3.2. Causal inference of DNAm at 11 shear stress associated genes on cardiovascular risk

Next, we used the 12 significant *cis*-mQTL-CpG pairs as genetic instruments to test the causal effect of promoter DNAm of shear stress associated genes on cardiovascular risk in human carotid plaques. (Fig. 3). We used the *cis*-mQTLs as proxies for the "exposure" DNAm of shear stress associated genes in carotid plaques and we used publicly available GWAS summary statistics of CAD [15] and IS [16] as "outcome" for cardiovascular risk. Three instrumental variables (rs2741188, rs4403843, rs637718) were identified and showed strong

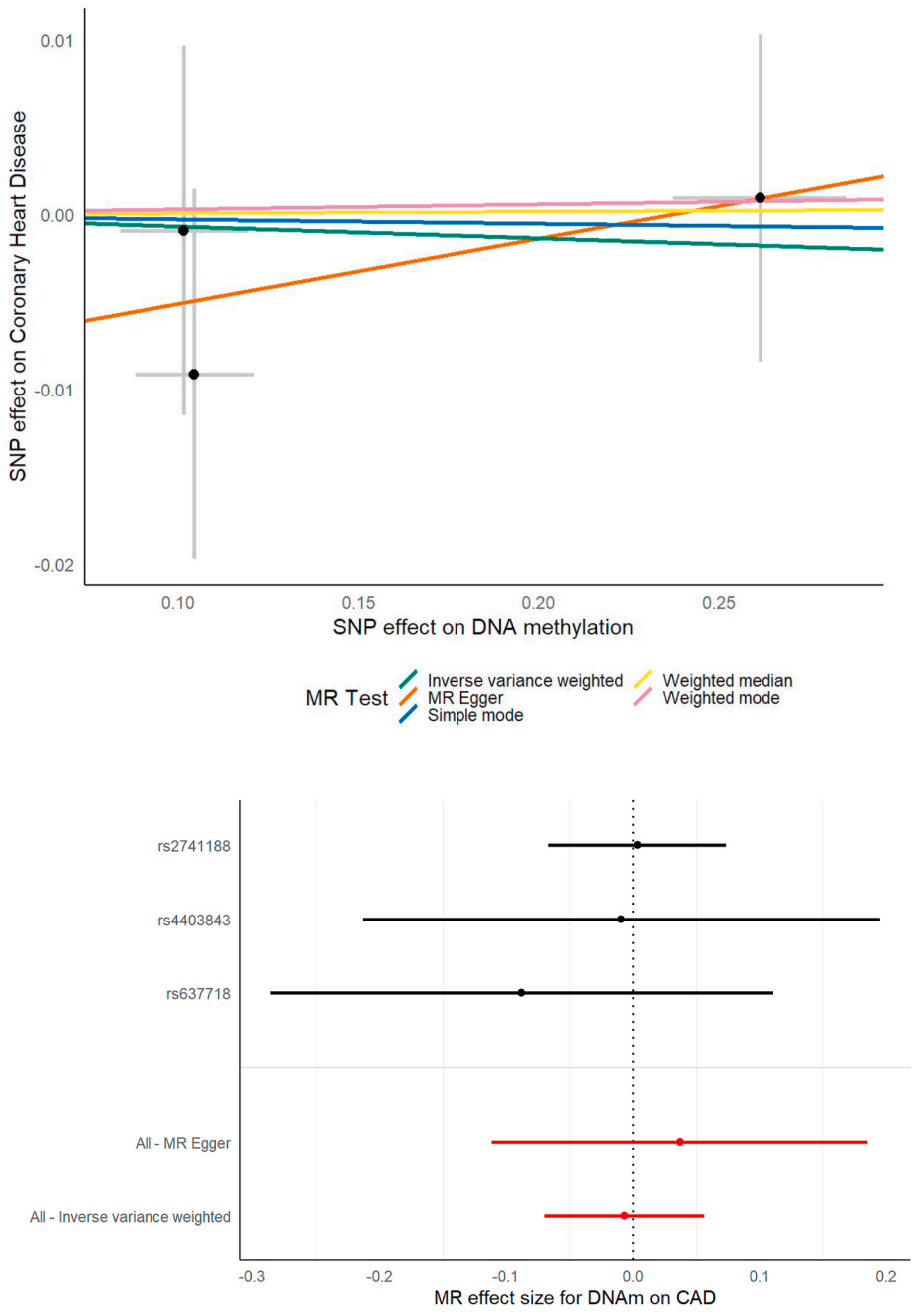

Fig. 3. (A) The causal effect of plaque DNA methylation in 11 shear stress associated genes on CAD. Two sample Mendelian randomisation (2SMR) analysis of DNAm in plaques at 11 shear stress associated genes on coronary artery disease. We performed 2SMR analysis with plaque mQTLs to test for a causal effect with CAD. Each coloured line corresponds to a test indicated by the legend above. The x-axis shows the effect of the SNPs on DNAm in plaques (mQTLs), the y-axis shows the effect of the SNPs on CAD risk. (B) Forest plot showing the per-SNP causal effects of plaque DNA methylation in 11 shear stress associated genes on CAD. Per-SNP 2SMR results for plaque DNAm in 11 shear stress associated genes on risk of CAD. The x-axis shows the causal effect of plaque DNAm on CAD.

Table 3
MR results of shear stress associated DNA methylation on CAD and IS.

Exposure	Outcome	Sample size	SNP	Beta	SE	*p*-value	F-statistic
DNAm	Coronary artery disease	184,305	rs2741188	0.004	0.036	0.919	116
			rs4403843	−0.009	0.104	0.931	33
			rs637718	−0.087	0.101	0.388	40
			All - Inverse variance weighted	−0.007	0.032	0.834	
			All - MR Egger	0.037	0.076	0.709	
			Intercept	*−0.009*		*0.637*	
DNAm	Ischemic stroke	29,633	rs4403843	−0.170	0.170	0.317	30

MR results of plaque DNAm (exposure) in 11 shear stress associated genes on the outcomes CAD and IS. Sample size indicates the total sample size in the respective outcome genome-wide association study. Per-SNP analyses were performed using the Wald Ratio test. Beta indicates the causal effect size of the effect allele (coding allele) with the corresponding standard error (*SE*), and *p*-value. F-statistics show no weak instruments.

F-statistics (Table 3). Inverse variance weighted (IVW) analyses show no causal relationship between DNAm of the 11 shear stress associated genes and CAD (inverse variance weighted (IVW): beta = −0.007 p = 0.834, Fig. 3A and Table 3). Similarly, our analyses showed no relationship between DNAm of these genes in plaque and IS (wald ratio: beta = −0.170 p = 0.317, Table 3). Horizontal pleiotropy was assessed using the MR Egger intercept and showed no pleiotropy (p = 0.637). Single SNP analyses of the causal effect of shear stress associated DNAm on CAD also showed no significant results (Fig. 3B and Table 3).

4. Discussion

Eleven genes are associated with shear stress and initiation of atherosclerosis in mice [3].

We sought to find a causal relationship between differential DNAm of these shear stress associated genes in advanced atherosclerotic plaques with cardiovascular disease risk, including CAD and IS. We performed a mQTL analysis and discovered 12 significant *cis*-mQTL-CpG pairs at 3 genes (*ENOSF1*, *CMKLR1*, *DOK4*). ENOSF1, enolase 1, facilitates L-fucose catabolism and genetic variants in *ENOSF1* are associated with gastric cancer [28,29]. We found no literature suggesting a relation between human atherosclerosis and ENOSF1, thus future research is necessary to investigate the role of *ENOSF1* in cardiovascular disease. *CMKLR1*, chemokine-like receptor 1, is a receptor for chemerin, an adipokine involved in metabolic disorders [30], whereas *DOK4*, docking protein 4, is a scaffolding protein that is associated with cancer [31], acute myeloid leukaemia [32], and insulin signalling [33]. *CMKLR1* and DOK4 both play a role in metabolism, hinting towards atherogenic handling of lipids and impaired insulin signalling in human atherosclerotic plaques.

Our MR analysis provides no evidence for a causal relation between DNAm of these shear stress associated genes in human plaque and increased risk of CAD and IS. This could be explained by the sample size of mQTL discovery (n = 442), although this is unlikely since our power calculation showed 85% power at allele frequencies > 6% (Supplemental Fig. 1) and the mQTLs we discovered have frequencies around 50%. Furthermore, no weak instruments (F-statistic < 10) were found, and no horizontal pleiotropy was detected, indicative for strong instrumental variables in our analysis.

We summit that although methylation of these genes could modulate the initiation of atherosclerosis, collectively it might not result in an increased risk of the ultimate clinical outcome, be it CAD or IS. The lack of replication studies of the original murine discovery studies, or a suboptimal representation of the human condition by the murine model systems used, could explain our results. Murine atherosclerosis models reveal little association with human atherosclerosis and most genes associated with a murine atherosclerotic phenotype do not translate to the human atherosclerotic phenotype [34,35]. Moreover, phenotypic effects and effect sizes of orthologous genes frequently differ between species.

Here, our MR analyses using mQTLs discovered in advanced human plaque provide no evidence for a causal relation with CVD. This could indicate that these specific 11 mouse genes might not translate to the human phenotype, however it does not imply that shear stress is unrelated to human atherosclerosis. Perhaps the underlying genes driving shear stress associated DNAm do not entail the same genes in humans as found in mouse models and/or murine genes do not share similar effect sizes and phenotypic effects as human orthologous genes.

In addition, CAD is a widespread multifactorial end-stage disease involving advanced plaques potentially rendering the influence of differential DNAm at these 11 shear stress associated genes during atherosclerosis initiation insignificant. Future studies using early-stage plaque, from *e.g.* accidental findings during autopsy, could yield more insight into the role of these 11 genes during the early stages of atherosclerosis.

Alternatively, future studies involving human endothelial cells, as these are flow-dependent and activation is responsible for atherosclerotic initiation [5,36], could provide more insight in the gene regulatory networks involved in humans and verify the earlier murine results. Such studies could include the design of a shear stress model based on endothelial cells to map of genome-wide differential DNA methylation.

In conclusion, we showed that differential promoter methylation in advanced atherosclerotic plaques of 11 shear stress associated genes, as discovered in mice models, is unlikely to increase cardiovascular disease risk in humans. Future research should focus on genome-wide discovery of shear stress associated genes in relevant *in vitro* models and early-stage human plaques.

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CRediT authorship contribution statement

Ruben Methorst: Conceptualization, Methodology, Investigation, Visualisation, Software, Writing-Original draft preparation. **Gerard Pasterkamp:** Supervision, Funding acquisition. **Sander W. van der Laan:** Conceptualization, Writing, Reviewing and Editing, Supervision. All authors approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2021.03.043.

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