DNA METHYLATION BIOMARKERS OF MYOCARDIAL INFARCTION AND CARDIOVASCULAR DISEASE

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ABSTRACT

Background: DNA methylation is associated with atherosclerosis and cardiovascular risk factors. However, little evidence regarding its association with cardiovascular diseases in large studies is currently available. We aimed to assess the association between DNA methylation and cardiovascular events, and to determine both the predictive capacity of the identified loci and the causality of those associations.

Methods: We defined two strategies: epigenome-wide (EWAS) and candidate-gene association studies. In both strategies, we designed one approach with prevalent cases of coronary heart disease (CHD) and another with incident cases of CHD and cardiovascular disease. We used data from three independent cohorts: the REgistre GIroní del COR (REGICOR) study, the Framingham Offspring Study and the Women's Health Initiative. We also assessed the association between the identified CpGs and cardiovascular risk factors in the three populations. Then, we developed methylation risk scores to evaluate whether their inclusion in the Framingham risk function improved its predictive capacity. Finally, we performed a Mendelian randomization study to determine the causality of the identified associations.

Results: We found 17 CpGs related to prevalent CHD and/or incident cardiovascular events, two of them observed using both approaches ($p < 1.21 \cdot 10^{-7}$ in the EWAS with prevalent events and $p < 1.07 \cdot 10^{-7}$ in the EWAS with incident events; $p < 3.57 \cdot 10^{-5}$ in the candidate-gene studies with prevalent events and $p < 3.25 \cdot 10^{-5}$ in the candidate-gene studies with incident events). Ten CpGs were also associated with at least one cardiovascular risk factor ($p < 4.63 \cdot 10^{-4}$ for those from EWAS, $p < 9.26 \cdot 10^{-4}$ for those from candidate-gene studies), most of them consistent with previous findings. Although the methylation risk scores based on the associations with incident cardiovascular events were related to cardiovascular incidence, none of them improved the predictive capacity of the Framingham risk function. We could perform Mendelian randomization studies for only two CpGs, using a single genetic variant; thus, these results were not conclusive.

Conclusion: We have identified novel cardiovascular-related CpGs and replicated some others. While some of them were related to cardiovascular risk factors, some others highlight less-explored mechanisms underlying cardiovascular diseases such as inflammation.

Keywords: DNA methylation; Epigenome-wide association study; Predictive biomarkers; Myocardial Infarction; Coronary heart disease; Cardiovascular disease; Cardiovascular risk.

BACKGROUND

Cardiovascular disease (CVD) and more specifically coronary heart disease (CHD) remains the number one cause of death and disease burden worldwide [1], and its burden increased in the period from 2007 to 2017 [2]. Therefore, cardiovascular prevention is one of the main global public health challenges. At the individual level, prevention is based on the estimation of cardiovascular risk (CVR) and the control of cardiovascular risk factors (CVRFs) [3]. However, the sensitivity of CVR estimation is low and a significant proportion of CHD events occur in individuals classified as having moderate or low risk [4]. On the other hand, the use of currently available drugs to control classical CVRFs does not prevent all CHD events, underlining the need to identify new strategies for reducing this residual CVR [5]. Thus, information encoded in biological mechanisms acting at different levels should be unravelled to find new predictive biomarkers and new potential therapeutic targets. Among these biomarkers, epigenetic mechanisms and DNA methylation arise as emerging candidates. DNA methylation in humans consists mostly of the chemical modification of cytosines followed by guanines (CpGs). DNA methylation marks can be inherited but also can change throughout the lifespan depending on lifestyle and external stimuli [6]. DNA methylation patterns have been found to be associated with multiple common chronic diseases [7]. However, the relationship between DNA methylation and CVD has been less explored. Thus, further insights are still needed to develop DNA methylation-based biomarkers related to these chronic diseases.

We hypothesized that DNA methylation is associated with CHD and CVD risk, and that some of these epigenetic marks could be predictive of future risk and causally related to cardiovascular outcomes. Thus, this study had three aims: 1) to unravel genomic methylation loci associated with cardiovascular events, 2) to assess their predictive capacity, and 3) to decipher the causality of those associations.

METHODS

Study design and populations

We designed a combination of two strategies and two approaches to identify differentially methylated CpG sites in the genome that are related to CVD or CHD. On the one hand, we used a hypothesis-free strategy, performing a two-stage epigenome-wide association study (EWAS). On the other hand, we used a candidate-gene strategy based on a list of genes previously identified as related to cardiovascular health (Additional file 1: Table S1) [8,9]. In both strategies, we defined two approaches: (a) analyses with prevalent cases (case-control studies with prevalent cases), and (b) analyses with incident cases (a case-control study with incident cases nested in a cohort and a prospective cohort study). Figure 1 shows the design and flow-chart of this study. A more detailed description of the methods is provided in the Additional file 2: Supplementary Material.

Three populations were included in this study:

a.- *REgistre GIroní del COR:* We designed a 1:1 case-control study (n=416) in the REgistre GIroní del COR (REGICOR) study. Cases were first-AMI (Acute Myocardial Infarction) patients attended in the reference hospital of the monitored area. Women were overrepresented to achieve their inclusion as 50% of our sample. Controls were matched by age and sex with cases, and were randomly selected from the population-based REGICOR cohort. The study was approved by the local ethics committee and participants provided informed written consent.

b.- Women's Health Initiative: We selected those participants in the Women's Health Initiative (WHI) with DNA methylation data (n=1,890) available in the database of Genotypes and Phenotypes (http://dbgap.ncbi.nlm.nih.gov; project number #9047). The women with DNA methylation data belong to a WHI case-control study nested in a cohort [10].

c.- Framingham Offspring Study: We included the cohort of participants in examination 8 (2005-2008) of the Framingham Offspring Study (FOS) [11] with DNA methylation data (n=2,568) available in the database of Genotypes and Phenotypes (http://dbgap.ncbi.nlm.nih.gov; project number #9047). Those participants with prevalent

CHD at exam 8 were included in the analysis of prevalent cases, and incident cases identified during the follow-up were included in the incident cases analysis.

Assessment of cardiovascular outcomes

The cardiovascular outcomes assessed were AMI in the REGICOR population and CHD and CVD in the WHI and FOS samples. In the REGICOR study, AMI was defined according to international World Health Organization criteria [12] when two or more of the following were present: abnormal new Q waves, increase in cardiac enzymes beyond twice the upper normal value, and chest pain lasting more than 20 min.

In the WHI sample, CHD was considered when the participant had experienced any of the following: clinical AMI, definite silent AMI, total AMI, angina, coronary revascularization procedure or CHD death. CVD included CHD and stroke or death due to cerebrovascular disease or other CVD. Clinical events were adjudicated in 2016.

In the FOS sample, data regarding CHD and CVD were extracted from follow-up for cardiovascular events through 2014 (until exam 12). We used exam 8 as the baseline visit and to identify prevalent cases.

Assessment of DNA methylation status

In the REGICOR study, DNA was extracted from whole peripheral blood drawn within the first 24 hours post-AMI for cases and at follow-up for controls. For the other samples, DNA was extracted from buffy coat obtained from whole peripheral blood samples collected at baseline in the WHI population [10] and at exam 8 in the FOS [13].

DNA methylation was assessed genome-wide with commercial arrays based on bisulfite conversion of unmethylated cytosines. In the REGICOR study, we used the Infinium MethylationEPIC BeadChip (Illumina, CA, USA). This array analyses over 850,000 CpGs per sample. In the WHI and FOS studies, the Infinium HumanMethylation450 BeadChip (Illumina, CA, USA) was used. This array analyses over 485,000 CpGs per sample [14], from which 439,562 are included in EPIC BeadChip [15].

A detailed quality control pipeline for the methylation data is available in Additional file 2: Supplementary Material. Methylation status at each CpG site was reported by β-values [16].

β-values range between 0 (completely unmethylated) and 1 (completely methylated). To remove potential sources of technical variation not related to the underlying biology, we standardized the β-values by batch, as described previously by our group [17]. Finally, to avoid the influence of extreme values, we excluded those CpGs with a β-value 4 standard deviations higher and lower from the mean.

Covariates

The following covariates were considered in our analyses: smoking, diabetes, hyperlipidaemia and hypertension (see definitions in Additional file 2: Supplementary Material). Moreover, we inferred the peripheral blood cell counts using DNA methylations signatures with the FlowSorted.Blood.450k R package available through the Bioconductor repository [18]. We also estimated two surrogate variables for unknown sources of potential technical or biological confounding using the sva R package [19].

Statistical analysis

All statistical analyses were performed using R in the computer cluster of the Hospital del Mar Medical Research Institute. The codes of the Singularity images are available in the repositories at https://github.com/regicor/methylation_ami/. A detailed description of the statistical methods is provided in Additional file 2: Supplementary Material.

Association between DNA methylation and cardiovascular outcomes

Logistic regression was used in the analyses in the samples of REGICOR, FOS with prevalent cases and WHI with incident cases, while Cox regression was used in the sample of FOS with incident cases. We considered the cardiovascular event (AMI, CHD or CVD) as the outcome and DNA methylation as the exposure or independent variable.

We defined three models for each approach. Model 1 was adjusted for estimated cell counts and two surrogate variables (plus age and ethnicity in the WHI sample, plus age and sex in the FOS samples). Model 2 was additionally adjusted for smoking status. Model 3 was further adjusted for diabetes, hyperlipidaemia and hypertension.

In order to avoid epigenomic inflation, we corrected the coefficients, the standard errors and the *p* values using the *bacon* R package available through the Bioconductor repository [20].

All analyses were performed in each study and afterwards we performed a fixed-effect meta-analysis of the bacon-corrected effect sizes observed in the EWAS weighted by the inverse of the variance. In approach A, we meta-analysed EWAS data from the REGICOR case-control and from the FOS sample with CHD-prevalent data. In approach B, we meta-analysed EWAS data from the WHI sample and from the FOS sample with incidence data (both for CHD and CVD events).

We defined as statistically significant any association fulfilling the Bonferroni criteria (0.05 divided by the number of probes analysed).

Association between the identified CpGs and CVRFs

We also analysed whether the methylation levels of the identified CpGs were associated with individual CVRFs in the three populations using multiple linear regression, and then meta-analysed the results.

Methylation risk scores (MRS) and predictive capacity

We developed weighted MRS based on the CpGs identified in each approach. We evaluated the association between these scores and CVD and CHD incidence in the FOS sample, using Cox regression. All analyses were adjusted for age, sex, diabetes, smoking status, systolic blood pressure (SBP), hypertensive treatment, and levels of total cholesterol and cholesterol transported in high-density lipoproteins (HDL-C), which are the CVRFs considered in the Framingham CVR function [21].

We also assessed the potential added predictive value of including the MRS in the Framingham CVR function. First, we evaluated the calibration of the models using the Hosmer-Lemeshow test [22]. Next, we assessed the discriminative capacity of the models using the concordance index (c-statistic) [23], applying the rcorr.cens function of the Hmisc R package. Last, we calculated the reclassification improvement using the net reclassification improvement (NRI) index [24] with the nricens R package. We also analysed the NRI in the group of individuals with intermediate CVR (clinical NRI). To correct for bias in the NRI estimation among individuals with intermediate risk, we used the method proposed by Paynter and Cook [25].

Causality of associations between DNA methylation and cardiovascular outcomes

We performed Mendelian Randomization (MR) studies to determine the direction of the association between the identified CpGs and cardiovascular events using the MR-based platform [26]. We selected methylation-level quantitative trait loci (meQTL) from the ARIES meQTL database [27] as instrumental variables, and summary statistic data from a meta-analysis of GWAS assessing CHD [28]. A more detailed description of the analysis is included in Additional file 2: Supplementary Material.

RESULTS

Quality control of DNA methylation data, cardiovascular outcomes and covariates

A detailed description of the results of the quality control analysis of the data is included in Additional file 2: Supplementary Material. Briefly, we included 391 individuals and 811,610 CpGs in the REGICOR case-control study, 1,863 women and 478,369 CpGs in the WHI sample and 2,540 participants and 483,656 CpGs in the FOS (Additional file 3: Figure S1). The main sociodemographic and clinical characteristics of the three populations are shown in Tables 1 and 2.

Association between DNA methylation and cardiovascular outcomes: EWAS strategy

A. Analyses with prevalent data

The meta-analysis of the REGICOR case-control and the FOS prevalent cases included 412,546 CpGs. We identified four and three CpGs associated with AMI and CHD ($p < 1.212 \cdot 10^{-7}$) in models 1 and 2, respectively, but none in model 3. In total, six differentially methylated CpGs were related to AMI and CHD. The magnitude of the association between the six CpGs and CHD had similar effect sizes in the three models (Table 3, Figure 2, Additional file 4: Table S2). These CpGs were located in four different genes and one intergenic region.

The most interesting results ($p < 10^{-5}$), Manhattan and Q-Q plots observed in each individual study are shown in Additional file 4: Tables S3-S4, and Additional file 5: Figures S2-S3.

B. Analyses with incident CHD and CVD events

The meta-analysis of the WHI and the FOS incident cases included 467,235 CpGs. We identified two CpGs associated with CHD (p <1.07·10^{-7}) in both model 1 and 2, and one in model 3. In the case of CVD, one, three and one CpGs were found in models 1, 2 and 3, respectively. In total, three and four differentially methylated CpGs were related to CHD and CVD, respectively; none of them related to both outcomes. The magnitud of the association between the seven CpGs and CHD or CVD had similar effect sizes in the three models (Table 3, Figure 3, Additional file 4: Table S5). These CpGs were located in five different genes and one intergenic region.

The most interesting results (p <10^{-5}), Manhattan and Q-Q plots observed in each individual study are shown in Additional file 4: Tables S6-S7, and Additional file 5: Figures S4-S5. The WHI results for CHD showed a genomic inflation (lambdas between 1.55-1.62) that were not corrected using the bacon method (lambdas between 1.70-1.84); thus, we used the coefficients and standard errors non-corrected with the bacon method for the CHD analyses. Due to this inflation, these results must be interpreted with caution.

Association between DNA methylation and cardiovascular outcomes: candidate-genes strategy

A. Analyses with prevalent data

This meta-analysis included 1,400 CpGs. Five and one CpGs were related to AMI and CHD (p <3.571·10^{-5}) in models 1 and 2, respectively, but none in model 3. In total, five unique CpGs were related to AMI and CHD, and their magnitude of association were similar in the three models (Additional File 6: Figure S6, and Additional File 7: Table S8). These CpGs were located in four different genes and one intergenic region.

The most interesting results (p <3.571·10^{-5}) in each individual study are shown in Additional File 7: Tables S9-S10.

B. Analyses with incident CHD and CVD events

We included 1,539 CpGs in this analysis. No CpG was associated with CHD in any model. Conversely, two and one CpGs were related to CVD (p <3.249·10^{-5}) in models 1 and 2, respectively, but none in model 3. In total, three differentially methylated CpGs were related

to CVD. The effect sizes of the associations betweenthe three CpGs and CVD were similar in the three models (Additional File 6: Figure S7, and Additional File 7: Table S11). They were located within the genes *AHRR* and *CPT1A* and one intergenic region. Two of these three CpGs were also identified in the EWAS analysis with prevalent CHD (cg21566642 and cg05575921).

We did not observe any significant association ($p < 3.249 \cdot 10^{-5}$) between any CpG and incident cardiovascular events in the individual analysis of each study.

Association between the identified CpGs and CVRFs

Table 4 shows the associations observed between the identified CpGs and classical CVRFs: smoking status, body mass index (BMI), systolic/diastolic blood pressure, and levels of glucose, triglycerides, total cholesterol, HDL-C and cholesterol transported in low-density lipoproteins (LDL-C). Six CpGs of the 13 identified in the EWAS strategy (Additional file 8: Table S12) and one of the six found in the candidate-gene strategy (Additional file 8: Table S13) were not related to any CVRF.

Association between MRS and incidence of CHD and CVD

The associations between MRS and the incidence of clinical coronary (n=94) and cardiovascular (n=222) events in the Framingham population are shown in Table 5. The median and interquartile range of the follow-up periods for CVD and CHD incidence were 7.67 (5.45, 9.89) and 7.87 (6.50, 9.25) years, respectively. Only the MRS for incident CVD events, including the CpGs identified in both the EWAS and candidate-gene strategies, were associated with higher CVR independently of the classical CVRFs.

The addition of any MRS to the Framingham risk function did not improve its capacity to discriminate either CVD or CHD in the Framingham cohort. Similarly, no increase in NRI was observed after including the MRS in the CVR function (Table 5).

Causality of the associations between DNA methylation and cardiovascular outcomes

We were able to assess causality of the association between CHD and two of the identified CpGs, cg21566642 (located in an intergenic region in chromosome 2) and cg03636183 (located within *F2RL3*). However, applying the criteria described in the Methods section, we

identified only one genetic variant associated with each of these CpGs. Therefore, only the Wald ratio method could be performed in the MR-Base web-app, and none of the associations was significant (Additional file 9: Table S14).

DISCUSSION

We have identified 17 methylation loci associated with CHD and CVD, 10 of them newly reported. Approximately 2/3 of these CpG sites were also related to traditional CVRFs, supporting the role of smoking and lipids in the development of these diseases, while 1/3 of the CpGs were not related to classical CVRFs, suggesting a key role of inflammation (Additional file 10: Table S15). We further constructed MRS and validated the association of some of these scores with CVD incidence independently of the traditional CVRFs. However, we did not observe an improvement of the predictive capacity of cardiovascular events when we included those risk scores in a CVR function. Additionally, our results about the causality of the association between the identified CpGs and CHD were not conclusive.

CpG sites consistently related to prevalent and incident CVD events

Two CpGs, cg21566642 and cg05575921, were significantly related to both prevalent CHD and incident CVD. cg21566642 maps to an intergenic region in chromosome 2. cg05575921 annotates to *AHRR*. To our knowledge, these CpGs were not associated with cardiovascular events in previous EWAS reports. Both CpGs were highly and inversely associated with smoking, which is supported by previous EWAS [29,30]. We have also previously reported both CpG sites as related to CVR independent of age [9]. cg05575921 was further associated directly with HDL-C and inversely with triglyceride levels in our study. This CpG has been related to all-cause mortality in a recent EWAS report [31] and was associated with both CHD prevalence and incidence in a candidate gene study [32].

Moreover, other four CpGs showed consistent association across the different outcomes of interest: cg06500161, cg21429551, cg19390658 and cg00574958. cg06500161 maps to *ABCG1,* which encodes a transporter involved in lipid homeostasis [33,34]. It has been previously related to CHD. Pfeiffer *et al* found this CpG in an EWAS assessment of lipid

profile, and reported its association with AMI in their discovery population but not in their replication sample [35]. Hedman *et al* found another CpG located within *ABCG1* that was related to lipids, as associated with incident CHD events [36]. Conversely, Campanella *et al* assessed the association between AMI and adiposity-related CpGs from their study, but did not associate cg06500161 with this outcome. We further found cg06500161 to be strongly and directly related to BMI and levels of triglycerides and inversely associated with HDL-C ($p < 2.2 \cdot 10^{-16}$). These associations have been previously observed in other studies [35–38]. cg06500161 methylation level has also been associated with ceramides and sphingomyelins levels [36], whose role in atherosclerosis and CHD has been reported [39,40].

cg21429551 and cg19390658 map to *GARS,* which encodes the ligase glycyl-tRNA synthetase. These CpGs were associated with smoking, BMI, triglycerides and HDL-C levels in our study and in previous reports [36,49,50]. Two previous candidate gene studies including both CpGs or cg21429551 alone have shown negative results regarding their association with CHD [32,36]. cg00574958, is located within *CPT1A*, which encodes an enzyme that regulates mitochondrial fatty acid oxidation [51]. It was positively associated with HDL-C levels and inversely associated with BMI and blood pressure, glucose and triglycerides levels. These findings are consistent with previous studies [9,36,38,52–54]. Genetic variants in *CPTA1* have also been associated with calcified atherosclerotic plaque in coronary artery [55].

All these results highlight the relevance of smoking (*AHRR*) and lipid metabolism (*ABCG1, GARS* and *CPTA1*) to atherosclerosis and CHD.

Other CpG sites related to prevalent CHD events

Six additional CpGs were related to prevalent cases of CHD. One of them, cg03636183, which maps to *F2RL3*, is widely reported to be associated with smoking [17], consistent with our findings. It is also related to all-cause and cardiovascular mortality [31,41,42].

Three additional CpGs of the six were also associated with CVRFs but their association with CHD has not been previously reported. cg00076653 (located within *C1QTNF7*) was directly

associated with glucose levels. *C1QTNF7* encodes a member of the CTRP family of adipokines related to metabolic dysfunction in the context of obesity [43]. cg11643285 and cg17238319 (both mapping to *RFTN1*) were associated with total and LDL cholesterol. Finally, cg14597545 and cg19314882, annotated to *ADPGK* and *DSCAML1*, respectively, were associated with prevalent CHD but not with CVRFs. Previous studies have found that cg14597545 is differentially methylated in the offspring of mothers with preeclampsia [44] cg19314882 is related to air pollution [45].

These six CpGs were not related to incident events, calling into question the temporal sequence of events and suggesting that these methylation signatures could be a consequence of the coronary event instead of a cause.

Additional CpG sites related to incident CHD or CVD events

Three CpGs (cg19893751, cg09165129 and cg07817505) were related to incident cases of CHD in the EWAS strategy. Neither was related to any CVRF. These three CpGs must be considered with caution as we could not correct the genomic inflation of the results in the WHI sample. Further information about these CpGs is provided in Additional file 10: Table S15.

Two additional CpGs were related to CVD incident events: cg08122652 and cg25103337. cg08122652 is located within *PARP9/DTX3L.* Increased expression of *PARP9/DTX3L* gene complex boosts immune response [46]. PARP9 is involved in the regulation of macrophage activation via pro-inflammatory IFN-γ signalling, which underlies the role of inflammation in the pathogenesis of CHD [47]. Interestingly, a recent study related methylation at this CpG to lower risk of incident CHD, consistent with our findings in incident CVD [48]. cg25103337, mapping to *H6PD*, which encodes one enzyme involved in the glucose metabolism (pentose phosphate pathway), was not related to any CVRF. Over-activation of this pathway was reported to cause the vascular inflammation associated to hyperglycaemia [56]. Experimental and observational studies have also reported an inverse relation between deficiency of the enzyme and atherosclerosis and CHD [57,58]. Overall, these evidences also suggest the relevance of inflammation to atherosclerosis and CHD.

Methylation risk scores as predictive CVD biomarkers

To assess the value of the identified CpGs as CVR biomarkers, we followed the AHA recommendations [59]. The risk scores based on CpGs associated with CVD incident cases were also associated with the incidence of CVD events in the FOS. However, we did not observe an improvement in the predictive capacity of the Framingham risk function when including any of the MRSs. This highlights the challenges of CVR prediction and novel biomarkers discovery, as well as the complex molecular network underlying CVD.

Causality of the associations between methylation loci and cardiovascular outcomes

MR studies based on public meQTLs could only be performed on the associations of cg21566642 and cg03636183 with AMI and CHD. Although a non-causal relationship was suggested, this must be interpreted with caution as only one genetic variable was used as genetic instrument. However, the association between CVRFs and some of the reported CpGs suggest that methylation at these loci might occur prior to a CVD event.

Strengths and limitations

The main strength of our study is that we included three independent populations and used two complementary strategies (hypothesis-free and hypothesis-based) to unravel methylation patterns related to cardiovascular events. We further assessed the relationships in both prevalent and incident cases.

On the one hand, we are aware of several limitations regarding the study design and the differences between the samples. First, we did not have access to information about AMI events in all populations and so could not compare methylation changes related to this specific CHD outcome. Second, our study is based on populations of European origin and has intrinsic EWAS limitations such as the type of sample used or the population size.

On the other hand, some analytical and technical limitations were also observed. First, one third of the CpGs analysed in the REGICOR population could not be assessed in the FOS population as the methylation arrays differed in the number of interrogated CpGs (EPIC VS 450k, respectively). Second, we could not reduce the inflation in the CHD results of the WHI sample; however, the two CpGs reported from this approach were also related to CHD in the

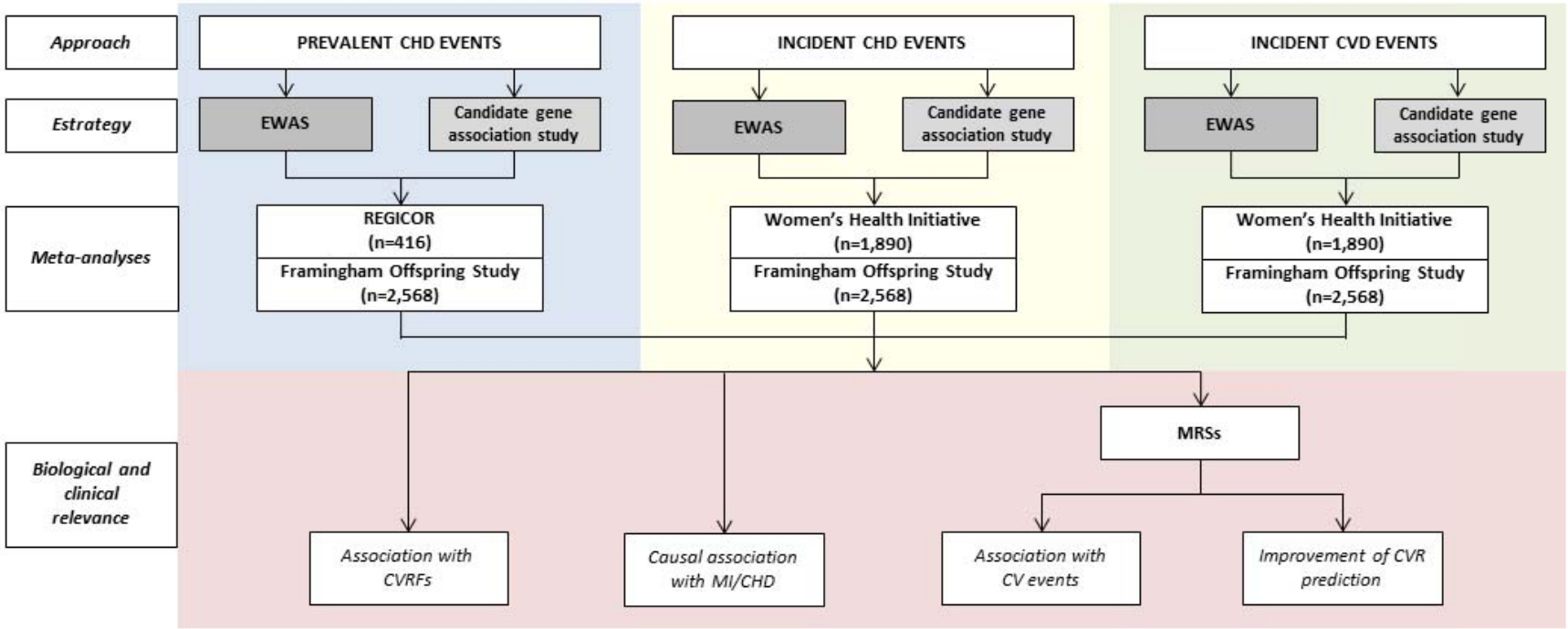
FOS population. Last, we cannot infer causality in the approach with prevalent cases, since changes in methylation could have occurred either prior to the event or as a consequence of the acute phase and disease management. We aimed to perform Mendelian randomization studies of the association between the identified CpGs and cardiovascular events, but available meQTL datasets are still limited.

Conclusion

Our study provides novel findings on DNA methylation loci related to the risk of CHD and CVD. Additionally, our results highlight the importance of traditional CVRFs in the development of these diseases, but also identify inflammation as a key pathway. Our results question the relevance of DNA methylation as a predictive biomarker. Overall, our findings contribute to understanding the molecular landscape of both prevalent and incident CHD/CVD and may be considered for further research and potential design of new preventive and therapeutic strategies.

Figures

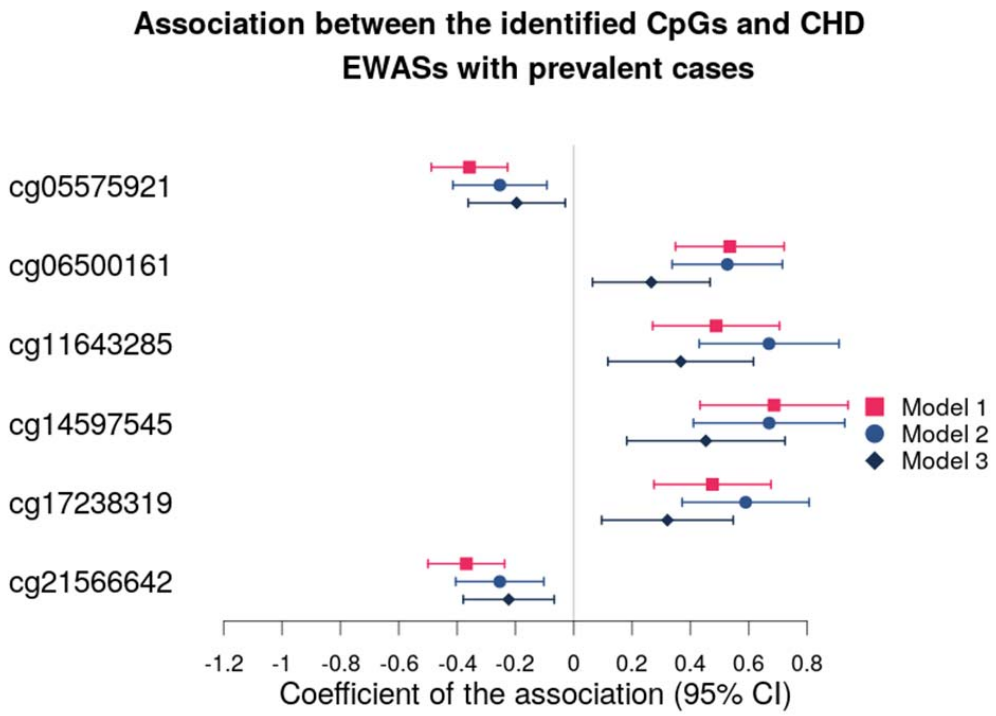
Figure 1. Overview of the study design.



We designed two strategies: epigenome-wide and candidate-gene association studies. In both strategies, we used three independent populations (REGICOR, Framingham Offspring Study and Women's Health Initiative) and we defined two complementary approaches based on their different design: (a) analyses with prevalent cases, and (b) analyses with incident cases. We performed meta-analyses of the

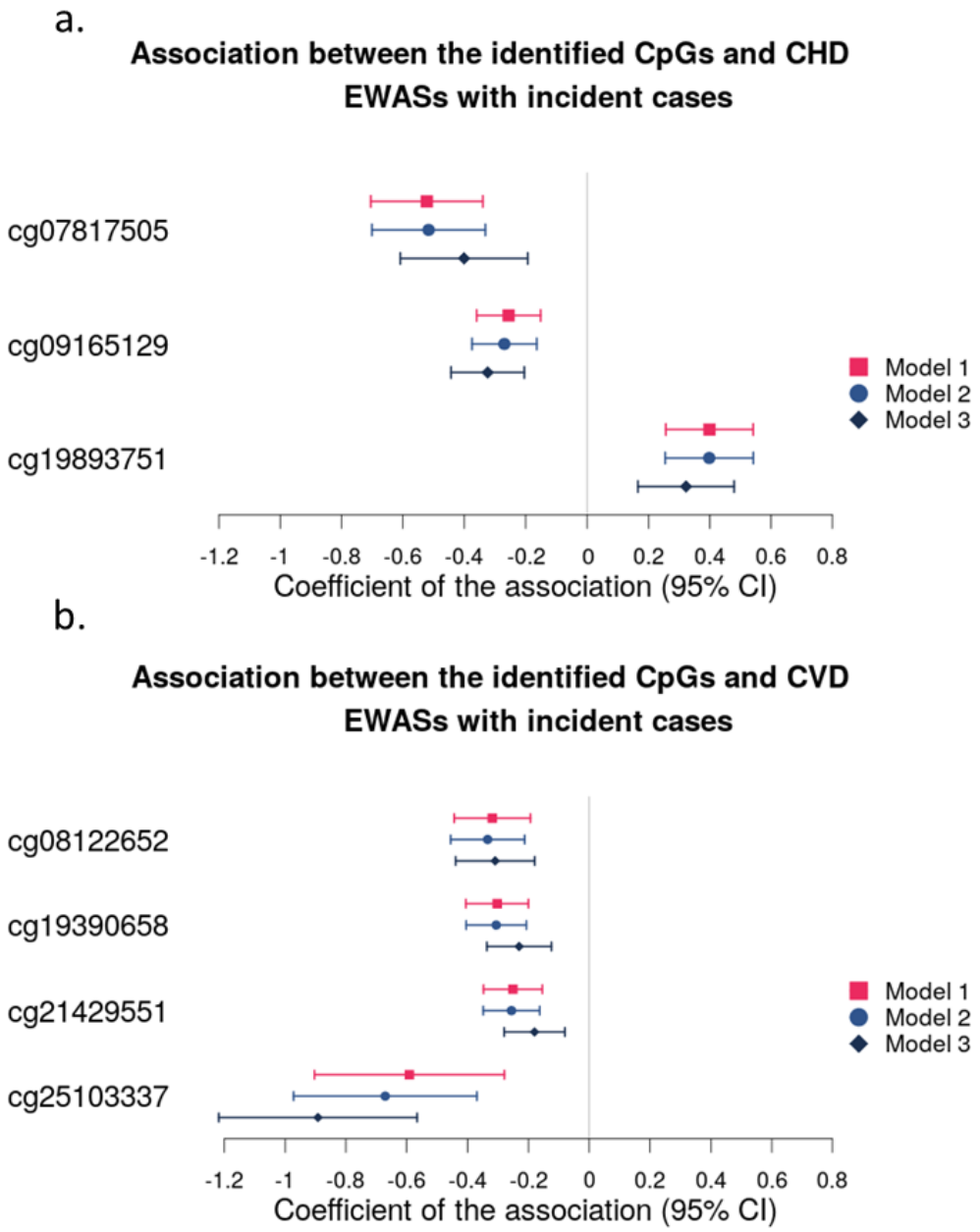
corresponding combinations. Those CpGs significant after correcting for multiple comparisons were analysed in association with classical cardiovascular risk factors (CVRFs). We also evaluated the causality of their association with acute myocardial infarction (MI) and coronary heart disease (CHD). Finally, we constructed risk scores based on those CpGs (MRSs) to assess their predictive value of incident cardiovascular events and to evaluate the predictive capacity of a cardiovascular risk (CVR) function including these MRSs.

Figure 2. Forest plot of the associations between the significant CpGs (p <1.21·10^{-7}) and CHD in the meta-analysis of the EWAS of approach A: REGICOR sample and Framingham Offspring Study (FOS) subset with prevalent data.



Model 1 was adjusted for estimated cell counts and two surrogate variables (plus age and sex in FOS). Model 2 was further adjusted for smoking status. Model 3 was additionally adjusted for diabetes, hyperlipidaemia and hypertension.

Figure 3. Forest plot of the associations between the significant CpGs (p <1.07·10^{-7}) and CHD (a) or CVD (b) in the meta-analysis of the EWAS of approach B: Women's Health Initiative (WHI) sample and Framingham Offspring Study (FOS) subset with incident data.



Model 1 was adjusted for age, estimated cell counts and two surrogate variables (plus ethnicity in WHI and sex in FOS). Model 2 was further adjusted for smoking status. Model 3 was additionally adjusted for diabetes, hyperlipidaemia and hypertension.

Tables

Table 1. Descriptive characteristics of the populations used in the approach with prevalent CHD events: REGICOR and Framingham Offspring Study (FOS) participants.

Variables	REGICOR		FOS - Prevalent CHD	
	N=391	NA	N=2540	NA
Age	63.2 (6.94)	0	66.3 (8.92)	0
Sex, female, n (%)	201 (51.4)	0	1376 (54.2)	0
AMI/CHD, n (%)‡	196 (50.1)	0	274 (10.8)	0
Smokers, n (%)	93 (24.4)	10	252 (9.97)	12
BMI, kg/m2*‡	28.5 (4.75)	52	28.3 (5.38)	8
Total cholesterol, mg/dl*	-	-	186 (37.2)	1
LDL-C, mg/dl*‡	-	-	105 (31.3)	2
HDL-C, mg/dl*‡	-	-	57.4 (18.2)	2
TG, mg/dl†	-	-	102 [73.0;142]	1
Cholesterol treatment, n (%)	110 (32.5)	53	1097 (43.3)	4
Hypercholesterolaemia, n (%)‡	179 (53.0)	53	1265 (49.9)	5
SBP, mmHg*‡	-	-	126 (17.1)	2
DBP, mmHg*‡	-	-	71.6 (10.2)	5
HTN, n (%)‡	212 (57.1)	20	1441 (57.0)	11
HTN treatment, n (%)‡	139 (40.8)	50	1235 (48.8)	10
Glucose, mg/dl†	-	-	107 (23.7)	2
Diabetes, n (%)	87 (24.7)	39	392 (16.3)	140
Diabetes treatment, n (%)	61 (17.9)	51	129 (5.34)	124

**Mean (Standard deviation). †Median (Interquartile Range). ‡AMI, Acute myocardial infarction; CHD, Coronary heart disease; BMI, Body mass index; LDL-C, Cholesterol in low-density lipoprotein; HDL-C, Cholesterol in high-density lipoprotein; TG, Triglycerides; Hypercholesterolaemia, defined as previous treatment or total cholesterol≥240 mmHg or LDL-C≥160 mmHg (or self-reported in the REGICOR case-control study); SBP, Systolic blood pressure; DBP, Diastolic blood pressure; HTN, Hypertension, defined as previous*

treatment or SBP≥140 mmHg or DBP≥90 mmHg (or self-reported in the REGICOR case-control study); Diabetes, defined as previous treatment or glycaemia≥126mg/dl (or self-reported in the REGICOR case-control study).

Table 2. Descriptive characteristics of the populations used in the approach with incident CVD and CHD events: Women's Health Initiative (WHI) and Framingham Offspring Study (FOS) participants.

Variables	WHI		FOS CHD		FOS CVD	
	N=1863	NA	N=2266	NA	N=2123	NA
Age	64.2 (7.03)	0	65.7 (8.82)	0	65.3 (8.66)	0
Sex, female, n (%)	1863 (100)	0	1284 (56.7)	0	1206 (56.8)	0
CHD, n (%)‡	914 (49.1)	0	106 (4.68)	0	_	_
CVD, n (%)‡	984 (52.8)	0	_	_	222 (10.5)	0
Smokers, n (%)	174 (9.46)	24	217 (9.62)	11	202 (9.56)	11
BMI, kg/m2*‡	29.8 (5.97)	11	28.1 (5.39)	8	28.0 (5.33)	6
Total cholesterol, mg/dl*	233 (42.3)	1	189 (36.2)	1	190 (35.8)	1
LDL-C, mg/dl*‡	152 (38.7)	31	107 (30.8)	2	108 (30.5)	2
HDL-C, mg/dl*‡	52.2 (13.2)	1	58.3 (18.3)	2	58.7 (18.3)	2
TG, mg/dl†	126 [93.0;176]	1	101 [73.0;140]	1	100 [73.0;139]	1
Cholesterol treatment, n (%)	166 (11.4)	403	871 (38.5)	3	787 (37.1)	3
Hypercholesterolaemia, n (%)‡	913 (55.8%)	227	1033 (45.7)	4	943 (44.5)	4
SBP, mmHg*‡	131 (17.5)	0	126 (16.9)	1	125 (16.7)	1
DBP, mmHg*‡	76.4 (9.27)	0	72.1 (9.99)	3	72.3 (9.92)	3
HTN, n (%)‡	952 (61.2)	308	1217 (53.9)	8	1110 (52.5)	7
HTN treatment, n (%)‡	672 (45.9)	400	1025 (45.4)	7	927 (43.8)	6
Glucose, mg/dl†	107 (38.3)	1	106 (21.8)	2	105 (21.5)	2
Diabetes, n (%)	319 (17.1)	0	307 (14.3)	126	266 (13.3)	119
Diabetes treatment, n (%)	178 (9.57)	3	87 (4.04)	110	72 (3.56)	102

**Mean (Standard deviation). †Median (Interquartile range). ‡ CHD, Coronary heart disease; CVD, Cardiovascular disease; BMI, Body mass index; LDL-C, Cholesterol in low-density lipoprotein; HDL-C, Cholesterol in high-density lipoprotein; TG, Triglycerides; Hypercholesterolaemia, defined as previous treatment or total cholesterol ≥240 mmHg or LDL-C ≥160 mmHg; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; HTN,*

Hypertension, defined as previous treatment or SBP ≥140 mmHg or DBP ≥90 mmHg;

Diabetes, defined as previous treatment or glycaemia ≥126mg/dl.

Table 3. Summary of the meta-analysis results: Differentially methylated CpGs associated with prevalent and incident events and consistency across the different outcomes. Significant associations from the EWAS and the candidate gene strategies are given for each approach. A. Significant CpGs from the approach with prevalent CHD. B. Significant CpGs from the approach with incident CHD. C. Significant CpGs from the approach with incident CVD. Results from the fixed-effects meta-analyses of model 1 are shown (adjusted for estimated cell counts and two surrogate variables, plus age and sex in the FOS, plus age and ethnicity in the WHI).

Genomic information				CHD prevalence			CHD incidence			CVD incidence		
CpG	**Chr***	**Position**	**Gene**	**Coef***	**SE***	***P***	**Coef***	**SE***	***P***	**Coef***	**SE***	***P***
A. CHD prevalence												
EWAS strategy												
cg21566642	2	233,284,661	Intergenic	**$-3.68\cdot10^{-1}$**	**$6.69\cdot10^{-2}$**	**$3.79\cdot10^{-8}$**	$-1.34\cdot10^{-1}$	$4.33\cdot10^{-2}$	$2.00\cdot10^{-3}$	$-1.72\cdot10^{-1}$	$4.38\cdot10^{-2}$	$8.29\cdot10^{-5}$
cg11643285	3	16,411,667	*RFTN1*	$4.87\cdot10^{-1}$	$1.11\cdot10^{-1}$	$1.08\cdot10^{-5}$	$-4.13\cdot10^{-4}$	$6.33\cdot10^{-2}$	$9.95\cdot10^{-1}$	$6.95\cdot10^{-2}$	$7.04\cdot10^{-2}$	$3.23\cdot10^{-1}$
cg17238319	3	16,428,391	*RFTN1*	$4.75\cdot10^{-1}$	$1.02\cdot10^{-1}$	$3.34\cdot10^{-6}$	$8.95\cdot10^{-2}$	$6.88\cdot10^{-2}$	$1.93\cdot10^{-1}$	$6.85\cdot10^{-2}$	$7.40\cdot10^{-2}$	$3.55\cdot10^{-1}$
cg05575921	5	373,378	*AHRR*	**$-3.58\cdot10^{-1}$**	**$6.66\cdot10^{-2}$**	**$7.86\cdot10^{-8}$**	$-1.80\cdot10^{-1}$	$4.73\cdot10^{-2}$	$1.38\cdot10^{-4}$	$-2.09\cdot10^{-1}$	$4.73\cdot10^{-2}$	$9.48\cdot10^{-6}$
cg14597545	15	73,074,210	*ADPGK*	**$6.86\cdot10^{-1}$**	**$1.29\cdot10^{-1}$**	**$1.12\cdot10^{-7}$**	$2.40\cdot10^{-1}$	$9.16\cdot10^{-2}$	$8.67\cdot10^{-3}$	$2.15\cdot10^{-1}$	$8.88\cdot10^{-2}$	$1.53\cdot10^{-2}$
cg06500161	21	43,656,587	*ABCG1*	**$5.35\cdot10^{-1}$**	**$9.49\cdot10^{-2}$**	**$1.79\cdot10^{-8}$**	$2.40\cdot10^{-1}$	$6.20\cdot10^{-2}$	$1.11\cdot10^{-4}$	$2.38\cdot10^{-1}$	$6.07\cdot10^{-2}$	$8.55\cdot10^{-5}$
Candidate gene strategy												
cg21566642	2	233,284,661	Intergenic				*Previously reported*					
cg00076653	4	15,341,878	*C1QTNF7*	**$3.47\cdot10^{-1}$**	**$8.28\cdot10^{-2}$**	**$2.78\cdot10^{-5}$**	$1.50\cdot10^{-1}$	$6.52\cdot10^{-2}$	$2.13\cdot10^{-2}$	$1.41\cdot10^{-1}$	$5.77\cdot10^{-2}$	$1.45\cdot10^{-2}$
cg05575921	5	373,378	*AHRR*				*Previously reported*					
cg19314882	11	117,391,953	*DSCAML1*	**$4.13\cdot10^{-1}$**	**$9.73\cdot10^{-2}$**	**$2.22\cdot10^{-5}$**	$-1.08\cdot10^{-1}$	$6.50\cdot10^{-2}$	$9.61\cdot10^{-2}$	$-5.38\cdot10^{-2}$	$6.01\cdot10^{-2}$	$3.71\cdot10^{-1}$
cg03636183	19	17,000,585	*F2RL3*	**$-3.34\cdot10^{-1}$**	**$6.60\cdot10^{-2}$**	**$4.15\cdot10^{-7}$**	$-8.60\cdot10^{-2}$	$4.88\cdot10^{-2}$	$7.77\cdot10^{-2}$	$-1.10\cdot10^{-1}$	$4.39\cdot10^{-2}$	$1.19\cdot10^{-2}$
B. CHD incidence												
EWAS strategy												

cg19893751	2	45,029,285	Intergenic	$-2.75\cdot10^{-2}$	$9.71\cdot10^{-2}$	$7.77\cdot10^{-1}$	**$3.99\cdot10^{-1}$**	**$7.26\cdot10^{-2}$**	**$3.90\cdot10^{-8}$**	$2.55\cdot10^{-1}$	$6.89\cdot10^{-2}$	$2.17\cdot10^{-4}$
cg09165129	7	137,524,369	*DGKI*	$-9.53\cdot10^{-2}$	$8.82\cdot10^{-2}$	$2.80\cdot10^{-1}$	$-2.56\cdot10^{-1}$	$5.32\cdot10^{-2}$	$1.52\cdot10^{-6}$	$-1.74\cdot10^{-1}$	$5.38\cdot10^{-2}$	$1.25\cdot10^{-3}$
cg07817505	19	17,972,324	*RPL18AP3; SNORA68; RPL18A*	$-1.98\cdot10^{-1}$	$1.37\cdot10^{-1}$	$1.47\cdot10^{-1}$	**$-5.22\cdot10^{-1}$**	**$9.31\cdot10^{-2}$**	**$2.06\cdot10^{-8}$**	$-3.65\cdot10^{-1}$	$9.16\cdot10^{-2}$	$6.73\cdot10^{-5}$
C. CVD incidence												
EWAS strategy												
cg25103337	1	9,293,583	*H6PD*	$6.35\cdot10^{-2}$	$2.20\cdot10^{-1}$	$7.73\cdot10^{-1}$	$-6.43\cdot10^{-1}$	$1.68\cdot10^{-1}$	$1.33\cdot10^{-4}$	$-5.91\cdot10^{-1}$	$1.59\cdot10^{-1}$	$2.06\cdot10^{-4}$
cg08122652	3	122,281,939	*PARP9; DTX3L*	$-4.05\cdot10^{-2}$	$9.83\cdot10^{-2}$	$6.81\cdot10^{-1}$	$-3.11\cdot10^{-1}$	$6.72\cdot10^{-2}$	$3.54\cdot10^{-6}$	**$-3.19\cdot10^{-1}$**	**$6.39\cdot10^{-2}$**	**$6.17\cdot10^{-7}$**
cg21429551	7	30,635,762	*GARS*	$-2.42\cdot10^{-1}$	$7.14\cdot10^{-2}$	$7.07\cdot10^{-4}$	$-1.83\cdot10^{-1}$	$4.95\cdot10^{-2}$	$2.14\cdot10^{-4}$	**$-2.51\cdot10^{-1}$**	**$4.93\cdot10^{-2}$**	**$3.75\cdot10^{-7}$**
cg19390658	7	30,636,176	*GARS*	$-2.58\cdot10^{-1}$	$7.49\cdot10^{-2}$	$5.75\cdot10^{-4}$	$-2.62\cdot10^{-1}$	$5.38\cdot10^{-2}$	$1.16\cdot10^{-6}$	**$-3.03\cdot10^{-1}$**	**$5.25\cdot10^{-2}$**	**$7.89\cdot10^{-9}$**
Candidate gene strategy												
cg21566642	2	233,284,661	Intergenic	*Previously reported*								
cg05575921	5	373,378	*AHRR*	*Previously reported*								
cg00574958	11	68,607,622	*CPT1A*	$-3.04\cdot10^{-1}$	$8.03\cdot10^{-2}$	$1.50\cdot10^{-4}$	$-1.76\cdot10^{-1}$	$5.35\cdot10^{-2}$	$1.01\cdot10^{-3}$	**$-2.01\cdot10^{-1}$**	**$4.96\cdot10^{-2}$**	**$5.07\cdot10^{-5}$**

**Chr, Chromosome; Coef; Coefficient of association; SE, Standard error.*

†Significant associations are highlighted in bold.

Table 4. Meta-analysis of the associations between the methylation level in the identified CVD-related CpGs and cardiovascular risk factors (CVRFs) in the REGICOR, the Women's Health Initiative (WHI) and the Framingham Offspring Study (FOS) samples. A. CpGs identified in both the candidate-gene and the EWAS strategies. B. CpGs identified in the EWAS strategy. C. CpGs identified in the candidate-gene strategy. Fixed-effects meta-analysis with the linear regression results from the REGICOR, WHI and FOS samples were performed. Only CpGs and CVRFs significantly associated with any CVRF or CpG, respectively, are shown.

a. CpGs from the candidate-gene and EWAS strategies

CpG	Smoking	BMI*	HDL-C*	TG*	Glucose	SBP	DBP*	
	-1.42	$5.48 \cdot 10^{-3}$	$4.24 \cdot 10^{-4}$	$-5.50 \cdot 10^{-4}$	$-9.27 \cdot 10^{-4}$	$1.60 \cdot 10^{-3}$	$7.37 \cdot 10^{-3}$	**Coefficient**
cg21566642	$4.25 \cdot 10^{-2}$	$2.66 \cdot 10^{-3}$	$9.87 \cdot 10^{-4}$	$2.02 \cdot 10^{-4}$	$4.87 \cdot 10^{-4}$	$8.79 \cdot 10^{-4}$	$1.55 \cdot 10^{-3}$	**SE***
	$<2.2 \cdot 10^{-16}$	$3.93 \cdot 10^{-2}$	$6.68 \cdot 10^{-1}$	$6.60 \cdot 10^{-3}$	$5.73 \cdot 10^{-2}$	$6.90 \cdot 10^{-2}$	**$1.85 \cdot 10^{-6}$**	***P***
	-1.80	$7.91 \cdot 10^{-3}$	$3.89 \cdot 10^{-3}$	$-7.50 \cdot 10^{-4}$	$-6.89 \cdot 10^{-4}$	$8.90 \cdot 10^{-4}$	$7.29 \cdot 10^{-3}$	**Coefficient**
cg05575921	$3.92 \cdot 10^{-2}$	$2.51 \cdot 10^{-3}$	$9.27 \cdot 10^{-4}$	$1.89 \cdot 10^{-4}$	$4.53 \cdot 10^{-4}$	$8.19 \cdot 10^{-4}$	$1.44 \cdot 10^{-3}$	**SE***
	$<2.2 \cdot 10^{-16}$	$1.61 \cdot 10^{-3}$	**$2.75 \cdot 10^{-5}$**	**$7.53 \cdot 10^{-5}$**	$1.29 \cdot 10^{-1}$	$2.77 \cdot 10^{-1}$	**$4.27 \cdot 10^{-7}$**	***P***

b. CpGs from the EWAS strategy

CpG	Smoking	BMI*	Total chol*	HDL-C*	LDL-C*	TG*	Glucose	SBP	DBP*	
	$3.10 \cdot 10^{-2}$	$2.36 \cdot 10^{-3}$	$-1.20 \cdot 10^{-3}$	$-1.44 \cdot 10^{-4}$	$-1.18 \cdot 10^{-3}$	$-3.52 \cdot 10^{-4}$	$8.72 \cdot 10^{-4}$	$-1.28 \cdot 10^{-3}$	$-3.89 \cdot 10^{-3}$	**Coef***
cg17238319	$3.08 \cdot 10^{-2}$	$1.78 \cdot 10^{-3}$	$2.68 \cdot 10^{-4}$	$6.16 \cdot 10^{-4}$	$3.03 \cdot 10^{-4}$	$1.40 \cdot 10^{-4}$	$3.65 \cdot 10^{-4}$	$5.85 \cdot 10^{-4}$	$1.01 \cdot 10^{-3}$	**SE***
	$3.14 \cdot 10^{-1}$	$1.84 \cdot 10^{-1}$	**$7.87 \cdot 10^{-6}$**	$8.15 \cdot 10^{-1}$	**$9.25 \cdot 10^{-5}$**	$1.17 \cdot 10^{-2}$	$1.67 \cdot 10^{-2}$	$2.94 \cdot 10^{-2}$	**$1.13 \cdot 10^{-4}$**	***P***
cg19390658	$-1.29 \cdot 10^{-1}$	$-1.12 \cdot 10^{-2}$	$-7.86 \cdot 10^{-4}$	$3.69 \cdot 10^{-3}$	$-3.28 \cdot 10^{-4}$	$-1.54 \cdot 10^{-3}$	$-1.52 \cdot 10^{-3}$	$-3.00 \cdot 10^{-3}$	$-2.49 \cdot 10^{-3}$	**Coef***

	$4.80 \cdot 10^{-2}$	$2.62 \cdot 10^{-3}$	$3.86 \cdot 10^{-4}$	$9.70 \cdot 10^{-4}$	$4.33 \cdot 10^{-4}$	$1.99 \cdot 10^{-4}$	$4.84 \cdot 10^{-4}$	$8.77 \cdot 10^{-4}$	$1.54 \cdot 10^{-3}$	**SE***
	$7.16 \cdot 10^{-3}$	**$1.81 \cdot 10^{-5}$**	$4.21 \cdot 10^{-2}$	**$1.40 \cdot 10^{-4}$**	$4.49 \cdot 10^{-1}$	**$8.88 \cdot 10^{-15}$**	$1.76 \cdot 10^{-3}$	$6.36 \cdot 10^{-4}$	$1.06 \cdot 10^{-1}$	***P***
	$1.47 \cdot 10^{-2}$	$1.87 \cdot 10^{-3}$	$-9.53 \cdot 10^{-4}$	$-4.00 \cdot 10^{-4}$	$-9.18 \cdot 10^{-4}$	$-2.30 \cdot 10^{-4}$	$6.31 \cdot 10^{-4}$	$-1.31 \cdot 10^{-3}$	$-3.59 \cdot 10^{-3}$	**Coef***
cg11643285	$2.46 \cdot 10^{-2}$	$1.42 \cdot 10^{-3}$	$2.15 \cdot 10^{-4}$	$4.77 \cdot 10^{-4}$	$2.43 \cdot 10^{-4}$	$1.11 \cdot 10^{-4}$	$3.01 \cdot 10^{-4}$	$4.63 \cdot 10^{-4}$	$7.92 \cdot 10^{-4}$	**SE***
	$5.51 \cdot 10^{-1}$	$1.87 \cdot 10^{-1}$	**$9.02 \cdot 10^{-6}$**	$4.01 \cdot 10^{-1}$	**$1.62 \cdot 10^{-4}$**	$3.91 \cdot 10^{-2}$	$3.62 \cdot 10^{-2}$	$4.63 \cdot 10^{-3}$	$5.86 \cdot 10^{-6}$	***P***
	$-2.30 \cdot 10^{-1}$	$-1.48 \cdot 10^{-2}$	$2.04 \cdot 10^{-4}$	$4.98 \cdot 10^{-3}$	$8.77 \cdot 10^{-4}$	$-1.82 \cdot 10^{-3}$	$-2.39 \cdot 10^{-3}$	$-3.51 \cdot 10^{-3}$	$-1.84 \cdot 10^{-3}$	**Coef***
cg21429551	$4.79 \cdot 10^{-2}$	$2.61 \cdot 10^{-3}$	$3.84 \cdot 10^{-4}$	$9.69 \cdot 10^{-4}$	$4.29 \cdot 10^{-4}$	$1.98 \cdot 10^{-4}$	$4.79 \cdot 10^{-4}$	$8.74 \cdot 10^{-4}$	$1.54 \cdot 10^{-3}$	**SE***
	$1.58 \cdot 10^{-6}$	**$1.30 \cdot 10^{-8}$**	$5.96 \cdot 10^{-1}$	**$2.76 \cdot 10^{-7}$**	$4.10 \cdot 10^{-2}$	**$<2.2 \cdot 10^{-16}$**	**$6.21 \cdot 10^{-7}$**	**$5.95 \cdot 10^{-5}$**	$2.33 \cdot 10^{-1}$	***P***
	$-5.89 \cdot 10^{-2}$	$2.83 \cdot 10^{-2}$	$-1.67 \cdot 10^{-3}$	$-1.19 \cdot 10^{-2}$	$-1.97 \cdot 10^{-3}$	$2.41 \cdot 10^{-3}$	$4.33 \cdot 10^{-3}$	$3.50 \cdot 10^{-4}$	$4.73 \cdot 10^{-4}$	**Coef***
cg06500161	$4.81 \cdot 10^{-2}$	$2.57 \cdot 10^{-3}$	$3.81 \cdot 10^{-4}$	$9.48 \cdot 10^{-4}$	$4.26 \cdot 10^{-4}$	$1.95 \cdot 10^{-4}$	$4.74 \cdot 10^{-4}$	$8.71 \cdot 10^{-4}$	$1.53 \cdot 10^{-3}$	**SE***
	$2.20 \cdot 10^{-1}$	**$<2.2 \cdot 10^{-16}$**	**$1.24 \cdot 10^{-5}$**	**$<2.2 \cdot 10^{-16}$**	**$4.00 \cdot 10^{-6}$**	**$<2.2 \cdot 10^{-16}$**	**$<2.2 \cdot 10^{-16}$**	$6.87 \cdot 10^{-1}$	$7.58 \cdot 10^{-1}$	***P***

c. CpGs from the candidate-gene strategy

CpG	Smoking	BMI*	HDL-C*	TG*	Glucose	SBP	DBP*	
	$-1.06 \cdot 10^{-1}$	$7.54 \cdot 10^{-3}$	$-2.69 \cdot 10^{-3}$	$3.14 \cdot 10^{-4}$	$1.63 \cdot 10^{-3}$	$-1.05 \cdot 10^{-3}$	$-8.36 \cdot 10^{-4}$	**Coefficient**
cg00076653	$4.65 \cdot 10^{-2}$	$2.54 \cdot 10^{-3}$	$9.38 \cdot 10^{-4}$	$1.94 \cdot 10^{-4}$	$4.69 \cdot 10^{-4}$	$8.47 \cdot 10^{-4}$	$1.49 \cdot 10^{-3}$	**SE***
	$2.34 \cdot 10^{-2}$	$2.97 \cdot 10^{-3}$	$4.07 \cdot 10^{-3}$	$1.05 \cdot 10^{-1}$	**$5.22 \cdot 10^{-4}$**	$2.16 \cdot 10^{-1}$	$5.74 \cdot 10^{-1}$	***P***
	$3.65 \cdot 10^{-2}$	$-3.11 \cdot 10^{-2}$	$6.25 \cdot 10^{-3}$	$-3.18 \cdot 10^{-3}$	$-5.20 \cdot 10^{-3}$	$-4.71 \cdot 10^{-3}$	$-5.19 \cdot 10^{-3}$	**Coefficient**
cg00574958	$4.77 \cdot 10^{-2}$	$2.55 \cdot 10^{-3}$	$9.57 \cdot 10^{-4}$	$1.92 \cdot 10^{-4}$	$4.70 \cdot 10^{-4}$	$8.66 \cdot 10^{-4}$	$1.52 \cdot 10^{-3}$	**SE***
	$4.43 \cdot 10^{-1}$	**$<2.2 \cdot 10^{-16}$**	**$6.44 \cdot 10^{-11}$**	**$<2.2 \cdot 10^{-16}$**	**$<2.2 \cdot 10^{-16}$**	**$5.33 \cdot 10^{-8}$**	**$6.65 \cdot 10^{-4}$**	***P***
	-1.34	$7.68 \cdot 10^{-3}$	$1.60 \cdot 10^{-3}$	$-4.65 \cdot 10^{-4}$	$1.82 \cdot 10^{-4}$	$2.80 \cdot 10^{-4}$	$6.23 \cdot 10^{-3}$	**Coefficient**
cg03636183	$4.44 \cdot 10^{-2}$	$2.64 \cdot 10^{-3}$	$9.78 \cdot 10^{-4}$	$2.00 \cdot 10^{-4}$	$4.81 \cdot 10^{-4}$	$8.75 \cdot 10^{-4}$	$1.54 \cdot 10^{-3}$	**SE***
	$<2.2 \cdot 10^{-16}$	$3.59 \cdot 10^{-3}$	$1.01 \cdot 10^{-1}$	$2.03 \cdot 10^{-2}$	$7.06 \cdot 10^{-1}$	$7.49 \cdot 10^{-1}$	**$5.20 \cdot 10^{-5}$**	***P***

**BMI, Body mass index; Total chol, Total cholesterol; HDL-C, Cholesterol in high-density lipoprotein; LDL-C, Cholesterol in low-density*

lipoprotein; TG, Triglycerides; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; Coef, Coefficient of association; SE, Standard error.

*†Significant p values considering the Bonferroni correction (0.05/(number of CpGs*9 CVRFs) are highlighted in bold (A. $p < 4.27 \cdot 10^{-4}$; B. $p < 9.26 \cdot 10^{-4}$; C. $p < 4.27 \cdot 10^{-4}$).*

Table 5. Utility of the methylation risk scores (MRS): association with CVD or CHD incidence and assessment of their predictive capacity. MRS were based on the results from approaches A and B of the EWAS and the candidate-gene strategies. MRS_1, MRS_2 and MRS_3 were calculated with CpGs from the EWAS strategy (approach A, approach B with CHD and approach B with CVD, respectively). MRS_4 and MRS_5 were calculated with CpGs from the candidate gene strategy (approach A and approach B with CVD, respectively). Analyses of the association with the CVD or CHD incidence were adjusted for age, sex, total cholesterol and HDL-C levels, diabetes, smoking status, systolic blood pressure, and hypertensive treatment, which are the CVRFs considered in the Framingham risk function. Analysis of the improvement in the predictive capacity of the Framingham function was performed with and without the corresponding MRS.

MRS	Association with CVD incidence		Association with CHD incidence		Improvement in the predictive capacity of Framingham risk function				
	HR* [95% CI*]	***P***	**HR* [95% CI*]**	***P***	**C-statistic Classical Function**	**C-statistic Classical Function + MRS**	**$P_{C\text{-}statistic}$***	**NRI* (%) [95% CI*]**	**Clinical NRI* (%) [95% CI*]**
MRS_1	1.18 [1;1.39]	0.056	1.09 [0.84;1.41]	0.509	0.688	0.691	0.392	-3.50 [-9.86;13.89]	-5.51 [-13.96;2.54]
MRS_2	0.98 [0.86;1.12]	0.774	1.14 [0.93;1.39]	0.205	0.692	0.696	0.449	1.47 [-7.41;11.68]	2.33 [-9.86;14.53]
MRS_3	**1.30 [1.13;1.50]**	**$2.86 \cdot 10^{-4}$**	1.19 [0.96;1.48]	0.111	0.726	0.734	0.242	-1.32· [-8.40;11.05]	-1.77 [-16.18;12.64]
MRS_4	1.17 [1;1.36]	0.047	0.97 [0.76;1.23]	0.785	0.690	0.689	0.770	1.94 [-7.07;15.11]	4.05 [-2.16;10.26]
MRS_5	**1.27 [1.08;1.49]**	**$3.20 \cdot 10^{-3}$**	1.04 [0.81;1.32]	0.776	0.731	0.738	0.185	4.97 [-4.10;13.15]	8.28 [-3.33;19.89]

***MRS, Methylation risk score; CVD, Cardiovascular disease; CHD, Coronary heart disease; HR, Hazard ratio; CI, Confidence interval; $P_{C\text{-}statistic}$, p value of the c-statistic comparison; NRI, Net-reclassification index.*

Additional Files

Additional File 1: Table S1: List of candidate CpGs and genes. (XLSX 13 kB)

Additional File 2: Supplementary Material. (PDF 523 kB)

Additional File 3: Figure S1: Flow chart of the quality control of the data. (PDF 28 kB)

Additional File 4: Table S2: Meta-analysis of the EWAS from approach A. **Table S3:** EWAS in the REGICOR sample. **Table S4:** EWAS in the FOS sample with prevalent CHD events. **Table S5:** Meta-analysis of the EWAS from approach B. **Table S6:** EWAS in the WHI sample. **Table S7:** EWAS in the FOS sample with incident events. (XLSX 125 kB)

Additional File 5: Figures S2-5: Manhattan and QQ plots of the EWAS in REGICOR, in FOS with prevalent events, in WHI and in FOS with incident events. (PDF 3.92 MB)

Additional File 6: Figure S6: Forest plot of the candidate-gene association studies from approach A. **Figure S7:** Forest plot of the candidate-gene association studies from approach B. (PDF 29 kB)

Additional File 7: Table S8: Meta-analysis of the candidate-gene association studies from approach A. **Table S9:** Candidate-gene association studies in the REGICOR sample. **Table S10:** Candidate-gene association studies in the FOS sample with prevalent CHD events. **Table S11:** Meta-analysis of the candidate-gene association studies from approach B. (XLSX 31 kB)

Additional File 8: Table S12: Association studies with CVRFs from the EWAS strategy. **Table S13:** Association studies with CVRFs from the candidate-gene strategy. (XLSX 65 kB)

Additional File 9: Table S14: Mendelian randomization studies of cg21566642 and cg03636183. (XLSX 13 kB)

Additional File 10: Table S15: Summary of the study and comparison with previous findings. (XLSX 26 kB)

List of abbreviations

AMI: Acute Myocardial Infarction; BP: Blood Pressure; BMI: Body Mass Index; CHD: Coronary Heart Disease; CVD: Cardiovascular Disease; CVR: Cardiovascular Risk; CVRF: Cardiovascular Risk Factor; DBP: Diastolic Blood Pressure; EWAS: Epigenome-Wide Association Studies; FOS: Framingham Offspring Study; HDL-C: High density lipoprotein cholesterol; LDL-C: Low density lipoprotein cholesterol; meQTLs: methylation-level Quantitative Trait Loci; MR: Mendelian Randomization; MRS: Methylation Risk Score; NRI: Net Reclassification Improvement; REGICOR: REgistre GIroní del COR; SBP: Systolic Blood Pressure; WHI: Women's Health Initiative.

Declarations

Ethics approval and consent to participate

The study was approved by the Parc de Salut Mar Ethics Committee (2015/6199/I; 2018/7855/I).

Consent for publication

Not applicable.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article (and its additional files). They are also available in our GitHub repository, at https://github.com/regicor/methylation_ami/. The original REGICOR datasets analysed during the current study are available from the corresponding author on reasonable request. WHI and FOS data are available from the Database of Genotypes and Phenotypes (http://dbgap.ncbi.nlm.nih.gov) but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All the authors have contributed to this manuscript according to the International Committee of Medical Journal Editors recommendations. The study was conceived and designed by RE and AF-S. Sample collection was directed by RE, JM and MS. MC and ME assessed DNA methylation of the REGICOR case-control. Data analyses were performed by AF-S with support from RE, S-SB, IS and SP-F. The manuscript was drafted by AF-S and RE, with the critical review of the rest of authors. All authors approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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