Association between DNA methylation and coronary heart disease or other atherosclerotic events: a systematic review.

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ABSTRACT

Background and aims: The aim of this study was to perform a systematic review of the association between DNA methylation and coronary heart disease (CHD) or related atherosclerotic traits.

Methods: A systematic review was designed. The condition of interest was DNA methylation, and the outcome was CHD or other atherosclerosis-related traits. Three DNA methylation approaches were considered: global methylation, candidate-gene, and epigenome-wide association studies (EWAS). A functional analysis was undertaken using the Ingenuity Pathway Analysis software.

Results: In total, 51 articles were included in the analysis: 12 global methylation, 34 candidate-gene and 11 EWAS, with six studies using more than one approach. The results of the global methylation studies were inconsistent. The candidate-gene results were consistent for some genes, suggesting that hypermethylation in *ESRα*, *ABCG1* and *FOXP3* and hypomethylation in *IL-6* were associated with CHD. The EWAS identified 84 genes showing differential methylation associated with CHD in more than one study. The probability of these findings was $<1.37 \cdot 10^{-5}$. One third of these genes have been related to obesity in genome-wide association studies. The functional analysis identified several diseases and functions related to these set of genes: inflammatory, metabolic and cardiovascular disease.

Conclusions: Global DNA methylation seems to be not associated with CHD. The evidence from candidate-gene studies was limited. The EWAS identified a set of 84 genes highlighting the relevance of obesity, inflammation, lipid and carbohydrate metabolism in CHD. This set of genes could be prioritized in future studies assessing the role of DNA methylation in CHD.

Keywords: DNA methylation, myocardial infarction, coronary heart disease, atherosclerosis, systematic review.

1. INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of mortality and morbidity, being responsible of 31% of global deaths in 2012 and 14% of all-age disability-adjusted life-years (DALY) in 2015 [1,2]. Coronary heart disease (CHD) is the leading individual cause of morbidity and mortality, and atherosclerosis is its main underlying mechanism.

Although the pathogenesis of CHD and its underlying atherosclerosis is not completely understood, a growing body of evidence suggests an important role for epigenetics in the development of atherosclerosis [3]. Epigenetic modifications can alter the expression of genes without changing their sequences. Deciphering the epigenomic signatures linked to CHD and atherosclerosis could contribute to better understanding of their mechanisms and to the definition of new therapeutic targets and preventive strategies.

DNA methylation, one of the most well-known epigenetic signatures, consists of the covalent methylation of the C5 position of cytosine residues when they are followed by guanine residues (CpG dinucleotides) [4]. It is heritable but it is also a dynamic process related to environmental stimuli. Both global methylation status of the genome and differentially methylated specific loci have been studied in several atherosclerotic conditions.

The aim of this study was to perform a systematic review to summarize all available evidence related to the association between DNA methylation and CHD. We selected studies that analyzed DNA methylation at either a global or gene-specific level, the latter using either a candidate-gene or epigenome-wide association approach.

2. MATERIALS AND METHODS

Data sources and searches

A systematic review of the articles included in PubMed database (www.ncbi.nlm.nih.gov/pubmed) until December 7th 2016 was designed. The search terms were as follows: “DNA methylation” AND (“Coronary heart disease” OR

“Ischemic heart disease” OR “Myocardial infarction” OR “Cardiovascular risk” OR “Vascular age”). No limits were defined on the basis of language, country or publication date. The reference lists of all relevant original research and review articles were also manually scanned to identify potentially missed studies (AF-S). (Figure 1).

Study selection

The condition of interest was the differential DNA methylation, and the outcome was CHD (myocardial infarction or angina), atherosclerosis or other CVD diseases related to atherosclerosis. DNA methylation was considered either global or at specific CpGs or at CpG islands (CGIs) as defined by each study, with no restriction regarding laboratory method or gene panel.

Studies were considered as eligible if they: a) were full-length original studies published in peer-reviewed journals; b) investigated the DNA methylation patterns in relationship with CHD or atherosclerosis traits, by using epidemiological observational designs or other experimental studies in human cell culture; c) the study was published in English. Those studies undertaken in specific populations of patients, such as familiar hypercholesterolemia or chronic kidney disease, were excluded. No restriction criteria were imposed with regard to: a) the type or size of the population studied; b) the tissue or anatomical site analyzed; c) the length of follow-up. Animal studies or non-human cell culture studies were excluded from the review.

The selection of eligible studies was performed in two phases. In the first one, studies that were not relevant for the aim of the review according to the content summary in the title or the abstract were excluded (AF-S). In the second phase, the articles were fully read for their eligibility according to the pre-defined inclusion/exclusion criteria (AF-S). In case of doubt, the inclusion of that study was discussed with a second reviewer (RE).

Data extraction and quality assessment

Studies were classified according to the DNA methylation approach as: (i) global methylation studies, (ii) candidate-gene methylation studies, and (iii) epigenome-wide

association studies (EWAS). In case of doubt, the classification of that study was discussed with a second reviewer. Some of the eligible studies included data obtained from more than a single approach. For each of them, the following data were collected: surname of first author, year of publication, journal, PMID, country, study design, population sample size, type of tissue analyzed, molecular technique used to assess DNA methylation, clinical outcome, adjustment of the measures of association and conclusions of the study. In the case of EWAS, population size of both discovery and validation phase were recorded. Data were extracted initially by one author (AF-S), followed by re-extraction of each paper by the co-author (RE). Discrepancies were resolved by discussion and consensus.

Two of the authors (AF-S, RE) conducted a quality assessment of the selected studies using the STrengthening the REporting of Genetic Association studies (STREGA) recommendations for reporting results of genetic association studies [5]. We considered all the questions in the checklist if applicable to each specific study. The final score for each study was the total number of checklist questions addressed divided by the total number of applicable questions.

Data synthesis and analysis

Due to the large heterogeneity in study aims, study designs, molecular techniques used to assess DNA methylation, analyzed tissues and evaluated outcomes, no quantitative meta-analysis of the results of the eligible studies was performed. The results are shown as a narrative synthesis and as summary tables according to the three defined DNA methylation approaches.

In the case of EWAS, we identified those CpGs that were reported in more than one study and we assessed whether the direction of the association was consistent across studies (set 1). We also identified those genes showing at least one CpG/CGI associated with CHD in more than one study, and also assessed whether the direction of the association was consistent (set 2) or not (set 3). Finally, we estimated the probability of finding a certain CpG and gene showing differential methylation associated with the traits of interest in at least two or three studies by chance. To calculate this probability we used a binomial approach and we made the following assumptions: 1) the arrays analyze 400,000 CpGs per individual, and there are

20,000 genes in the human genome, and 2) the different arrays include 10 CpGs by gene. We used the following equation:

$\mathrm{B(x, n, P)} = \binom{n}{x} p^x (1-p)^{n-x}$, where

- *b(x, n, P)* = probability that a *n*-trial binomial experiment results in *x* successes, assuming the probability of success to be *p;*
- *x* = the number of successes that result from the binomial experiment. In our case two and three studies;
- *n* = the number of trials in the binomial experiment. In our study corresponds to the number of EWAS studies;
- *p* = probability of success on an individual trial. In our case was stated as $2.5 \cdot 10^{-6}$ (1/400.000 CpGs) per CpGs and $5.0 \cdot 10^{-4}$ [(1/20.000 genes)*10 CpGs per gene] per genes.

Functional studies and pathway analysis

We performed functional analyses of the genes identified as differentially methylated in more than one of the EWAS. On one hand, we searched in the Genome-wide Association Studies (GWAS) Catalog (https://www.ebi.ac.uk/gwas/) if any of those genes had previously been related to a cardiovascular or atherosclerotic trait in a GWAS. If such was the case, the traits were recorded.

On the other hand, we performed a pathway and network analysis of three sets of genes found in more than one of the EWAS: set 1, genes including the same CpG methylation direction; set 2, genes with the same methylation direction at gene level; and set 3, differentially methylated genes independently of the methylation consistency. In that purpose, we used the Ingenuity Pathway Analysis (IPA) software (http://www.ingenuity.com/; QIAGEN, Redwood City, CA, USA). We uploaded the Gene symbol identifiers of those genes. The database underlying IPA is referred to as the Ingenuity Knowledge Base (Genes only). Only human annotations were considered. Pathway analyses were performed with IPA's Core Analysis module. "Canonical pathways" and "Diseases and functions" terms with a $p < 0.05$ in the Fisher's exact test or after Benjamini-Hochberg multiple testing correction were defined as a statistically significant overrepresentation of input genes in a given process.

In the “Canonical pathway” analysis, we selected all “Metabolic pathways” and “Signalling pathways” related to “Cardiovascular signalling”, “Cell cycle regulation”, “Cellular growth, proliferation and development”, “Cellular immune response”, “Cellular stress and injury”, “Humoral immune response”, “Intracellular and second messenger signalling”, “Nuclear receptor signalling” and “Transcription regulation”. In the “Diseases and functions” analysis, we selected CHD and atherosclerosis-relevant terms to create functional networks linking the input genes to functions or diseases. These networks are based on the information contained in the IPA database.

3. RESULTS

We identified 96 potentially relevant publications in the PubMed database (www.ncbi.nlm.nih.gov/pubmed) (Supplementary Figure 1). Based on the titles and abstracts, full texts of 43 articles were selected for further evaluation. Of those, 31 were original studies, while the remaining 12 articles were reviews. The examination of their reference lists allowed us to identify 20 additional original studies. In total, 51 articles met our eligibility criteria (Supplementary Table 1) and were included in the analysis.

3.1. Characteristics of the included studies

Detailed characteristics of the eligible studies are summarized in Tables 2-4. The outcomes of interest ranged widely, from CHD clinical events to atherosclerosis-related traits such as smooth muscle cell phenotype (proliferative vs contractile), carotid intima-media thickness, arterial stiffness, atherosclerotic plaque and cardiovascular risk, cardiac fibrosis induced by ischemia, endothelial progenitor cells function, and T regulatory cells function.

Of the 51 selected studies, 17 included participants from Europe [6–22] 14 from China [23–36], six from USA [37–42], three from India [43–45], two from Canada [46,47], and one each from Japan[48], Malaysia [49], Iran [50], Russia [51], Brazil [52] and Mexico [53]. One article did not clearly specify the origin of the samples [54] and two studies used commercial samples [55,56].

Seven studies analyzed global DNA methylation [6,7,23,34,36,42,52], 31 followed a candidate-gene approach [8–14,20,22,24–33,35,38–41,45,46,49,50,54–56], seven were epigenome-wide association studies (EWAS) [16,18,19,21,44,48,53], two used both global DNA methylation and candidate-gene approaches [37,43], three used EWAS and global DNA methylation approaches [15,17,51], and one used EWAS and candidate-gene approaches [47]. Most of the studies had a case-control design (n=29) [6,8,15,18,20,21,24–26,28–36,44–51,53,54,56]; the remaining study designs were case-control nested in a cohort (n=5) [9–11,17,43], cohort (n=3) [13,22,41], cross-sectional (n=3) [16,40,52], case-control and *in vitro* (n=3) [27,38,39], case-control in a survey (n=2) [12,19], *in vitro* (n=2) [37,55], case-cohort (n=1) [14], cross-sectional in a cohort (n=1) [42], cross-sectional and *in vitro* (n=1) [7] and cohort and case-control in a survey (n=1) [23].

3.2 Global methylation studies

Twelve studies analyzing the association between global methylation and CHD or atherosclerosis were selected. High heterogeneity was observed among the molecular techniques used to assess global DNA methylation. Almost half of the studies used the methylation measurement of repetitive elements (LINE-1 or Alu) as a proxy for assessing global methylation [17,23,34,36,42] and the remaining studies used other methods [6,7,15,37,43,51,52] (Supplementary Table 2a).

Most of the eligible global methylation studies analyzed this feature in blood samples (n=8) [6,17,23,34,36,42,43,52] and the rest used vascular tissues [7,15,37,51] (Supplementary Table 2a). Six of the studies included a sample larger than 100 individuals [17,23,34,36,42,43], two studies analyzed samples from fewer than 10 individuals [15,51] and one study used a cell line derived from the aorta but the number of aortas isolated was not mentioned [37] (Supplementary Table 2a).

The results of the 12 eligible global DNA methylation studies were inconsistent, with hypomethylation associated to CHD or atherosclerosis in six studies [6,17,34,36,42,52], and hypermethylation associated to those same traits in four studies [15,23,43,51] and to cardiac fibrotic burden in another [7]. The remaining study found no association between global DNA methylation and the phenotype of

smooth muscle cell (proliferative vs differentiated) [37] (Table 1, Supplementary Table 2a).

3.3. Candidate-gene methylation studies

Thirty-four studies analyzing the association between DNA methylation in candidate-genes and CHD or atherosclerosis were selected (Table 2). In the analyzed studies, several molecular techniques were used to assess DNA methylation at the gene level (Supplementary Table 2b).

The vast majority of the selected candidate-gene methylation studies analyzed blood samples (n=28) [8–12,14,20,22,24–32,35,40,41,43,46,47,49,50,56]. The other tissues isolated were aortic [37,39,55] or other vascular tissues [13,38,54] (Supplementary Table 2b). Five of the studies included more than 1,000 participants [12,14,22,31,41], and 13 studies included a sample size between 100 and 999 [9–11,13,25,27,30,32,33,40,45,49,50]. One study analyzed a commercial cell line derived from one individual [55] (Supplementary Table 2b).

Four studies analyzed *ESRα* [8,24,37,38], while *ABCA1* [9,46], *ABCG1* [12,30], *APOE* [11,43], *FOXP3* [27,33], *IL-6* [31,32], *MTHFR* [11,49], and *PON1* [11,20] were analyzed in two studies. Two studies analyzed the association between epigenetic aging and mortality due to CHD [41] or CVD [14]. The full list of candidate-genes and the reported results are shown in Supplementary Table 3.

We found consistent results for *ESRα*, *ABCG1*, *APOE*, *FOXP3* and *IL-6*. Hypermethylation in the promoters of *ESRα* [8,24,37,38] and *ABCG1* [12,30] and in *FOXP3* [27,33], and hypomethylation in the promoter of *IL-6* [31,32] were associated with CHD, whereas *APOE* methylation was not [11,43]. However, a discrepancy was observed between studies that found an association between CHD or atherosclerosis and DNA methylation in the promoter of *ABCA1* [46], *MTHFR* [49] and *PON1* [11] and others that found no association [9,11,20], respectively.

3.4. Epigenome-wide association studies

Eleven EWAS analyzing the association between DNA methylation and CHD or atherosclerosis were selected (Table 3, Supplementary Table 2c). The Infinium Human Methylation 450 BeadChip Array (Illumina) was the most commonly used

array (Supplementary Table 2c) [15–17,19,47,48]. Most of the selected EWAS analyzed blood samples (n=7) [16–19,21,43,47], and the others analyzed vascular tissues [15,48,53] (Supplementary Table 2c).

Four EWAS only showed results of the discovery cohort without any validation of the main findings [18,19,47,48]. Samples from more than 100 individuals were analyzed in the discovery phase of three EWAS [16,17,19] and in the validation phase of three EWAS [16,17,21]. Two EWAS included samples from fewer than 10 individuals in the discovery phase [18,51]. Some studies discovered differentially methylated CpG sites, while others found regions ("islands") with a high frequency of CpG sites (Supplementary Table 4).

Not all studies provided the full list of CpGs identified in the discovery phase. From the available information, we identified 2,625 CpGs and 111 CpG islands (CGIs) showing differential methylation associated with CHD or atherosclerosis (Supplementary Table 4). The reported CpG sites or CGIs were located within 1,540 different genes (n=2,057) and hundreds of intergenic regions (n=673) (Supplementary Table 5). Six CGIs did not match any pairwise sequence. Of the 2,625 reported CpGs, 14 were identified in more than one of the EWAS, and 8 of these were located in different genes, showing a consistent direction of the association (set 1). Of the 1,540 genes identified in those sequences, 75 were found in two studies and only one gene was identified by three studies. Moreover, 8 additional genes were reported in two of the EWAS when considering genes located upstream or downstream of intergenic CpGs/CGIs. These 84 genes (set 3) and the direction of the association between methylation at gene level and CHD are shown in table 4. Of those 84 genes, 52 have shown consistency in the direction of the association between methylation and atherosclerosis-related traits (set 2). The probability of finding the same gene or CpG in two of the 11 studies was $1.37 \cdot 10^{-5}$ and $3.44 \cdot 10^{-10}$, and in three studies was $2.05 \cdot 10^{-8}$ and $2.58 \cdot 10^{-15}$, respectively.

3.5. Functional studies and pathway analysis

We analyzed the biological function of the genes identified as differentially methylated in more than one of the EWAS (n=84). First, we reviewed the Genome-Wide Association Studies catalog and found none of the listed traits identified in 9 of the genes and no CVD- or atherosclerosis-related trait in 19 other genes. The CVD-

or atherosclerosis-related traits associated with the remaining 56 genes are shown in Supplementary Table 6. Most of these genes showed several associations, the most common being obesity-related traits (33%).

We next used the IPA software to identify enriched canonical pathways and the "diseases and functions" terms related to the three sets of genes established according to the consistency on the direction of the association between methylation and CHD: set 1, 8 genes with consistency on CpG methylation status; set 2, 52 genes with consistent methylation at gene level; and set 3, 84 differentially methylated genes. The main results are shown in table 5. The top canonical pathway related to CHD or atherosclerosis, consistently identified by Fisher's exact test in all three sets, was RhoA signaling (Supplementary Figure 2). Statistical significance declined when adjusting by Benjamini-Hochberg. Using this multiple comparison adjustment, we observed five consistent cardiovascular-related terms in all three sets: endocrine system disorders, cardiovascular system development and function, inflammatory response, immune cell trafficking, and inflammatory disease. Assessment of the more extended sets of genes (set 2 and 3) also identified the following: cardiovascular and nutritional diseases; lipid, carbohydrate and vitamin and mineral metabolism; connective tissue development and function or disorders, and cell-mediated immune response (Supplementary Figures 3 and 4). As an example, the defined cardiovascular disease network is shown in Figure 1.

4. DISCUSSION

In this review, we systematically analyzed the currently available evidence on the association between DNA methylation and CHD and its underlying atherosclerosis. Overall, the scarce evidence suggests no consistent association between global DNA methylation and CHD. On the other hand, several studies have shown a relationship between differential methylation at specific loci and CHD or atherosclerosis outcomes, suggesting a role of epigenetics in the etiopathogenesis of CHD. In total, 84 genes were found in more than one of the EWAS, and this set was enriched in genes related to obesity and metabolism.

The 12 studies that examined the association between global methylation and CHD reported contradictory results. These findings are consistent with other reviews of DNA methylation and CVD studies [57]. Inconsistencies among the results were also reported in systematic reviews examining the relationship between DNA methylation and type 2 diabetes [58], and dyslipidaemia [59]. There are several explanations for this inconsistency: first, methodological issues related to differences in sample size and heterogeneous outcomes; second, the applied assay, the DNA source and the DNA isolation method are critical in the interpretation of global DNA methylation patterns but the analyzed studies had applied heterogeneous methods [60,61]; and third, global methylation refers to the overall level of methylated cytosines within CpG sites in the genome but does not take into account differential methylation at specific loci. The effect of DNA methylation on gene expression is known to be dependent on the location within the genomic sequence where it occurs. Consequently, gene- and loci-specific methylation may be a more informative measure to determine the association between DNA methylation and gene expression and health-related outcomes [62].

Most of the selected studies analyzed DNA methylation using either a candidate gene or an EWAS approach. Although the overall results are more consistent than those from global methylation studies, the outcomes, sample sizes, methylation assays, and DNA sources and isolation methods are diverse across studies, limiting the interpretation of the overall results. The majority of the reviewed studies used a candidate-gene approach. These genes were selected based on previous genetic studies or their biological function. Only eight genes were examined in more than one study; therefore, most of the findings must still be replicated. Considering the replicated candidate-genes, hypermethylation in gene promoters of *ESRα* and *ABCG1* and in the first intron of *FOXP3* and hypomethylation in the promoter of *IL-6* were associated with CHD.

ESRα acts dually on cardiovascular cells and tissues: if this receptor bonds to estrogen, the result is vascular protective effects, while the unligated form promotes an inflammatory phenotype in vascular cells [63]. If hypermethylation of its gene promoter inactivates its expression, it could be that no estrogen will bind the receptor and thus, its protective effects would be lost. *ABCG1* encodes a transporter involved

in reverse cholesterol transport [64]. Thus, if hypermethylation in its promoter led to its downregulated expression, this would be expected to influence atherogenesis through a lower reverse cholesterol transport capacity. On the other hand, *IL-6*, which encodes a pro-inflammatory cytokine, is related to CHD [65]. Therefore, under atherosclerotic conditions, hypomethylation in its promoter could entail the upregulation of its expression, leading to a higher production of the cytokine by macrophages. Finally, *FOXP3* encodes a transcriptional regulator crucial for the regulatory T-cells (Tregs), which modulate inflammation and immunity, and their dysregulation was associated with CVD and atherosclerosis [66]. Demethylation at a highly conserved CpG-enriched element within *FOXP3* intron 1 (*FOXP3-i-l*) has been found to be restricted specifically to this type of Tregs [67]. A final consideration is the important role of *Apo E* in the clearance of triglyceride- and cholesterol-rich lipoproteins from the circulation, slowing down the process of atherosclerosis [68]. The results of four studies support the lack of association between DNA methylation in *APOE* and CHD, suggesting that this relationship may be not influenced through this epigenetic mechanism.

EWAS also examine gene-specific DNA methylation. They have increasingly replaced the candidate-gene approach as they are useful to identify novel CpG sites related to disease phenotypes. Large sample sizes are required and the results obtained must be validated in a replication cohort [69]. Most of the reviewed EWAS validated the obtained results in a replication cohort, but the sample size was relatively small in all of them. Among the differentially methylated translating loci, 84 were identified in more than one of the EWAS, suggesting an epigenetic regulation of those genes in CHD or atherosclerotic conditions. However, the direction of the association between methylation at the gene level and CHD or atherosclerosis is difficult to ascertain, as the effects of methylation on gene expression depend on where the methylation occurs in the genomic sequence [62]. Using three distinct approaches, we analyzed this issue first at the CpG level (8 CpGs showing consistent association); second, at the gene level (52 genes); and third, selecting all the genes showing differential methylation (84 genes).

Among the 84 genes found in more than one of the EWAS, differential methylation of *MCL1* was observed in three independent EWAS. It encodes isoforms of a member

of the Bcl-2 family involved in the regulation of apoptosis, both those maintaining cell viability and inducing apoptosis [70,71]. Apoptosis is associated with the progression of atherosclerosis, with opposing roles depending on the cell type, plaque stage and apoptotic cell localization [72]. Interestingly, anti-inflammatory *IL-10* was shown to enhance both lipid accumulation in macrophages from ACS patients and *MCL1* expression with an anti-apoptotic effect [73]. Thus, *MCL1* may have an important role in atherosclerosis development and it could be regulated differently in each stage through a different mechanism, including DNA methylation.

From the 8 genes showing consistency in the direction of the association between methylation at CpG level and CHD or atherosclerosis, *CRELD2* and *KCNJ14* (a potassium channel) have not been related to any atherosclerotic trait. *AIM2* encodes a cytoplasmic DNA sensor that triggers the inflammasome in macrophages and is upregulated in advanced coronary plaques [74]. *TNS1* is differentially expressed in atherosclerotic plaques [75,76]. *NGEF* is associated with abdominal visceral fat and overall adiposity [77,78]. *PKD2* has a role in monocyte migration and atherosclerosis development [79]. *HRH2* encodes an histamine receptor and is involved in hyperlipidemia-induced atherosclerosis [80]. Finally, *GRIP1* has been shown to play a role in platelet function and thrombosis [81].

In addition, from the other 75 genes found in at least two EWAS, *HOXA3*, whose expression is associated with high abdominal adiposity [82], has been recently identified as differentially methylated in relationship with HDL functionality. Its hypomethylation is associated with a high cholesterol efflux capacity and, thus, a lower atherosclerosis burden [83].

A significant proportion of the genes identified as differentially methylated in more than one of the EWAS were previously identified in genome-wide association studies as associated with CVD or its risk factors and related conditions. The most frequent one was obesity, which is a well-known coronary risk factor [84]. Similarly, in the IPA for “diseases and functions” terms overrepresented among those genes, endocrine system disorders, metabolic disease, and carbohydrate and lipid metabolism were some of the most significant terms. These risk factors for atherosclerotic CVD have obesity as a major driver [85].

In conclusion, the present systematic review suggests that differential DNA methylation at specific genes is associated with CHD or atherosclerosis. The findings describe a set of genes that could be prioritized in future EWAS analysis. The EWAS identify a set of genes highlighting the importance of obesity, lipid and carbohydrate metabolism, inflammation and other pathways in CHD. Further studies are needed to validate the reported results in larger sample sizes. In addition, a consensus method for DNA methylation analysis could be useful to allow data comparisons, especially regarding the type of tissue examined, the molecular technique used and the confounder adjustment for statistical analysis. Finally, functional assays are required to understand the molecular mechanisms that relate DNA methylation to CHD or atherosclerosis and determine its causal, mediating or reversal of causality role.

Conflict of interest

The authors declare they do not have any conflict of interest.

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Author contributions

A.F-S., R.E., I.S., S.S-B. participated in the conception or design of the work. A.F-S. and R.E. were responsible of the review of the manuscripts included in this systematic review and the extraction of the data. S.S-B. and I.S. performed the analysis of the data. All the authors participated in the interpretation of data for the work. A.F-S. and R.E. wrote the draft of the manuscript and the rest of authors revised it critically for important intellectual content. All authors have approved the final version of the manuscript. All authors 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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References

[1] WHO. Global status report on noncommunicable diseases. 2014. http://www.who.int/nmh/publications/ncd-status-report-2014/en/.

[2] GBD 2015 DALYs and HALE Collaborators, M. Arora, R.M. Barber, et al, Global, regional, and national disability-adjusted life-years (DALYs) for 315 diseases and injuries and healthy life expectancy (HALE), 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016;388:1603–1658.

[3] M. Neidhart. DNA Methylation in Cardiology. In: DNA Methylation Complex Human Disease. 2016:261–272.

[4] A.P. Wolffe, M.A. Matzke. Epigenetics: regulation through repression. Science. 1999;286:481–6.

[5] J. Little, J.P.. Higgins, J.P.. Ioannidis, et al. STrengthening the REporting of Genetic Association Studies (STREGA)— An Extension of the STROBE Statement. PLoS Med. 2009;6:e1000022.

[6] R. Castro, I. Rivera, E.A. Struys, et al. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. Clin. Chem. 2003;49:1292–1296.

[7] C.J. Watson, P. Collier, I. Tea, et al. Hypoxia-induced epigenetic modifications are associated with cardiac tissue fibrosis and the development of a myofibroblast-like phenotype. Hum. Mol. Genet. 2014;23:2176–88.

[8] I. Huica, A. Botezatu, I. V. Iancu, et al. Genetic and epigenetic aspects in cardio-vascular disease and ageing, Rom. Biotechnol. Lett. 2011;16:6488–6496.

[9] R.P. Talens, J.W. Jukema, S. Trompet, et al. Hypermethylation at loci sensitive to the prenatal environment is associated with increased incidence of myocardial infarction. Int. J. Epidemiol. 2012;41:106–115.

[10] S. Friso, V. Lotto, S.-W. Choi, et al. Promoter methylation in coagulation F7 gene influences plasma FVII concentrations and relates to coronary artery disease. J. Med. Genet. 2012;49:192–9.

[11] G. Fiorito, S. Guarrera, C. Valle, et al. B-vitamins intake, DNA-methylation of One Carbon Metabolism and homocysteine pathway genes and myocardial infarction risk: The EPICOR study. Nutr. Metab. Cardiovasc. Dis. 2014;24:483–488.

[12] L. Pfeiffer, S. Wahl, L.C. Pilling, et al. DNA Methylation of Lipid-Related Genes Affects Blood Lipid Levels. Circ. Cardiovasc. Genet. 2015;8:334–342.

[13] R. Murray, J. Bryant, P. Titcombe, et al. DNA methylation at birth within the promoter of ANRIL predicts markers of cardiovascular risk at 9 years. Clin. Epigenetics. 2016;8:90.
[14] L. Perna, Y. Zhang, U. Mons, et al. Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. Clin. Epigenetics. 2016;8:64.
[15] S. Zaina, H. Heyn, F.J. Carmona, et al. DNA methylation map of human atherosclerosis. Circ. Cardiovasc. Genet. 2014;7:692–700.
[16] W.E. Ek, A.K. Hedman, S. Enroth, et al. Genome-wide DNA methylation study identifies genes associated with the cardiovascular biomarker GDF-15. Hum. Mol. Genet. 2016;25:817–27.
[17] S. Guarrera, G. Fiorito, N.C. Onland-Moret, et al. Gene-specific DNA methylation profiles and LINE-1 hypomethylation are associated with myocardial infarction risk. Clin. Epigenetics. 2015;7:133.
[18] C. Oudejans, A. Poutsma, O. Michel, et al. Genome-wide identification of epigenetic hotspots potentially related to cardiovascular risk in adult women after a complicated pregnancy. PLoS One. 2016;11:e0148313.
[19] M. Rask-Andersen, D. Martinsson, M. Ahsan, et al. Epigenome-wide association study reveals differential DNA methylation in individuals with a history of myocardial infarction, Hum. Mol. Genet. 2016. doi:10.1093/hmg/ddw302.
[20] A.M. Gómez-Uriz, E. Goyenechea, J. Campión, et al. Epigenetic patterns of two gene promoters (TNF-α and PON) in stroke considering obesity condition and dietary intake. J. Physiol. Biochem. 2014;70:603–14.
[21] A.M. Gómez-Úriz, F.I. Milagro, M.L. Mansego, et al. Obesity and ischemic stroke modulate the methylation levels of KCNQ1 in white blood cells. Hum. Mol. Genet. 2015;24:1432–40.
[22] Y. Zhang, R. Yang, B. Burwinkel, et al. F2RL3 methylation in blood DNA is a strong predictor of mortality. Int. J. Epidemiol. 2014;43:1215–1225.
[23] M. Kim, T.I. Long, K. Arakawa, et al. DNA methylation as a biomarker for cardiovascular disease risk. PLoS One. 2010;5:1–8.
[24] Y.S. Huang, Y.F. Zhi, S.R. Wang. Hypermethylation of estrogen receptor-alpha gene in atheromatosis patients and its correlation with homocysteine. Pathophysiology. 2009;16:259–265.

[25] J. Zhuang, W. Peng, H. Li, et al. Methylation of p15INK4b and Expression of ANRIL on Chromosome 9p21 Are Associated with Coronary Artery Disease, PLoS One. 2012;7:e47193.
[26] D. Jiang, D. Zheng, L. Wang, et al. Elevated PLA2G7 Gene Promoter Methylation as a Gender-Specific Marker of Aging Increases the Risk of Coronary Heart Disease in Females, PLoS One. 2013;8:1–7.
[27] C. Lü, R. Xu, M. Cao, et al. FOXP3 demethylation as a means of identifying quantitative defects in regulatory T cells in acute coronary syndrome., Atherosclerosis. 2013;229:263–70.
[28] L. Xu, D. Zheng, L. Wang, et al. GCK gene-body hypomethylation is associated with the risk of coronary heart disease, Biomed Res. Int. 2014. Article ID 151723.
[29] P.-P. Niu, Y. Cao, T. Gong, et al. Hypermethylation of DDAH2 promoter contributes to the dysfunction of endothelial progenitor cells in coronary artery disease patients. J. Transl. Med. 2014;12:170.
[30] P. Peng, L. Wang, X. Yang, et al. A preliminary study of the relationship between promoter methylation of the ABCG1, GALNT2 and HMGCR genes and coronary heart disease. PLoS One. 2014;9:8–15.
[31] Q. Yang, Y. Zhao, Z. Zhang, J. Chen. Association of interleukin-6 methylation in leukocyte DNA with serum level and the risk of ischemic heart disease. Scand. J. Clin. Lab. Invest. 2016;76:291–295.
[32] H. Zuo, Y. Guo, L. Che, X. Wu, Hypomethylation of Interleukin-6 Promoter is Associated with the Risk of Coronary Heart Disease., Arq. Bras. Cardiol. 2016;107:131–136.
[33] L. Jia, L. Zhu, J.Z. Wang, et al. Methylation of FOXP3 in regulatory T cells is related to the severity of coronary artery disease, Atherosclerosis. 2013;228:346–352.
[34] L. Wei, S. Liu, Z. Su, et al. LINE-1 hypomethylation is associated with the risk of coronary heart disease in Chinese population. Arq. Bras. Cardiol. 2014;102:481–8.
[35] J. Zhong, X. Chen, N. Wu, et al. Catechol-O-methyltransferase promoter hypomethylation is associated with the risk of coronary heart disease. Exp. Ther. Med. 2016;12:3445–3449.
[36] R.-T. Lin, E. Hsi, H.-F. Lin, et al. LINE-1 methylation is associated with an increased risk of ischemic stroke in men. Curr. Neurovasc. Res. 2014;11:4–9.

[37] A.K. Ying, H.H. Hassanain, C.M. Roos, et al. Methylation of the estrogen receptor-a gene promoter is selectively increased in proliferating human aortic smooth muscle cells. Cardiovasc. Res. 2000;46:172–179.
[38] W.S. Post, P.J. Goldschmidt-Clermont, C.C. Wilhide, et al. Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. Cardiovasc. Res. 1999;43:985–991.
[39] S. Zhu, P.J. Goldschmidt-Clermont, C. Dong. Inactivation of monocarboxylate transporter MCT3 by DNA methylation in atherosclerosis. Circulation. 2005;112:1353–1361.
[40] J. Zhao, C.W. Forsberg, J. Goldberg, et al. MAOA promoter methylation and susceptibility to carotid atherosclerosis: role of familial factors in a monozygotic twin sample. BMC Med. Genet. 2012;13:100.
[41] S. Horvath, M. Gurven, M.E. Levine, et al. An epigenetic clock analysis of race/ethnicity, sex, and coronary heart disease. Genome Biol. 2016;17:171.
[42] A. Baccarelli, R. Wright, V. Bollati, et al. Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology. 2010;21:819–28.
[43] P. Sharma, J. Kumar, G. Garg, et al. Detection of Altered Global DNA Methylation in Coronary Artery Disease Patients. DNA Cell Biol. 2008;27:357–365.
[44] P. Sharma, G. Garg, A. Kumar, et al. Genome wide DNA methylation profiling for epigenetic alteration in coronary artery disease patients. Gene. 2014;541:31–40.
[45] S.V.V. Lakshmi, S.M. Naushad, C.A. Reddy, et al. Oxidative stress in coronary artery disease: epigenetic perspective. Mol. Cell. Biochem. 2013;374:203–211.
[46] S.-P. Guay, C. Légaré, A.-A. Houde, et al. Acetylsalicylic acid, aging and coronary artery disease are associated with ABCA1 DNA methylation in men. Clin. Epigenetics. 2014;6:14.
[47] A. Nguyen, M. Mamarbachi, V. Turcot, et al. Lower methylation of the ANGPTL2 gene in leukocytes from post-acute coronary syndrome patients. PLoS One. 2016;11:1–17.
[48] Y. Yamada, T. Nishida, H. Horibe. Identification of hypo- and hypermethylated genes related to atherosclerosis by a genome-wide analysis of DNA methylation. Int. J. Mol. Med. 2014;33:1355–1363.
[49] L.K. Wei, H. Sutherland, A. Au, et al. A potential epigenetic marker mediating serum folate and vitamin B12 levels contributes to the risk of ischemic stroke. Biomed Res. Int. 2015. Article ID 167976.

[50] M. Afzali, A. Nakhaee, S.P. Tabatabaei, et al. Aberrant promoter methylation profile of niemann-pick type C1 gene in cardiovascular disease, Iran. Biomed. J. 2013;17:77–83.
[51] M.S. Nazarenko, A. V. Markov, I.N. Lebedev, et al. A comparison of genome-wide DNA methylation patterns between different vascular tissues from patients with coronary heart disease. PLoS One. 2015;10:1–15.
[52] R.B. Ramos, V. Fabris, S.B. Lecke, et al. Association between global leukocyte DNA methylation and cardiovascular risk in postmenopausal women. BMC Med. Genet. 2016;17:71.
[53] S.A. Castillo-Díaz, M.E. Garay-Sevilla, M.A. Hernández-González, et al. Extensive demethylation of normally hypermethylated CpG islands occurs in human atherosclerotic arteries. Int. J. Mol. Med. 2010;26:691–700.
[54] J. Kim, J.Y. Kim, K.S. Song, et al. Epigenetic changes in estrogen receptor beta gene in atherosclerotic cardiovascular tissues and in-vitro vascular senescence. Biochim. Biophys. Acta. 2007;1772:72–80.
[55] J.J. Connelly, O.A. Cherepanova, J.F. Doss, et al. Epigenetic regulation of COL15A1 in smooth muscle cell replicative aging and atherosclerosis. Hum. Mol. Genet. 2013;22:5107–5120.
[56] A.A. Baccarelli, H.-M. Byun, Platelet mitochondrial DNA methylation: a potential new marker of cardiovascular disease. Clin. Epigenetics. 2015;7:44.
[57] T. Muka, F. Koromani, E. Portilla, et al. The role of epigenetic modifications in cardiovascular disease: A systematic review. Int. J. Cardiol. 2016;212:174–183.
[58] T. Muka, J. Nano, T. Voortman, et al. The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: A systematic review. Nutr. Metab. Cardiovasc. Dis. 2016;26:553–66.
[59] K.V.E. Braun, T. Voortman, K. Dhana, et al. The role of DNA methylation in dyslipidaemia: A systematic review. Prog. Lipid Res. 2016;64:178–191.
[60] H.C. Wu, L. Delgado-Cruzata, J.D. Flom, et al. Global methylation profles in DNA from different blood cell types. Epigenetics. 2011;6:76–85.
[61] C. Soriano-Tárraga, J. Jiménez-Conde, E. Giralt-Steinhauer, et al. DNA Isolation Method Is a Source of Global DNA Methylation Variability Measured with LUMA. Experimental Analysis and a Systematic Review. PLoS One. 2013;8:e60750.
[62] P.A. Jones. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat. Rev. Genet. 2012;13484–92.

[63] Q. Lu, G.R. Schnitzler, C.S. Vallaster, et al. Unliganded estrogen receptor alpha regulates vascular cell function and gene expression. Mol. Cell. Endocrinol. 2017;442:12–23.
[64] R.S. Rosenson, H.B. Brewer, B.J. Ansell, et al. Dysfunctional HDL and atherosclerotic cardiovascular disease. Nat. Rev. Cardiol. 2016;13:48–60.
[65] D.R. Anderson, J.T. Poterucha, T.R. Mikuls, et al. IL-6 and its receptors in coronary artery disease and acute myocardial infarction. Cytokine. 2013;62:395–400.
[66] X. Meng, J. Yang, M. Dong, et al. Regulatory T cells in cardiovascular diseases. Nat. Rev. Cardiol. 2016;13:167–79.
[67] U. Baron, S. Floess, G. Wieczorek, et al. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. Eur. J. Immunol. 2007;37:2378–89.
[68] R.W. Mahley. Apolipoprotein E: from cardiovascular disease to neurodegenerative disorders. J. Mol. Med. 2016;94:739–46.
[69] K.B. Michels, A.M. Binder, S. Dedeurwaerder, et al. Recommendations for the design and analysis of epigenome-wide association studies. Nat. Methods. 2013;10:949–55.
[70] J. Bae, C.P. Leo, S.Y. Hsu, A.J. Hsueh. MCL-1S, a splicing variant of the antiapoptotic BCL-2 family member MCL-1, encodes a proapoptotic protein possessing only the BH3 domain. J. Biol. Chem. 2000;275:25255–61.
[71] C.D. Bingle, R.W. Craig, B.M. Swales, et al. Exon skipping in Mcl-1 results in a bcl-2 homology domain 3 only gene product that promotes cell death. J. Biol. Chem. 2000;275:22136–46.
[72] E.A. Van Vré, H. Ait-Oufella, A. Tedgui, Z. Mallat. Apoptotic Cell Death and Efferocytosis in Atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 2012;32:887–93.
[73] B. Halvorsen, T. Waehre, H. Scholz, et al. Interleukin-10 enhances the oxidized LDL-induced foam cell formation of macrophages by antiapoptotic mechanisms. J. Lipid Res. 2005;46:211–9.
[74] M. Hakimi, A. Peters, A. Becker, D. Böckler, S. Dihlmann. Inflammation-related induction of absent in melanoma 2 (AIM2) in vascular cells and atherosclerotic lesions suggests a role in vascular pathogenesis. J. Vasc. Surg. 2014;59:794–803.e2.
[75] M. Papaspyridonos, A. Smith, K.G. Burnand et al. Novel candidate genes in unstable areas of human atherosclerotic plaques. Arterioscler. Thromb. Vasc. Biol.

2006;26:1837–44.
[76] O. Puig, J. Yuan, S. Stepaniants, et al. A Gene Expression Signature That Classifies Human Atherosclerotic Plaque by Relative Inflammation Status. Circ. Cardiovasc. Genet. 2011;4:595–604.
[77] J.M. Norris, C.D. Langefeld, M.E. Talbert, et al. Genome-wide Association Study and Follow-up Analysis of Adiposity Traits in Hispanic Americans: The IRAS Family Study. Obesity. 2009;17:1932–1941.
[78] H.-J. Kim, J.-H. Park, S. Lee, et al. A Common Variant of NGEF Is Associated with Abdominal Visceral Fat in Korean Men. PLoS One. 2015;10: e0137564.
[79] M. Tan, F. Hao, X. Xu, G.M. Chisolm, M.-Z. Cui. Lysophosphatidylcholine activates a novel PKD2-mediated signaling pathway that controls monocyte migration. Arterioscler. Thromb. Vasc. Biol. 2009;29:1376–82.
[80] S. Yamada, K.-Y. Wang, A. Tanimoto, Y. Sasaguri. Novel function of histamine signaling in hyperlipidemia-induced atherosclerosis: Histamine H1 receptors protect and H2 receptors accelerate atherosclerosis. Pathol. Int. 2015;65:67–80.
[81] K.L. Modjeski, S.K. Ture, D.J. Field, et al. Glutamate Receptor Interacting Protein 1 Mediates Platelet Adhesion and Thrombus Formation. PLoS One. 2016;11:e0160638.
[82] F. Karpe, K.E. Pinnick. Biology of upper-body and lower-body adipose tissue--link to whole-body phenotypes. Nat. Rev. Endocrinol. 2015;11:90–100.
[83] S. Sayols-Baixeras, A. Hernáez, I. Subirana, et al. DNA Methylation and High-Density Lipoprotein Functionality. Arterioscler. Thromb. Vasc. Biol. 2017;37:567-569.
[84] T. Mandviwala, U. Khalid, A. Deswal. Obesity and Cardiovascular Disease: a Risk Factor or a Risk Marker? Curr. Atheroscler. Rep. 2016;18:21.
[85] S.M. Grundy. Metabolic syndrome update. Trends Cardiovasc. Med. 2016;26:364–73.

FIGURES AND TABLES

Figure 1. Downstream effect analysis of specific differentially methylated genes associated with Cardiovascular disease (CVD), using the set 2 of genes (those reported as methylated in the same direction by more than one of the EWAS, independently of the CpG). Gene products (in the upper part) and CVDs (in yellow) are represented as nodes, and the biological relationship between two nodes is represented as an edge. Edge arrows indicate causation, while simple edges indicate correlation. All edges are supported by at least one publication in the Ingenuity Knowledge Database.

Table 1. Main results of the studies using a global methylation approach.

Reference	Conclusion
Ying 2000[37]	Non-significant increase in global DNA hypermethylation in proliferating SMCs vs differentiated SMCs.
Castro 2003[6]	Global DNA hypomethylation was associated with CHD.
Sharma 2008[43]	Global DNA hypermethylation was associated with CHD, especially in aged patients.
Kim 2010[23]	Global DNA hypermethylation was associated with prevalence and incidence of CHD and its risk factors (MI, stroke, hypertension, diabetes) in males.
Baccarelli 2010[42]	Global DNA hypomethylation assessed in LINE-1 was associated with CHD and stroke.
Lin 2014[36]	Global DNA hypomethylation assessed in LINE-1 was associated with ischemic stroke in men.
Watson 2014[7]	Global DNA hypermethylation in hypoxic cardiac fibroblast was associated to the fibrotic burden in hypoxia.
Wei 2014[34]	Global DNA hypomethylation measured in LINE-1 repeats was associated with CHD in the Chinese population.
Zaina 2014[15]	Global DNA hypermethylation was associated with atherosclerosis.
Nazarenko 2015[51]	Global DNA hypermethylation was associated with coronary atherosclerosis.
Guarrera 2015[17]	Global DNA hypomethylation measured in LINE-1 repeats was associated with CHD and MI risk in men, being more pronounced in cases with shorter time to disease.
Ramos 2016[52]	Global DNA hypomethylation was associated with higher cardiovascular risk in postmenopausal women.

CHD: coronary heart disease; CVD: cardiovascular disease. MI: myocardial infarction. SMCs: smooth muscle cells.

Table 2. Main results of the studies using a candidate-gene approach.

Reference	Conclusion
Post 1999[38]	Hypermethylation in *ESRα* promoter was associated with atherosclerosis and aging of the vascular system.
Ying 2000[37]	Hypermethylation in *ESRα* promoter was associated with proliferating SMCs.
Zhu 2005[39]	Hypermethylation in exon 2 of *MTC3* was associated with atherosclerosis burden.
Kim 2007[54]	Hypermethylation in *ESRβ* promoter was associated with atherosclerosis.
Sharma 2008[43]	No significant differences in methylation in *APOE* promoter between patients and controls.
Huang 2009[24]	Hypermethylation in *ESRα* promoter was associated with atherosclerosis.
Huica 2011[8]	Hypermethylation of *TIMP1* and *ESRα* was associated with CVD and aging.
Talens 2012[9]	Hypermethylation of *INS* and *GNASAS* was associated with the incidence in MI in women.
Friso 2012[10]	Hypomethylation in *F7* promoter was associated with CHD in wild-type A1A1 genotypes.
Zhuang 2012[25]	Hypermethylation of *p15*^{INK4b} was associated with CHD and may have been mediated by altered expression of *ANRIL*.
Zhao 2012[40]	Hypermethylation in *MAOA* promoter was associated with decreased carotid intima-media thickness when twins were analyzed as individuals, but in match pair analysis no association was observed.
Lakshmi 2013[45]	Hypomethylation in *BNIP3* promoter and hypermethylation in *EC-SOD* promoter were associated with CHD.
Jiang 2013[26]	Hypermethylation in *PLA2G7* promoter was associated with CHD and aging in women independently of classical risk factors.
Jia 2013[33]	Epigenetic suppression of *FOXP3* might have led to downregulation of Treg cells and, in turn, increased the risk of CHD.

Afzali 2013[50]	Hypermethylation in *NPC1* promoter was associated with CHD independently of other parameters.
Lü 2013[27]	Hypomethylation of *FOXP3*, which is a characteristic of Treg cells, was associated with non-CHD.
Connelly 2013[55]	Hypomethylation of *COL15A1* occurs during SMC proliferation and the subsequent increased gene expression may impact SMC phenotype and atherosclerosis formation.
Fiorito 2014[11]	Hypermethylation in *TCN2* promoter and *AMT* gene body in males, *PON1* gene body in females, and *CBS* 5'UTR in both genders was associated with CHD.
Gómez-Úriz 2014[20]	Hypomethylation in *TNF-α* promoter was associated with stroke.
Zhang 2014[22]	Hypomethylation of *F2RL3* was associated with higher CVD mortality, as well as all-cause and other mortality.
Xu 2014[28]	Hypomethylation in *GCK* gene-body was associated with high risk of CHD, while its hypermethylation is associated with aging in healthy individuals.
Niu 2014[29]	Hypermethylation in *DDAH2* promoter was associated with CHD and with the dysfunction of endothelial progenitor cells in CHD patients.
Peng 2014[30]	Hypermethylation in *ABCG1* and *GALNT2* promoters was associated with an increased risk of CHD.
Guay 2014[46]	Hypermethylation in *ABCA1* promoter was associated with CHD and aging in men.
Pfeiffer 2015[12]	Hypermethylation in *ABCG1* was associated with CHD.
Wei 2015[49]	Hypermethylation in *MTHFR* was associated with ischemic stroke.
Baccarelli 2015[56]	Hypermethylation of *MT-CO1*, *MT-CO2*, *MT-CO3* and *MT-TL1* was associated with CVD.
Yang 2016[31]	Hypomethylation in *IL-6* promoter was associated with increased risk of CHD.
Nguyen 2016[47]	Hypomethylation in *ANGPTL2* promoter was associated with the pro-inflammatory environment in post-ACS patients.

Perna 2016[14]	Epigenetic age acceleration was associated with higher CVD mortality.
Horvath 2016[41]	Epigenetic aging rates were not associated with incident CHD outcomes.
Zuo 2016[32]	Hypomethylation in *IL-6* promoter was associated with increased risk of CHD, especially MI.
Murray 2016[13]	Hypermethylation in *ANRIL* promoter was associated with increased arterial stiffness, which indicated greater cardiovascular risk.
Zhong 2016[35]	Hypomethylation in *COMT* promoter was associated with CHD in males and with aging in controls.

CHD: coronary heart disease; CVD: cardiovascular disease. MI: myocardial infarction. ACS: acute coronary syndrome. SMCs: smooth muscle cells.

Table 3. Main results of the studies using an epigenome-wide approach.

Reference	Conclusion
Castillo-Díaz 2010[53]	Hypomethylation at 142 CGIs and hypermethylation at 17 CGIs were associated with atherosclerosis.
Sharma 2014[44]	Hypermethylation at 72 DMRs was associated with CHD.
Yamada 2014[48]	Hypomethylation at 15 CpGs in 14 genes and hypermethylation at 30 CpGs in 22 genes were associated with atherosclerosis.
Zaina 2014[15]	1858 dm-CpGs were associated with atherosclerosis.
Gómez-Úriz 2015[21]	80 dm-CpGs and hypermethylation in the promoter of *PM20D1* were associated with stroke.
Nazarenko 2015[51]	Hypomethylated CpGs in atherosclerotic samples were located within genes involved in inflammation, immune processes and development.
Ek 2015[16]	16 CpGs at 11 genes out of 31 discovered and 66 CpGs identified by meta-analysis were associated with CHD.
Guarrera 2015[17]	Hypomethylation at a DMR within *ZBTB12* gene body was associated with CHD, being more pronounced in cases with shorter time to disease.
Oudejans 2016[18]	12 DMRs were identified in the twin sisters at risk of CVD.
Nguyen 2016[47]	No statistical difference in methylation between controls and CHD patients.
Rask-Andersen 2016[19]	211 dm-CpGs in 196 genes were associated with MI, of which 42 had been related to cardiac function and development, CVD and recovery after ischemic episode.

CHD: coronary heart disease; CVD: cardiovascular disease. MI: myocardial infarction. CGI: CpG island. dm-CpG: differentially methylated CpG. DMR: differentially methylated region.

Table 4. Differentially methylated genes identified in more than one epigenome-wide association study (EWAS).

(A) Set 1, genes with one CpG reported as methylated in the same direction by two EWAS (n=8)			
Hypermethylated			**Hypo-methylated**
GRIP1[15,51]	*KCNJ14*[15,51]	*PKD2*[15,51]	*AIM2*[16,51]
HRH2[15,51]	*NGEF*[15,51]	*TNS1*[15,51]	*CRELD2*[16,48,a.]

(B) Set 2, genes reported as methylated in the same direction by more than one EWAS, independently of the CpG (n=52)				
Hypermethylated				**Hypo-methylated**
ABCB4[15,51]	*DLC1*[19,48]	*KCNJ14*[15,51]	*SFRP4*[15,19]	*AIM2*[16,51]
ACOT2[15,51]	*DLG2*[15,51]	*MECOM*[15,19]	*SH2D4B*[15,51]	*CRELD2*[16,48,a.]
AR[44,51,a.]	*DOCK5*[15,19]	*NGEF*[15,51]	*SLC6A6*[15,51]	*F2RL3*[16,51]
C1QTNF7[15,51]	*DSCAML1*[15,44]	*OLFML3*[15,51]	*SMOC2*[15,19]	*GPR143*[51,53]
C4orf48[18,19]	*DYSF*[15,19]	*PART1*[15,51]	*SYTL3*[15,19]	*HOXC5*[15,51]
CALD1[15,51]	*FOXJ3*[15,16]	*PDZD2*[15,19]	*THSD4*[15,51]	*MLC1*[15,19,51]
CAMTA1[15,48]	*FYN*[15,48]	*PHACTR2*[15,19]	*TNS1*[15,51]	
CBFA2T3[15,17]	*GATA3-AS1*[44,51]	*PKD2*[15,51]	*TRANK1*[15,19]	
CORT[15,51]	*GRIP1*[15,51]	*PKNOX2*[15,44]	*VWC2*[15,19]	
CTNNA3[15,44]	*HAND2*[19,44]	*RASGRF1*[15,19]	*ZBTB16*[15,16]	
DFNA5[15,19]	*HMCN1*[15,19]	*RNF216*[15,48]		
DIP2C[15,19]	*HRH2*[15,51]	*SEPT9*[15,48]		

(C) Set 3, genes reported as differentially methylated by more than one EWAS, independently of the CpG or the methylation direction (Set 2 + 32 additional genes)			
ABCC1[15,51]	*CARS*[15,16]	*GSC*[19,53]	*PNLIP*[15,51,b.]
ABR[15,48]	*CLIC4*[15,53,a.]	*HOXA3*[15,51]	*PNLIPRP1*[15,51,b.]
ALX4[19,51]	*ESRRG*[19,53,a.]	*HOXC11*[15,53,a.]	*SCEL*[15,51]
ARHGEF10[15,16]	*FAM109B*[19,53]	*HOXD4*[51,53,a.]	*TAS2R9*[15,51,b.]
ARID1B[15,48]	*FMNL2*[15,19]	*IL5RA*[15,51]	*TEAD1*[15,51]
ART4[15,19,b.]	*FOXP1*[15,53]	*MIR2054*[15,19]	*TGFBR3*[15,44]
BEND6[15,19]	*GCNT2*[15,51]	*MRPS9*[15,53,a.]	*TICAM1*[15,53,a.]
CAPZB[16,53]	*GDF6*[15,19]	*NRG1*[16,19]	*WT1*[19,51]

[a.]genes that are upstream or downstream of one of the reported CpGs; [b.]genes within which one CpG/CGI was reported by two EWAS.

Table 5. Ingenuity pathway analysis: functional classification of the three sets of genes identified as differentially methylated in more than one independent EWAS. Set 1 includes the 8 genes with methylation consistency at CpG level. Set 2 contains the 52 genes with methylation consistency at gene level. Set 3 comprises the 84 genes found as differentially methylated genes in more than one EWAS, independently of the consistency of the methylation direction. **(A)** Ingenuity pathway analysis of "Canonical pathways" using Fisher's exact test. **(B)** Ingenuity pathway analysis of "Diseases and functions" using Fisher's exact test and Benjamini-Hochberg multiple testing correction. Note that those "Diseases and functions" terms in italic separated by a dash line are only significant when using Fisher's exact test. Terms in bold are those found as enriched for the three sets of genes.

Set 1	Set 2	Set 3
(A) Canonical pathways		
- Inflammasome pathway - FAK Signaling - Gαs Signaling - **RhoA Signaling**	- **RhoA Signaling** - FAK Signaling - Acyl-CoA Hydrolysis	- Retinol Biosynthesis - Triacylglycerol Degradation - **RhoA Signaling** - Ephrin A Signaling - RhoGDI Signaling - Acyl-CoA Hydrolysis
(B) Diseases and functions		
- **Endocrine System Disorders** - **Inflammatory Disease** - **Cardiovascular System Development and Function** - **Inflammatory Response** - **Immune Cell Trafficking**	- **Endocrine System Disorders** - **Cardiovascular Disease** - Carbohydrate Metabolism - Connective Tissue Disorders - **Inflammatory Disease** - Lipid Metabolism - Metabolic Disease - Nutritional Disease	- **Endocrine System Disorders** - Metabolic Disease - Carbohydrate Metabolism - **Cardiovascular Disease** - **Cardiovascular System Development and Function** - Connective Tissue Disorders - **Inflammatory Disease** - Lipid Metabolism - Nutritional Disease - **Vitamin and Mineral Metabolism** - Connective Tissue Development and Function

- ***Vitamin and Mineral Metabolism*** - ***Cardiovascular Disease***	- *Cell-mediated Immune Response* - ***Immune Cell Trafficking*** - ***Inflammatory Response*** - ***Connective Tissue Development and Function*** - ***Cardiovascular System Development and Function*** - ***Vitamin and Mineral Metabolism***	- *Cell-mediated Immune Response* - ***Immune Cell Trafficking*** - ***Inflammatory Response***