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The role of epigenetic modifications in cardiovascular disease: A systematic review

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ABSTRACT

Background: Epigenetic modifications of the genome, such as DNA methylation and histone modifications, have been reported to play a role in processes underlying cardiovascular disease (CVD), including atherosclerosis, inflammation, hypertension and diabetes.
Methods: Eleven databases were searched for studies investigating the association between epigenetic marks (either global, site-specific or genome-wide methylation of DNA and histone modifications) and CVD.
Results: Of the 3459 searched references, 31 studies met our inclusion criteria (26 cross-sectional studies and 5 prospective studies). Overall, 12,648 individuals were included, with total of 4037 CVD events. The global DNA methylation assessed at long-interspersed nuclear element (LINE-1) was inversely associated with CVD, independent of established cardiovascular risk factors. Conversely, a higher degree of global DNA methylation measured at Alu repeats or by the LUMA method was associated with the presence of CVD. The studies reported epigenetic regulation of 34 metabolic genes (involved in fetal growth, glucose and lipid metabolism, inflammation, atherosclerosis and oxidative stress) in blood cells to be related with CVD. Among them, 5 loci were validated and methylation at F2RL3 was reported in two large prospective studies to predict cardiovascular disease beyond the traditional risk factors.
Conclusions: Current evidence supports an association between genomic DNA methylation and CVD. However, this review highlights important gaps in the existing evidences including lack of large-scale epigenetic investigations, needed to reliably identify genomic loci where DNA methylation is related to risk of CVD.

1. Introduction

There is a worldwide epidemic of cardiovascular disease (CVD) causing one-third of all deaths worldwide and counting for trillions of dollars of health care expenditure [1,2]. This figure will surely increase in both developing and developed countries as risk factors for the disease, such as dyslipidemia, hypertension, obesity and diabetes continue to increase [2].

Current scientific knowledge does not completely explain the complex pathophysiology underlying CVD and therefore a search for other pathways is constantly being conducted. Epigenetic modifications of the genome might constitute an additional pathway leading to CVD [3]. Epigenetics refers to various dynamic features that modify the genome's functionality under exogenous influence and also provide a molecular substrate that allows for the stable propagation of gene expression states from one generation of cells to the next [4]. DNA methylation and histone modifications are the best understood of the epigenetic mechanisms thus far [4], and have been suggested to regulate gene expression and affect CVD risk factors including atherosclerosis, inflammation, hypertension and diabetes [5–7]. Unlike mutations and other genetic abnormalities, epigenetic modifications are dynamic and could be modified by lifestyle and perhaps other therapeutic approaches [8,9]. Therefore, it has been suggested that these epigenetic mechanisms can be important regulatory key players not only in understanding CVD's pathophysiology but also in both its diagnosis and treatment [10]. To date, however, little work has been done to systematically appraise the current evidence for the role of DNA methylation and histone modifications on the risk of CVD.

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We aimed to conduct a systematic review and meta-analysis of all available evidences in humans to quantify the association of DNA methylation and histone modifications with cardiovascular outcomes.

2. Material and methods

2.1. Literature search

This review was conducted using a predefined protocol and in accordance with the PRISMA [11] and MOOSE [12] guidelines (eAppendix 1 and 2). Eleven bibliographic databases (Embase.com, Medline (Ovid), Web-of-Science, Scopus, PubMed, Cinahl (EBSCOhost), Cochrane Central, ProQuest, Lilacs, Scielo and Google Scholar) were searched until May 27th 2015 (date last searched) without any language restrictions, with the help of an experienced medical information specialist. The search strategy combined terms related to exposure (e.g., epigenetics, DNA methylation, histone, and CpG) and outcomes (e.g., cardiovascular disease, coronary disease, heart disease, cerebrovascular disease, myocardial infarction, stroke, ischemia, and carotid artery disease). In databases where a thesaurus was available (Embase, Medline and Cinahl) articles were searched by thesaurus terms and by title and/or abstract, and in other databases only by title and/or abstract. We restricted the search to studies on humans. The full search strategies of all databases are provided in eAppendix 3. After eliminating duplications, in total, we identified 3459 potentially relevant citations.

2.2. Study selection and inclusion criteria

Studies to be included either described an association between epigenetic marks (global, site specific or genome-wide methylation of DNA or histone modifications) and cardiovascular outcomes defined as fatal or non-fatal coronary heart disease (CHD) and stroke. CHD events included myocardial infarction, coronary artery bypass graft, ischemic heart disease or sudden cardiac death if caused by myocardial infarction and CHD deaths. Stroke included both hemorrhagic and ischemic cerebrovascular events. Two independent reviewers, screened the retrieved titles and abstracts and selected eligible studies. In case of disagreement, decision was made through consensus or consultation with a third independent reviewer. Full texts were retrieved for studies that satisfied all selection criteria.

2.3. Data extraction

A predesigned data collection form was prepared to extract the relevant information from the selected studies, including study design, study population, location, age range, duration of follow up (for longitudinal studies), and degree of adjustment. The degree of adjustment was defined as '+' when the measures of association were adjusted for age and non-established cardiovascular risk factors (e.g., education, income, and ethnicity) and "++" when further adjustment was done for established vascular risk factors and potential mediators (e.g., smoking status, body mass index, lipids, and hypertension), tissue sample and method used to assess epigenetic marks, for type and numbers of cardiovascular outcomes and reported measures of associations (e.g., correlation analysis, odds ratio, and relative risks).

2.4. Assessing the risk of bias

Bias within each individual study was evaluated using the validated Newcastle–Ottawa Scale, a semi-quantitative scale designed to evaluate the quality of nonrandomized studies [13]. Study quality was judged on the selection criteria of participants, comparability of cases and controls, and exposure and outcome assessment. Studies that received a score of nine stars were judged to be at low risk of bias; studies that scored seven or eight stars were considered to be at medium risk; those that scored six or less were considered to be at high risk of bias.

2.5. Outcome assessment and statistical methods

For each study, we defined whether an association was reported, and when applicable, direction effect sizes were reported. Heterogeneity permitting, we sought to pool the results using a random effects meta-analysis model. If pooled, results were expressed as the pooled estimate and the corresponding 95% confidence intervals.

3. Results

In total, after deduplication, we identified 3459 potentially relevant citations (Fig. 1). Based on the title and abstracts, full texts of 35 articles were selected for detailed evaluation. Of those, 31 articles met our eligibility criteria and were therefore included in the analysis (Supplementary Tables S1–S2).

3.1. Summary of included studies

Overall, 12,648 individuals were included within the systematic review, with a total of 4037 CVD outcomes (3599 prevalent CVD outcomes and 439 incident CVD events) (Supplementary Tables S1–S2). Of the 31 studies included, 6 studies assessed the global DNA-methylation (4 case control studies and 2 prospective studies), 20 studies assessed the DNA methylation in specific candidate genes (17 cross-sectional studies and 3 longitudinal studies), 3 studies (all case control studies) used genome-wide approaches, one study assessed histone modifications and one study (case–control) examined both DNA methylation and histone modifications in specific candidate genes in relation to CVD (Tables 1, 2 and Supplementary Tables S1–S2). Seven studies included participants from China, 4 studies from India and the rest included participants from Canada, Germany, Italy, Spain, Scotland, Sweden, Romania, Iran and the USA (Supplementary Tables S1–S2). All available studies were cross-sectional, case control or prospective cohorts in design and were judged as low or medium-quality studies, with only one study to be judged as a high quality (Supplementary Tables S1–S2).

3.2. Global DNA methylation and cardiovascular disease

Global methylation refers to the overall level of methylcytosine in the genome, expressed as percentage of total cytosine. A large portion of methylation sites within the genome are found in repeat sequences and transposable elements, such as Alu and long-interspersed nuclear element (LINE-1) and correlate with total genomic methylation content

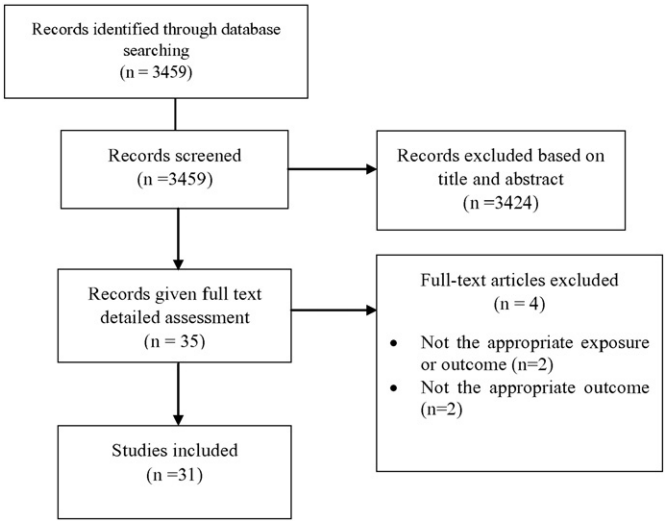

Fig. 1. Flowchart of studies investigating epigenetic marks in relation to cardiovascular disease.

Table 1
Global DNA methylation and cardiovascular disease.[a]

CVD outcome	Tissue type	Population	Association, reference	Comment
LINE-1 methylation				
CHD, n = 344	PBL	M and F, n = 1122	Inverse association [20]	LINE-1 methylation level was inversely associated with the risk of CHD (relative to the subjects in the fourth quartile of LINE-1 methylation ORs for CHD were 0.9 (95% CI, 0.6–1.4), 1.9 (95% CI, 1.3–2.9), and 2.3 (95% CI, 1.6–3.5) for the subjects with methylation in the third, second and first quartile; $P_{trend} < 0.001$). The association tended to be stronger among subjects with higher levels of homocysteine ($P_{interaction} = 0.04$) and those with diagnosis of hypertension ($P_{interaction} = 0.01$)
Ischemic stroke, n = 280)	PB	M and F, n = 560	Inverse association in men; no association in women [21]	In men, a decrease of 1% methylation level in men was associated with an increased risk of stroke (OR = 1.2; 95% CIs: 1.1–1.32).
-Non-fatal IHD (prevalent, n = 212, incident, n = 36) -Non-fatal stroke (prevalent, n = 51; incident, n = 8) -IHD mortality (n = 35) -Stroke mortality (n = 10) -Combined IHD and Stroke	WB	M, n = 712	Inverse association [22]	The associations were significant in both cross-sectional (combined incident non-fatal IHD and stroke: 1st quartile vs. 4th quartile: OR = 2.2 95% CI = 1.2–3.9) and prospective analysis (combined incident non-fatal IHD and stroke: (<median vs. >median: HR = 4.1, 95% CI = 1.9–8.7); combined incident fatal IHD and stroke: (<median vs. >median: OR = 2.9, 95% CI = 1.3–6.2))
ALU				
MI and/or stroke, n = 14	PBL	M and F, n = 286	Positive association [23]	Subject with a MI and/or stroke at baseline showed a significantly higher mean of combined methylation in ALU and SAT 2 repetitive elements (geometric mean = 201 and 95% CI = 145–180, $p = 0.045$). This association was prominent in men ($p = 0.02$) but not in women ($p = 0.66$).
HpaII/MspI ratio				
CAD, n = 137	PBL[b]	M and F, n = 287	Positive association [24]	Global DNA methylation was positively associated with CAD ($p = 0.02$). The results differ by the levels of plasma homocysteine.
CVD-mortality, n = 13	PBL	M and F, n = 56	Positive association [25]	Global DNA hypermethylation (HpaII/MspI ratio < median) was associated with increases risk of CVD mortality (HR = 13.9, 95% CIs: 1.8–109.3.)

CAD, coronary artery disease; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease; F, female; IHD, ischemic heart disease; M, male; MI, myocardial infarction; n, number; OR, odds ratio; PB, peripheral blood; PBL, peripheral blood leukocytes.
[a] Significant inverse association with IHD and IHD and stroke combined but not with stroke.
[b] Levels of methylations estimated in terms of [3H] dCTP following MspI and HpaII.

[14–16]. Methylation of these repetitive elements is thus used as a surrogate for the overall methylation of the genome [17,18]. Other methods (e.g., Luminometric Methylation Assay, LUMA and the [^{3}H]-methyl acceptance based method) to asses global genomic DNA methylation are primarily based on the digestion of genomic DNA by restriction enzymes HpaII and MspI [19].

All six studies that examined global DNA methylation and risk of CVD used blood samples to assess DNA methylation (Table 1). Three studies used LINE-1 methylation [20–22], one study used Alu methylation [23] and two studies used the LUMA method and the [^{3}H]-methyl acceptance based method to estimate the global genomic methylation [24,25]. Four studies were case control [20,21,23,24], one study was prospective [25] and one study used both cross-sectional and longitudinal designs [22]. Five studies [20–22,24,25] adjusted for established CVD risk factors whereas one study [23] did not (Supplementary Table S1).

3.2.1. LINE-1 methylation and cardiovascular disease

One cross-sectional study showed that lower levels of LINE-1 methylation were associated with the presence of CHD in both men and women (comparing 1st quartile vs. 4th quartile: odds ratio (OR) = 2.3, 95% confidence interval (CI) = 1.6–3.5), and this association tended to be stronger among subjects with higher levels of homocysteine and among hypertensive subjects [20]. Another study, using both a cross-sectional and longitudinal design demonstrated that a lower degree of LINE-1 methylation was associated with both prevalent and incident ischemic heart disease and stroke (the longitudinal analysis, <median vs. ≥ median global DNA-methylation: hazard ratio (HR) = 2.9, 95% CI = 1.3–6.2) [22]. Finally, a cross-sectional study reported an inverse association between LINE-1 methylation and ischemic stroke in men (per decrease of 1% in DNA-methylation level, OR = 1.2, 95% CI = 1.1–3.2) but not in women [21].

3.2.2. Alu methylation and cardiovascular disease

One study of cross-sectional design examined Alu methylation in relation to myocardial infarction and reported a higher degree of methylation in cases compared to healthy controls [23].

3.2.3. DNA methylation as assessed by LUMA or [^{3}H]-methyl acceptance and cardiovascular disease

One cross-sectional study reported global DNA hypermethylation to be associated with the presence of coronary artery disease (CAD) within chronic kidney disease patients who underwent hemodialysis [24]. Similarly, a prospective study showed that global DNA hypermethylation was associated with increased risk of CVD-mortality (<median vs. ≥ median global DNA-methylation, HR = 13.9, 95% CI = 1.8–10.3) [25].

3.3. Gene specific DNA methylation and cardiovascular disease

DNA methylation, the addition of a methyl group to the 5 position of cytosine in a dinucleotide CpG site, is an important mechanism in gene expression regulation [26]. Loss of DNA methylation promotes gene expression [27], however, the association of DNA methylation with gene expression depends on where within the gene sequence the methylation occurs. DNA methylation in the promoter region of the gene

Table 2
Specific gene methylation and cardiovascular disease: gene and genome-wide approaches.

Author	CVD outcome	Tissue type	Population	Methylation sites/method	Main Finding
Candidate gene approach					
Afzali M. et al. (2013) [31]	CVD (n = 50)	PBL	M and F, n = 100	NPC1 promoter/nested methylation specific polymerase	The frequency of semi-methylated NPC1 promoter (methylated/un-methylated) is higher in a CVD patient than in controls (OR = 6.521, 95% CIs, 2.211–19.215). The prevalence of methylated allele was elevated in CVD patients than healthy subjects (OR = 2.011, 95% CIs, 1.116–3.594)
Baccarelli, A. et al. (2015) [45]	CVD (n = 10)	Platelet	M and F, n = 27	Mitochondrial genes (cytochrome *c* oxidase (MT-CO1, MT-CO2, MT-CO3), tRNA leucine (MT-TL1), ATP synthase (MT-ATP6 and MT-ATP8) and NADH dehydrogenase (MT-MD5)/bisulphite sequencing	Mean DNA methylation of MT-CO1, MT-CO2, MT-CO2 MT-TL1 were higher in CVD cases than in non-CVD subjects ($p < 0.001$). No differences were observed in DNA methylation at MT-ATP6, MT-ATP8 or MT-ND5 between CVD cases and non-CVD patients.
Breitling, L.P. et al. (2012) [39]	CVD-mortality (n = 64)	WB	M and F, n = 1206	F2RL3/6 CpGs/Sequenom SpectroACQUIRE and MassARRAY EpiTyper.	No association was observed between F2RL3 CpG_4 methylation levels and CVD-mortality comparing the 1st quartile with the fourth (HR = 2.32, 95% CIs: 0.97–5.58). Per 10% less methylation: HR = 1.30, 95% CIs: 1.04–1.63.
Fiorito G. et al. (2014) [43]	MI (n = 206)	PB	M and F, n = 412	33 genes involved in homocystein metabolism and one-carbon metabolism pathway, 575 CpG sites/HumanMethylation450 BeadChip	3 differentially methylated regions in males (TCN2 promoter, CBS 5′UTR, AMT gene-body) and 2 in females (PON1 gene-body, CBS 5′UTR) were identified, each of them characterized by an increased methylation in MI subjects. Four clusters of distinct methylation profile were identified, which were differently associated with the risk of MI (high risk vs. low risk methylation profile groups: OR = 3.49, $p = 1.87 \times 10^{-4}$ and OR = 3.94, $p = 0.0317$ in males and females respectively).
Friso S. et al. (2012) [38]	CAD (n = 165)	PBMC	M and F, n = 253	F7 gene promoter, 6 CpGs in SNPs/methyl specific PCR primers and bisulphite sequencing	CAD-free subject showed a higher F7 methylation index compared to CAD patients (32.12 ± 9.80, $p = 0.012$). Among the 6 CpGs, the CpG2, 3 and 6 accounted for the largest difference in methylation.
Gomez-Uriz A.M. et al. (2014) [35]	Stroke (n = 12)	WB	M and F, n = 12	TNF-α promoter, 19 CpGs/Sequenom EpiTyper MassARRAY	Lower values of total TNF-α promoter methylation, using the median value as cut-off (median 0.918) was associated with higher odds of having stroke (OR = 9.0, 95% CIs, 1.4–57.1). The binding pattern of H3K4me3 and H3K9ac in a region of TNF-α was similar when comparing non-stroke and stroke conditions.
Guay S.P. et al. (2012) [32]	CAD (n = 71)	PBL	M and F, n = 97	ABCA1 gene promoter, 26 CpGs/bisulfite-pyrosequencing	CAD subjects had higher ABCA1 DNA methylation levels compared with those without CAD (34.3 ± 8.4 versus 4.2 ± 15.2, $p = 0.003$)
Guay S.P. et al. (2014) [33]	CAD (n = 88)	PBL	M, n = 88	ABCA1 gene, 8 CpGs/bisulfite-pyrosequencing	Subjects with a previous history of CAD showed higher mean ABCA1 DNA methylation levels than subjects without CAD (38.7 ± 1.2 versus 36.0 ± 1.0, $p = 0.04$). These results differ by age (older CAD-subjects had higher methylation levels than young CAD-subjects and non-CAD subjects).
Guay S.P. et al. (2014) [33]	CAD (n = 22)	WB	M and F, n = 44	ABCG1-CpGC3, LIPC-CpGA2, PLTP-CpGC/bisulfite-pyrosequencing	Subjects with a previous history of CAD showed lower mean LIPC-CpGA2DNA methylation levels than subjects without CAD (83.8 ± 1.9 versus 85.2 ± 1.9, $p = 0.02$). No differences were observed in ABCG1-CpGC3 and PLTP-CpGC DNA methylation.
Huica I. et al. (2011) [36]	CVD (n = 37)	WB	ND, n = 62	Estrogen receptor alpha (ERα) and tissue inhibitors of metalloproteinases (TIMP-1) specific PCR primers and bisulphite sequencing	ERα and TIMP1 presented a statistically significant frequency of hypermethylation in CVD cases compared to non-CVD cases ($p < 0.001$ for each gene). Hypermethylation of ERα: OR = 43.1, 95% CIs: 9.8–192.3. Hypermethylation of TIMP1: OR = 15.3, 95% CIs: 3.8–61.3
Jiang D. et al. (2013) [34]	CHD (n = 36)	PBL	M and F, n = 72	CF1 region of PLA2G7 gene promoter, 4 CpGs/pyrosequencing	There was a higher promoter DNA methylation of PLA2G7 gene in the CHD cases than in non-CHD controls (6.41 ± 2.62 versus 4.98 ± 3.06, $p = 0.025$). The stratified analysis by gender, showed that the significant association was found in females ($p = 0.003$) but not in males ($p = 0.096$). Receiver operating characteristic curves showed that LA2G7 methylation could predict the risk of CHD in females (area under the curve = 0.912,

(continued on next page)

Table 2 (*continued*)

Author	CVD outcome	Tissue type	Population	Methylation sites/method	Main Finding
					$p = 2.40E-5$).
Lakshmi S.V.V. et al. (2013) [42]	CAD (n = 94)	PB	ND, n = 177	BCL2/E1B adenovisur interacting protein 3 (BNIP3), extracellular superoxide dismutase (EC-SOD) and glutathione-S-transferase P1 (GSTP1), methyl specific PCR primers and bisulphite sequencing	Hypomethylation of BNIP3 promoter in CAD cases compared to controls (41.95 ± 26.91% vs. 53.51 ± 42.78%, $p = 0.03$). EC-SOD promoter hypermethylation was observed in CAD compared to controls (62.23 ± 33.36% vs. 32.35 ± 24.76%, $p < 0.0001$). GSTP1 promoter hypermethylation was observed in CAD compared to controls, but was not significant (45.63 ± 22.74% vs. 41.67 ± 25.26%, $p = 0.28$)
Lu C.X. et al. (2013) [2]	ACS (n = 89)	$CD4^+CD25^+$ T-cells	M and F, n = 124	FOXP3 gene, 2 CpGs/pyrosequencing	Demethylation of DNA at FOXP3 gene in ACS subjects was significantly lower than in non-ACS subjects ($p < 0.0001$). Analysis of operating characteristic curve showed an area under the curve of 0.916 ($p < 0.001$), supporting the notion that FOXP3 demethylation can distinguish ACS subjects from non-ACS subjects.
Peng P. et al. (2014) [85]	CHD (n = 85)	PBL	M and F, n = 139	ABCG1, GALNT2 and HMGCR gene promoter/bisulphite-specific PCR	Promoter hypomethylation of the ABCG1 gene was associated with the risk of CHD (OR = 19.966, 95% CI: 7.319–54.468). Methylation status of the GALNT2 gene promoter was associated with the risk of CHD (OR = 2.978, 95% CI: 1.335–6.649). There was no association between methylation status of the HMGCR gene promoter and the risk of CHD (OR = 2.978, 95% CI: 1.335–6.649).
Sharma, P. et al. (2008) [24]	CAD (n = 137)	PBL	M and F, n = 287	ApoE, 25CpGs/bisulphite sequencing	No significant difference in methylation patterns between CAD and non-CAD subjects.
Talens R.P. et al. (2011) [29]	MI (n = 122)	PBL	M and F, n = 248	IL10, LEP, ABCA1, IGF2, INS and GNASA, 49 CpG sites/Sequenom EpiTyper MassARRAY	Overall, DNA methylation was modestly higher in MI cases at GNASAS compared with the control group ($p = 0.03$). No differences in DNA methylation were observed at the other loci. Sex differences were observed for INS (p-interaction = 0.014) and GNASAS (p-interaction = 0.031). In women, DNA methylation at INS ($p = 0.002$) and GNASAS ($p = 0.009$) were higher in MI cases compared to control. In men, no differences in DNA methylation were observed. Overall, hypermethylation at one locus (INS or GNASAS) and at both loci (INS and GNASAS) was associated with OR = 1.7 (95% CI: 2.7–27.9) and 2.8 (95% CI = 1.4–5.9) respectively. In men, no associations were observed.
Wei L.K. et al. (2015) [44]	Ischemic stroke (n = 297)	WB	M and F, n = 407	MTHFR, CpG A and B/bisulphite-specific PCR	CpG A methylation levels were associated with a higher risk for ischemic stroke (OR = 4.73, 95% CI: 2.56–8.75). CpG B methylation levels were not associated with ischemic stroke (OR = 0.90, 95% CI: 0.56–1.45).
Xu L. et al. (2014) [30]	CHD (n = 36)	PB	M and F, n = 72	GCK gene-body, 4 CpGs/bisulfite-pyrosequencing	CHD cases had a significantly lower methylation level (49.77 ± 6.43%) compared with controls (54.47 ± 7.65%, $p = 0.018$). Similar trends were observed in three CpGs (CpG2, 3 and 4; p for all <0.05).
Xu L. et al. (2015) [37]	CHD (n = 784)	PB	M and F, n = 1530	4 CpGs of the vascular-related genes (VEGFA, CST3, AGTR1, ACE)/Sequenom EpiTyper MassARRAY	None of the four CpG-SNPs in the vascular related genes was associated with the risk of CHD.
Zhang Y. et al. (2014) [40]	CVD-mortality (n = 151)	WB	M and F, n = 3572	F2RL3, 4CpG [2 to 5]/Sequenom EpiTyper MassARRAY	Lower methylation was associated with increased risk of CVD mortality (comparing the lowest vs. highest quartile of F2RL3 CpG_4 methylation, HR = 2.94, 95% CIs: 2.45–4.68). Similar results were observed for other CpGs. Per 10% less methylation: HR = 1.38, 95% CIs: 1.14–1.66.
Zhuang J. et al. (2012) [41]	CAD (n = 95)	PBL	M and F, n = 205	BAX, BCL-2, TIMP3, $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$,seven CpGs at $p15^{INK4b}$/MethyLight	$p15^{INK4b}$ was associated with the presence of CAD (OR = 2.55, 95% CIs, 1.26–5.01). No association was observed between $p16^{INK4a}$ and CAD (OR = 1.14, 95% CIs, 0.59–2.36). CpGs +314 and +332 at $p15^{INK4b}$ site, were significantly increased in CAD patients compared with controls. No difference was observed for CpGs +269, +272, +280, +303 and +321

Table 2 (*continued*)

Author	CVD outcome	Tissue type	Population	Methylation sites/method	Main Finding
Genome-wide approaches					
Gomez-Uriz A.M. et al. (2015) [46]	Ischemic stroke (Discovery study: n = 12. Replication study: n = 60	PBL	M and F, Discovery study: n = 24. Replication study: n = 115	27,578 CpG sites, 14,495 genes/Illumina human methylation 27 Beads and MassARRAY EpiTyper	80 CpG sites differentially methylated in patients who suffered an ischemic stroke compared to those who did not ($p < 0.05$). 59 CpG sites presented an interaction between stroke and obesity. Among 21 CpG sites and 15 genes selected as candidates: • CpG sites 19 and 20 of the gene Wilm's tumor 1 (WT1) showed higher methylation levels in stroke patients compared to non-stroke subjects ($p < 0.05$). These results were not replicated in the validation study. • The promoter region of peptidase M20 domain containing 1 (PM20D1) gene was significantly hypermethylated in stroke patients ($p < 0.05$). Differences in this region were significant at the CpG sites 1_2_10_11_12_13_14_16_17_18_22. These results were not replicated in the validation study. • CpGs 8_9 of KQT-like subfamily, member 1 (KCNQ1) explained 31 and 33% respectively of the variability for the case stroke in the validation study.
Sharma P. et al. (2014) [47]	CAD (Discovery study: n = 18. Replication study: n = 48)	WB	M, Discovery study: n = 132. Replication study: n = 96	Bisulphite sequencing by 454 platform	19 differentially methylated regions were significantly hypermethylated in CAD subjects compared to controls. In the validation study: out of the 12 differentially methylated regions selected, 6 CpG sites in 4 regions falling within three differentially methylated regions had significantly higher methylation in CAD patients: of the 6 sites, 3 were in the intronic region of STRADA gene (*Homo sapiens* STE20-related kinase adaptor alpha) flanking CCDC47 and LID2, 2 were in the first exon of C1QL4 gene flanking TROAP and FLJI3236 while the other was in the intronic region of HSP90B3P gene flanking CD27 and TGFBR3
Guay S.P. et al. (2015) [48]	CAD (Discovery study: n = 6. Replication study: n = 45)	WB	M, Discovery study: n = 12. Replication Study: n = 161	27,578 CpG sites, bisulphite sequencing by 454 platform	There were 1765 CpG dinucleotides with potential DNA methylation differences between CAD and non-CAD men ($P < 0.05$). Among 1765 CpGs, 369 CpG dinucleotides were considered as the most promising differentially methylated loci. The gene ontology analysis revealed a significant enrichment of genes with epigenetic changes in biological pathways relevant to CVD development, such as cellular homeostasis, proliferation of connective tissue cells, angiogenesis and cardiovascular system. New candidate genes emerged: COL14A1 (hypomethylation) and MMP9 (hypermethylation) were associated with CAD (however, the findings were not replicated in the validation study).

ABCA1, ATP-binding cassette sub-family A; ACS, acute coronary syndrome; CAD, coronary artery disease; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease; EC-SOD, extracellular superoxide dismutase; ERα, estrogen receptor alpha; F, female; GSTP1, glutathione-S-transferase P1; HR, hazard ratio; M, male; MI, myocardial infarction; MTHFR, methylenetetrahydrofolate reductase; MTHFR, methylnetetrahydrofolate reductase; NCP1, Niemann-Pick disease type 1; Niemann-pick type C1; NPC1; PB, peripheral blood; PBL, peripheral blood leucocyte; PBMC, peripheral blood mononuclear cells; TIMP-1, tissue inhibitors of metalloproteinase; WB, whole blood.

down-regulates its expression whereas higher methylation in the gene-body promotes the expression of the gene [28]. This can be evaluated using candidate gene studies and genome-wide approaches:

3.3.1. Candidate gene studies

There were 21 studies (18 cross-sectional studies and 3 prospective studies) that examined methylation sites in, or near, known candidate genes for CVD susceptibility in relation to CVD outcomes (Supplementary Table S2). Most of the studies used a hypothesis-driven approach, whereas in others, the choice of genes was based on prior analysis of gene expression differences in the same subjects. The candidate gene methylation studies examined a range of genes involved in fetal growth [29], glucose [30] and lipid metabolism [31–34], inflammation [34,35], vascular reactivity [36,37], coagulation [38–40], atherosclerosis [34,36,41], obesity, oxidative stress [35,42] and in the homocysteine and folate metabolic pathways [43,44] (Table 2). Adjustment for established CVD risk factors were done in 13 studies (Supplementary Table S2). DNA methylation assessment was only done in the promoter region of the gene in 18 studies. One study assessed DNA methylation in the body of gene [30] and one study assessed DNA methylation in both sites [43] (Table 2).

Overall, these studies showed that compared to subjects without CVD, subjects with an established CVD have higher methylation levels of Niemann–Pick disease type 1 (*NCP1*), ATP-binding cassette sub-

family A (*ABCA1*), *PLA2G7*, *GALNT2*, *INS*, *p15*^{INK4b} and *GNASAS* in peripheral blood leucocyte, extracellular superoxide dismutase (*EC-SOD*), estrogen receptor alpha (*ERα*), tissue inhibitors of metalloproteinase (*TIMP-1*), methylenetetrahydrofolate reductase (*MTHFR*) in whole blood, F7 in peripheral blood mononuclear cells, glutathione-S-transferase P1 (*GSTP1*) in PB, *FOXP3* in $CD4^+CD25^+$ T-cells and of genes involved in homocysteine metabolism and one-carbon metabolism pathway (*TCN2*, *CBS 5′UTR* and *AMT* in males and *PON1* and *CBS 5′UTR* in females) in peripheral blood, and lower methylation levels of *ABCG1* in peripheral blood leucocytes, adenovirus interacting protein 3 (*BNIP3*) in peripheral blood, and of *F2RL3*, tumor necrosis alpha (*TNF-α*), *LIPC*, *GCK*, *F2RL3* and *BCL2/E1B* in whole blood (Table 2 and Supplementary Table S3). Furthermore, one study that analyzed platelet mitochondrial DNA methylation levels of genes associated with ATP synthesis and of tRNA leucine gene 1 (*MT-TL1*) showed protein-encoding cytochrome *c* oxidase genes (*MT-CO1*, *MT-CO2* and *MT-CO3*) and *MT-TL1* to be hypermethylated in CVD cases compared to healthy controls [45]. The most consistently reported epigenetic association was that of methylation at the F2RL3 in whole blood with the risk of CVD mortality which was reported in 2 prospective studies, one that included CVD-free participants and the other that included participants with established CVD [39,40]. Both studies showed that hypomethylation at F2RL3 was associated with increased risk of CVD-mortality (per 10% less methylation, Breitling et al.: HR = 1.30, 95% CI, 1.04–1.63; Zhang et al.: HR = 1.38, 95% CI, 1.14–1.66). Three studies showed sex-differences in the association between gene-specific DNA methylation and CVD [29,34,43]. Collectively, these studies suggest that altered epigenetic regulation of a number of metabolic genes (Supplementary Table S3) could be involved in cardiovascular disease etiopathogenesis.

3.3.2. Genome-wide analysis for cardiovascular disease

Due to the advent of genome-wide arrays for quantifying site-specific DNA methylation, several studies have investigated differentially methylated regions in the genome in a hypothesis-free approach. Three studies looked for CVD-associated differentially methylated sites in peripheral blood cells [46–48]. All three studies used a replication study to validate their findings. Collectively, up to 1675 CpG dinucleotides were identified with potential DNA methylation related to risk of CVD. The identified genes were enriched for genes (known from genome-wide association studies) with epigenetic changes in biological pathways relevant to CVD-development, such as cellular homeostasis, proliferation of connective tissue cells, angiogenesis and cardiovascular system (Table 2). Among them, 4 loci were validated (*STRADA*, *C1QL4*, *HSP90B3P* and *KCNQ1*) (Table 2 and Supplementary Table S3). Also, new candidate genes emerged such as *COL14A1* (hypomethylation) and *MMP9* (hypermethylation) which were reported to be associated with CAD [48].

3.4. Histone modifications and cardiovascular disease

Two studies examined the association between histone modifications and CVD (Supplementary Table S4) [35,49]. One study showed that the levels of acetylated histone H3 in the peripheral blood mononuclear cells of acute ischemic stroke patients were lower than normal controls [49] whereas the other study reported no difference in the binding pattern of H3K4me3 and H3K9ac in a region of TNF-α when comparing non-stroke with stroke patients [35].

4. Discussion

The present work is the first to systematically review the current evidence for the role of epigenetic marks in CVD. Our findings indicate that global DNA methylation might influence CVD risk and this could occur beyond the traditional cardiovascular risk factors. Furthermore, DNA methylation at 34 genes seems to be associated with the risk of CVD through mechanisms including inflammation, hyperlipidemia and oxidative stress.

4.1. Global DNA methylation

The results of the present review support global DNA methylation measured in LINE-1 repeats to be inversely associated with the risk of CVD, independent of established cardiovascular risk factors. LINE-1 methylation was used in several studies as a marker of global DNA methylation. Given that LINE-1 is the most common repetitive sequence in the human genome and one third of DNA methylation in the genome occurs in these elements, the use of LINE-1 methylation as a marker of global DNA methylation seems justified [17,50]. Moreover, LINE-1 methylation correlates with other methods including genomic 5-methyl cytosine content and luminometric methylation assay (LUMA) [18,51].

However, little is known about the biological function of LINE-1. The majority of LINE-1 copies are found to be inactive, however, multiple somatic cells express it which triggers senescence [52]. LINE-1 hypomethylation in the peripheral blood cells has been associated with diabetes, obesity, lower levels of HDL-cholesterol, elevated levels of total cholesterol and inflammation and a higher risk of metabolic status worsening [20,53–57]. Also, higher plasma glucose levels and blood pressure, along with greater risk for metabolic syndrome [58] have been reported to inversely associate with LINE-1 methylation levels in other tissues, such as visceral fat. All these suggest that LINE-1 hypomethylation is associated with an unfavorable cardiovascular risk profile [57,59]. Furthermore, it has been previously shown that suppression of LINE-1 expression improves the outcomes after MI by ameliorating post-ischemic functional recovery and decreasing infarct size through Akt/PKB signaling [60].

In contrast to studies that used LINE-1 as an index of global DNA methylation, other studies reported a higher degree of global DNA-methylation to be associated with CVD. These studies assessed global DNA methylation in other repetitive elements such as Alu repeats, and/or used other methods to asses DNA methylation rather than bisulphate pyrosequencing. LINE-1 and Alu repeats represent distinct measures of dispersed DNA methylation, and might have different functions [61]. The quantitative assessment of DNA methylation at ALU is about one-third to one-fourth of methylation at LINE-1, which may suggest that epigenetic changes at LINE-1 and ALU might measure different traits [61]. Moreover, the assay used and the source of DNA are important determinants in the interpretation of global DNA methylation patterns. For instance, global DNA methylation assessed by LUMA modestly correlates with LINE-1 methylation [62]. Furthermore, DNA methylation occurs throughout the genome in a sequence-context-dependent fashion, and the extent to which regional sequence context might affect different measures of DNA methylation is unknown. Finally, similar opposing effects between LINE-1 and Alu methylation are observed with cardio-metabolic risk factors and other diseases, such as cancer and Alzheimer [57,63–65].

The contradicting observations with different markers of global DNA methylation may raise a question on how functionally such a measure could be useful. It should be noted that methylation has a different effect depending on its position towards coding genes. Hypermethylation of the promoter CpG island is usually associated with gene transcriptional silencing and their hypomethylation of CpG islands is generally associated with increased gene expression [28]. In contrast, hypermethylation at the gene-body is associated with increased gene expression [66]. Therefore, global DNA methylation provides an oversimplified assessment of epigenetic dysregulation, as it neither quantitatively nor qualitatively acknowledges the co-existence of hypo- and hypermethylation within a gene or distinct genes within the same cell. Thus, further efforts are needed to dissect the molecular phenotype of these alterations, their link to disease processes and methodologies capable of distinguishing

differences in methylation extent from inherent genomic variability of these elements.

4.2. Epigenetic wide-association studies and candidate gene approach

Our study denotes 34 genes to be differentially methylated according to the presence of CVD. Among the 34 sites reported to be differentially methylated, the DNA methylation at F2RL3 and the risk of CVD mortality was the most consistently epigenetic association found in this review. Furthermore, 5 loci, including ABCA1, KCNQ1 and C1QL4 were validated. It is hypothesized that the epigenome regulates gene expression, cardiovascular risk factors and eventually risk of CVD [42]. Of note, these gene/genes regions are known to affect biological processes related to CVD, such as homeostasis, endothelial dysfunction, inflammation and oxidative stress. Also, some of these genes are implicated in lipid metabolisms. For example, ABCA1 gene is critical in promoting the efflux of cellular cholesterol and phospholipids onto small pre-beta 1 high-density lipoprotein cholesterol (HDL-C) practices and in converting them to larger alpha migrating HDL-C practices [67]. ABCA1 gene variants are associated with the levels of HDL-C and the risk of developing coronary heart disease [68]. Also, KCNQ1 gene encodes for a voltage-gated potassium channel required for the repolarization phase of cardiac action potential and mutations at this gene are an uncommon cause of atrial fibrillation [69]. Also, methylation of some of these genes has been associated with cardio-metabolic risk factors, e.g., epigenetic changes at the *ABCA1* gene promoter region contributes to the inter-individual variability in plasma HDL-cholesterol and methylation at *F7* and *TNF-α* promoter regions has been associated with plasma factor VII concentrations and body weight respectively [32,35,38]. The association between DNA methylation at F3RL3 and CVD mortality was beyond the traditional risk factors for CVD. Methylation at F2RL3 has been associated with smoking, a well-established risk factor for CVD [70]. Also, the gene product of F2RL3 is implicated in platelet activation, intimal hyperplasia and inflammation [71]. It could be expected that the identified regions constitute only a small fraction of the epigenome related to CVD. Further research is therefore needed to identify such regions and establish a cause-and-effect relation between methylation of these loci and development of CVD and elucidate the underlying mechanisms, including eventual possibilities of developing epigenetic risk assessments for CVD as well as prevention strategies.

4.3. Histone modifications

This review underscores a number of gaps in the literature concerning CVD and histone modifications, an important epigenetic mechanism that can be involved in CVD. Modifications in histone H3 in smooth muscle cells in atherosclerotic regions compared to normal arteries have been uncovered [72,73]. Also, recent studies have shown that histone modifications may have an impact in adipogenesis, energy homeostasis, inflammation and diabetes [74–77].

4.4. Study design, causality, bias and confounding

In the current investigation we found that epigenetic markers were associated with CVD; however, identifying causality is difficult due to the unstable nature of the epigenome and the cross-sectional design of the majority of studies included. In the current review, except for five studies, the epigenetic marks and CVD were assessed at the same time point, making it difficult to conclude whether specific epigenetic marks are a cause or consequence of CVD. Longitudinal studies with repeated measurements of DNA methylation before disease development and after it may help provide stronger evidence towards causation. Moreover, statistical approaches such as Mendelian Randomization may provide another opportunity to study the direction of causation from cross-sectional data, but this approach requires a large sample size. Moreover, the majority of the studies in our review lack the adjustment for basic covariates such as sex and age and also for the established cardiovascular risk factors. All the included studies, except one, were classified as low quality, mainly due to the lack of proper adjustment. Unlike genetic association studies which are resistant to confounding, controlling for different confounders and mediators is of importance in epigenetic analysis.

4.5. Strengths and limitations

The strengths and limitations of the findings from this study merit careful consideration. The present analysis, involving data from nearly 13,000 individuals, is the first systematic review on the subject that critically appraised the literature following an a priori designed protocol with clearly defined inclusion and exclusion criteria. However, as mentioned above, on the majority of studies included are cross-sectional assessments, making it difficult to conclude whether specific epigenetic marks are a cause or consequence of CVD. Also, the included studies were limited in sample size and while individual studies attempted to adjust for established cardiovascular risk factors, the levels of adjustment was inconsistent across the studies. Although every effort has been made to undertake a comprehensive search of the literature, we cannot exclude the possibility of publication bias from underreporting negative findings. Furthermore, a meaningful quantitative pooling of the existing data was unfeasible due to heterogeneity in the input parameters, assumptions and the study design.

4.6. Perspective and conclusion

The study of epigenetic markers is emerging as one of the most promising molecular strategies for risk stratification for complex disease, including CVD, and when implemented will have a sizable public health impact. Peripheral blood is easy to access and reflects multiple metabolic and inflammatory pathways [78,79]. Therefore, methylation profiling in peripheral blood to identify CVD-related methylated regions is of great interest since they would allow clinicians to identify high-risk individuals who may benefit from preventive and therapeutic interventions, promising high potential clinical utility [80]. Thus, large-scale epigenetic investigations are needed to characterize the identified site-specific DNA methylation in this review and other methylated sites that will predict CVD. Also, as epigenetic DNA modifications are potentially reversible and may be influenced by nutritional–environmental factors and through gene–environment interactions, future therapies targeting the epigenome can be a novel preventive strategy and treatment for CVD. For example, some studies show that supplementation with methyl donors such as folate, choline and vitamin B12 may influence DNA methylation and may have a beneficial effect on CVD risk, but results are still inconsistent [81–84]. Also, epigenetic drugs have been shown to successfully reverse several epigenetic marks and disease symptoms and have been approved by FDA for use in cancer [9]. Therefore, in the background of the high burden of CVD despite great advances in its prevention and treatment, transfer of these novel therapeutic avenues on the field of CVD should become a research priority in the future. However, epigenetic therapeutics should aim to modify various epigenetic elements in a complex and intricate cross-talk without disturbing further pathways. Also, to constitute potential therapeutic and preventive strategies in epigenetic medicine, in addition to folate, vitamin B6 and B12, a better understanding of other C1-metabolites (e.g., S-adenosylmethionine and S-adenosylhomocysteine) in CVD is of great interest in the forthcoming years. Also, because small differences in methylation values determine a diseased or disease-free state, the need for harmonizing an appropriate method for quantitative DNA methylation detection is critical. Only the application of sophisticated methods for analysis of DNA methylation, which may be standardized across different laboratories, will provide reliable data that may confirm the importance of epigenetics in CVD. Furthermore, future studies should explore further areas of epigenetic regulatory

mechanisms beyond DNA methylation, including histone modifications, which remain very poorly characterized in the context of CVD.

Competing interest

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Role of the funder/sponsor

Metagenics Inc. with the steering committee was involved in study design; collection, analysis, and interpretation of data; writing of the report; and decision to submit for publication. The funder/sponsor did not have the ability to veto publication of study results.

Contributors

The contributions of the authors are as follows: TM and OHF conceived and designed the study. TM, FK, EP and AO screened the titles/abstracts. TM obtained the full text, determined the eligibility of articles and participated in data extraction. FK and EP assessed the quality of the included studies. TM participated in data synthesis/analysis and interpretation of the data. TM, AD and OHF drafted the final manuscript. All authors contributed to the critical revision of the manuscript and approved the final version.

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Appendix A. Supplementary data

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