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Sexually Dimorphic DNA-methylation in Cardiometabolic Health: a Systematic Review

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ABSTRACT

Sex is a major determinant of cardiometabolic risk. DNA methylation (DNAm), an important epigenetic mechanism that differ between sexes, has been associated with cardiometabolic diseases. Therefore, we aimed to systematically review studies in adults investigating sex-specific associations of DNAm with intermediate cardiometabolic traits and incident cardiovascular disease including stroke, myocardial infarction (MI) and coronary heart disease (CHD). Five bibliographic databases were searched from inception to 15 July 2019. We selected 35 articles (based on 30 unique studies) from 17,023 references identified; including a total of 14,020 participants from European, North American and Asian ancestries. Four studies reported sex differences between global DNAm and blood lipid levels and stroke risk. In 25 genome wide and candidate gene approach studies, DNAm at 31 gene sites were associated with sex differences in cardiometabolic diseases. The identified genes were *PLA2G7, BCL11A, KDM6A, LIPC, ABCG1, PLTP, CETP, ADD1, CNN1B, HOOK2, GFBP-7,PTPN1, GCK, PTX3, ABCG1, GALNT2, CDKN2B, APOE, CTH, GNASAS, INS, PON1, TCN2, CBS, AMT, KDMA6A, FTO, MAP3K13, CCDC8, MMP-2* and *ER-α*. Prioritized pathway connectivity analysis associated these genes with biological pathways such as vitamin B12 metabolism, statin pathway, plasma lipoprotein, plasma lipoprotein assembly, remodeling and clearance and cholesterol metabolism. Our findings suggest that DNAm might be a promising molecular strategy for understanding sex differences in the pathophysiology of cardiometabolic diseases and that the future studies should investigate the effects of sex on epigenetic mechanisms in cardiometabolic risk. In addition, we emphasize the gap between the translational potential and the clinical utilization of cardiometabolic epigenetics.

Key words: DNA methylation; type 2 diabetes; coronary disease; myocardial infarction; stroke.

Introduction

Cardiometabolic diseases include cardiovascular diseases (CVD), type 2 diabetes (T2D) and their associated risk factors including components of the metabolic syndrome and obesity[1]. Aging is associated with development of unfavorable cardiometabolic profile which in large contributes to increased incidence of major cardiovascular events and mortality[2]. Intermediate cardiometabolic risk factors are unequally distributed among sexes, and sex differences are also described in cardiometabolic diseases prevalence, clinical characteristics and prognosis[3, 4],[5]. Generally, before menopause women have better cardiometabolic risk profiles than same aged men; however, this sex advantage gradually disappears with advancing age, particularly after menopause[6]. Mechanisms underlying sex differences in CVD have been extensively studies in the past two decades and signaling pathways including the epigenetic modifications of the genome emerged as possible pathway leading to sexual dimorphism in cardiometabolic diseases [7].

Epigenetics modifications comprise dynamic changes in the genome engaged in modification of important cellular processes such as gene expression, chromosomal stability, genomic imprinting [8, 9]. DNA methylation (DNAm) is the best understood and most extensively studied epigenetic mechanisms in regard to CVD risk [8, 9]. DNAm refers to the transfer of a methyl group onto the C5 position of the cytosine to form 5-methylcytosine (5mC) and increases or decreases in genomic 5mC are referred as DNA hyper- and hypomethylation respectively [8, 9]. The global DNAm assessed at long-interspersed nuclear element (LINE-1) was inversely associated with intermediate CVD risk factors and higher risk of metabolic status worsening[10]. Conversely, a higher degree of global DNA methylation measured at Alu repeats or by the LUMA method was associated with the presence of CVD[10]. Also, gene specific hyper- or DNA hypomethilation was associated with changes in gene expression and was shown to affect cardiometabolic risk including atherosclerosis, inflammation, blood pressure, serum lipid and glucose levels, subsequently leading to increased risk of developing T2D, stroke and myocardial infarction[11]. Also, sex-specific differences in DNAm have been found in brain, human pancreatic islets and blood[12, 13]. Men in general seem to have lower levels of methylation in their genome as compared to women[14, 15], indicating that similarly to sex chromosomes, methylation at the autosomes might be subject to sex differences. Despite this, only a relatively small number of studies in the ample field of cardiometabolic epigenetics stratify according to sex or focus in sex differences. Although a few studies[11, 16, 17] have summarized the existing literature on this complex topic, they did not focus on epigenetically induced sex differences in CVD, intermediate CVD risk factors or T2D, with the exception of a recent review that focused only on major CVD events[16].

Therefore, we aimed to systematically review the available evidence in human studies exploring the association between sex-specific DNAm and cardiometabolic diseases.

Methods

Data Sources and Searches

This review was conducted using a predefined protocol (which was not registered at online platforms) and following a recently published guideline on how to perform systematic reviews[18] and in accordance with PRISMA guidelines[19]. A literature search was done using 5 electronic databases (Medline ALL via Ovid (1946-current), EMBASE (1974-current) via embase.com, Web of Science Core Collection (1900-current), Cochrane CENTRAL registry of trials (issue 7 2019) via Wiley and Google Scholar) until 15 July 2019 (date last searched) with the help of an experienced medical information specialist (WMB). Details on the search strategy are provided in Supplemental Table 1. All references were imported in an EndNote library and deduplicated with the algorithm developed by Bramer et al[20]. Additionally, we searched the reference lists of the included studies and relevant reviews. Two independent reviewers screened the titles and abstracts of all studies identified initially, according to the selection criteria. Full texts were retrieved from studies that satisfied all selection criteria. All disagreements were resolved through consensus or consultation with two other independent reviewers.

1. *Study Selection*

Observational (cross-sectional, case-control, prospective) studies conducted in adults and investigating the associations of global or gene-specific DNAm with cardiometabolic outcomes were selected. Studies were included if they investigated sex-stratified associations between DNAm and intermediate cardio-metabolic traits (blood lipids, glucose, blood pressure, inflammatory markers, atherosclerosis, T2D) and CVD (MI, CHD, stroke). Also, we included studies that reported a significant interaction term with sex but did not stratify by sex in their analysis. Furthermore, studies conducted only in men/women were not included in the current review.

2. *Data Extraction*

A predesigned electronic data abstraction form was used to extract relevant information. This included questions on study location, percentage of men and women included in the study, participants' age, cardio-metabolic outcome, tissue type, DNAm technique used and general and sex-specific findings. Two authors independently extracted the data and a consensus was reached in case of any inconsistency with involvement of an additional author.

3. *Assessing the Risk of Bias*

Two independent investigators used the Newcastle-Ottawa Scale[21] to assess the risk of bias of the included observational studies. The Newcastle-Ottawa Scale uses a star system (maximum of nine stars) to evaluate three domains: selection of participants; comparability of study groups; and the ascertainment of outcomes and exposures of interest. Studies that received a score of nine stars were judged to be of low risk of bias; a score of seven or eight stars was medium risk and those that scored six or less were considered at high risk of bias.

4. *Pathway connectivity analysis*

To identify biological pathways of the differentially methylated genes previously linked to CVD, we used the CPDB (ConsensusPathDB-human) tool from the Max Plank Institute for Molecular Genetics[22]. This tool integrates interaction networks in humans (in this study) and includes information on binary and complex protein-protein, genetic, metabolic, signaling, gene regulatory and drug-target interactions, as well as biochemical pathways[22]. Data that explains interactions was derived from 32 public resources.

Results

1. *Search Results and Study Characteristics*

The search strategy identified 17,023 potentially relevant studies; after titles and abstracts were screened 16, 814 references were excluded (**Figure 1**). For the remaining 209 references, full-text articles were reviewed, 174 of which were excluded for various reasons outlined in **Figure 1**. A total of 35 articles based on 30 unique studies were included in this systematic review including a total of 14,020 non-overlapping participants, of whom approximately 53% were women. The studies included population with European (n=13), North American (n=3) and Asian (n=14) ancestries with age ranging from 32 to 75 years. Due to differences in the epigenetics marks and outcomes investigated, as well as different study designs of the included studies, we were not able to quantitatively pool the estimates from various studies. Therefore we report in this review a detailed descriptive summary of the available published literature. The characteristics of the included studies are described in **Table 1**. The median Newcastle-Ottawa quality score for the included studies was 7 of 9 possible points. The **Table 1** depicts the methodologic quality of all included studies.

The Role of Sex-Specific DNAm in Intermediate Cardio-metabolic Traits

2. *Blood Lipids*

Global DNA methylation is a frequent used marker for epigenetic screening since it captures the DNA methylation also at unknown genetic locations while the results of average DNA methylation correlate with the methylation of some trait-relevant genes[23, 24]. Ten articles[14, 25-33] investigated sex-specific associations between DNAm and blood lipid concentrations applying global (n=2), epi-genome wide (n=1) and candidate gene (n=7) approaches. In total 2,443 non-overlapping participants (1,174 women and 1,269 men) from USA, Canada, Finland, UK and China were included in these studies. The summary of the sex-specific findings is described in **Supplemental figure 1**.

Two cross-sectional studies[14, 25] investigating global DNAm and blood lipid levels reported sex differences. In the study conducted by Cash et al, LINE-1 methylation was significantly higher in men than in women, and among the entire sample, lower levels of *LINE-1* methylation was associated with higher levels of fasting low-density lipoprotein-cholesterol (LDL) and lower levels of fasting high-density lipoprotein-cholesterol (HDL)[14]. However, when stratifying by sex, the inverse association between global LINE-1 methylation, LDL and HDL remained significant only in men[14]. Malipatil et al, in their study reported that an increase of 10% in LINE-1 methylation was associated with decreased cholesterol/HDL ratio by 0.4 mmol/L in the overall sample of men and women. However, when stratifying by sex, the inverse association remained significant only in women[25]. In an EWAS performed by Garcia-Calzon et al, female samples displayed on average higher methylation in the X-chromosome, whereas males presented higher methylation in the autosomes. Further, women showed higher HDL levels, which were associated with higher *KDM6A* expression and epigenetic differences in human liver[31]. The results were not replicated. Further, the authors integrated DNA regulatory regions and other epigenetic factors for CpGs in the autosomal and X-chromosome based on sex ($q < 0.05$) for only four liver donors. Particularly, 42% of the autosomal CpG sites (13,817 CpGs) and 27% of the X-chromosome sites (2601 CpGs) differentially methylated by sex overlapped with histone marks related to active chromatin and enhancer regions (H3K4me1), whereas 14% of the autosomal sites (4760 CpGs) and 11% of the X-chromosome sites overlapped with histone marks related to heterochromatin (H3K27me3)[31].

In three candidate gene-studies *PTPN1*[32], *PLA2G7*[26] and *BCL11A*[27] DNAm was positively associated with serum lipids in women, but not in men. Another study reported that methylation at *ABCG1* was negatively associated with triglycerides in women only[28], whereas methylation at *LIPC* was negatively associated with triglycerides only in men[27]. This latter study also reported sex-specific associations for total cholesterol: whereas

ABCG1 was associated with triglycerides only in women, methylation at this CpG site was inversely associated with total cholesterol only in men[28]. For some CpG sites there were specific associations with HDL for males; methylation at *PLTP*[28], *CETP*[29], and *LIPC*[28] were negatively associated with HDL in men, but not in women. Moreover, a male-specific association was found between *GCK* CpG4 methylation at *GCK* and total cholesterol concentration[33]. However, a single study performed among 739 African Americans in the Genetic Epidemiology Network of Arteriopathy (GENOA) did not find overall or sex-specific significant associations between DNA methylation and lipid levels[30].

3. Blood pressure

Seven articles[25, 34-39] based on five unique studies reported associations between sex-specific DNAm and essential hypertension (EH), while one study investigated the cross-sectional association between DNAm and blood pressure[25]. Among these, five studies investigated candidate gene methylation[34-36, 38], while another study investigated epi-genome wide methylation[40] in regard to hypertension. Studies included 3,561 non-overlapping participants (2,029 women and 1,373 men) from China and UK. The summary of sex-specific findings is reported in **Supplemental figure 1**.

In the study conducted by Malipatil et al, in the overall sample of men and women, a 10% increase in LINE-1 methylation was associated with a 2.5 mmHg lower baseline diastolic blood pressure. The stratified analysis by sex did not show any significant influence of sex on this association[25]. One candidate gene study reported higher methylation levels of the two CpG sites at *SCNN18* in women compared with men as controls (CpG1: t=-2.283, P=0.025; CpG2: t=-2.568, P=0.012) and incident EH cases (CpG1: t=-2.694, P=0.009; CpG2: t=-2.583, P=0.011)[35]. However, for these two CpG sites no significant difference was observed between males and females in the prevalent cases group (CpG1: t=0.409, P=0.068; CpG2: t=0.621, P=0.536)[35]. These results indicated a significant association between EH and *SCNN1B* methylation, which was affected by age, sex and antihypertensive therapy. Similarly, in one other study, higher *ADD1* DNAm levels were observed in females as compared to males (CpG1: P = 0.016; CpG2-5: P = 0.021)[34]. Further, the study showed that lower *ADD1* CpG1 methylation levels were significantly associated with EH in females (cases versus controls (%, SD): 10.00±1.41 versus 11.36±3.63, adjusted P =0.042) but not in males (adjusted P = 0.133). In contrast, lower levels of *ADD1* CpG2-5 methylation were associated with an increased risk of EH in males (cases versus controls: 22.48% versus 31.86%, adjusted P =

0.008) but not in females[34]. The prediction potential of EH for *ADD1* CpG1 and CpG2-5 methylation levels was assessed by the ROC curves. CpG2-5 methylation was reported as a significant predictor of EH in males (area under curve (AUC) = 0.855, P = 0.001), while CpG1 methylation showed a trend toward being an EH indicator in females (AUC= 0.699, P =0.054)[34]. In the same population, *AGTR1* CpG1 methylation was a significant predictor of EH in both sexes[36] and hypomethylation of CpG3 site at *IL-6* promotor was significantly associated with EH risk in both, men and women. Further, sex-specific DNAm levels were observed only at CpG1 and CpG2 sites of *IL-6* promoter (males were hypomethylated as compared to females)[38]. Another study by Han et al, investigated the interactions between alcohol consumption and DNA methylation of the *ADD1* gene promoter and its association with EH, involving 2040 cases and controls[36]. The researchers concluded that CpG1 methylation was associated with EH in females while CpG2-5 methylation was significantly associated with EH in males, suggesting that these interactions in the *ADD1* gene promoter might play a role in modifying EH susceptibility[36]. Finally, Bao et al reported that hypomethylation of the *IFNG* promotor region was associated with a higher risk of EH. However, the authors did not observe any sex differences overall, except that in the control group DNAm levels were found to be higher in males when compared to females[39].

4. *Inflammation and Atherosclerosis*

Three articles[41-43], investigated the associations between epi-genome wide DNAm (n=2), candidate gene methylation and inflammatory markers. Also, we did not identify any study investigating the sex-specific role of DNAm in atherosclerosis. Studies in inflammatory markers included 2,771 non-overlapping participants (713 women and 317 men and one study did not report the number of men and women separately[41]) from Germany, China and USA. None of the epi-genome wide studies reported sex specific associations between global DNAm and inflammatory markers[41, 42]. Nevertheless, Guo et al, reported men-specific association between lower *PTX3* promoter methylation levels and higher neutrophil to lymphocyte ratio. Also, the level of *PTX3* promoter methylation in the coronary artery disease group (mean, SD: 62.69% ± 20.57%) was significantly lower than that of the group free of coronary artery disease (mean, SD: 72.45% ±11.84%), suggesting an important role of this gene in developing coronary artery disease[43] (**Supplemental figure 1**).

5. *Glycemic Traits and T2D*

Eight articles[12, 27, 32, 33, 44-47] reported sex-specific associations between DNAm and glycemic traits and T2D. Five studies were candidate gene studies and three were epi-genome wide studies. Among them, six studies

focused on DNAm and T2D, one investigated the association between DNAm and metabolic syndrome and another investigated insulin metabolism. Studies included 2,239 non-overlapping participants (353 women and 554 men, with one study not specifying the number of men and women[46]) from Israel, Spain, Sweden, China and USA. Summary of the sex-specific findings is shown in **Supplemental figure 1**.

In a case-control study including 1,169 individuals, individual methylation levels at the *FTO* gene showed that CpG sites in the first intron were slightly (3.35%) hypomethylated in T2D cases relative to controls[46]. The odds of developing T2D increased by 6.1% for every 1% decrease in DNAm. Men were hypomethylated relatively to women and the effect of DNAm was stronger in males compared to females (P =0.034 for sex interaction, AUC = 0.675 among men and 0.609 among women)[46]. Also, in another case-control study association between *PTPN1* promoter methylation and the risk of T2D was observed in the overall population and in females[32] .

In the study by Burghardt et al., a significant increase in *CDH22* gene methylation in subjects with metabolic syndrome was identified in the overall sample[44]. However, when investigating males and females separately; differential methylation levels were observed within the *MAP3K13* gene in females and the *CCDC8* gene in males with metabolic syndrome. In the validation sample a significant difference in methylation was again observed for the *CDH22* and *MAP3K13* genes, but not for *CCDC8* gene[44]. Another study investigated the impact of sex on the genome-wide DNAm pattern in human pancreatic islets from 53 males and 34 females, and relate the methylome to changes in expression and insulin secretion[12]. The study identified both chromosome-wide and site-specific sex differences in DNA methylation at the X chromosome of human pancreatic islets. However, the autosomal chromosomes showed differences in DNA methylation only on the level of individual CpG sites between sexes. Importantly, they found higher insulin secretion in pancreatic islets from females compared with males, as well as sex differences in gene expression[12]. Additionally, the authors did not find any difference in β-cell number between females and males. This could suggest that the DNA methylation differences seen between males and females might not be due to altered β-cell composition in the islets[12].

In a case-control study conducted by Rodriguez-Rodero et al[45], hypermethylation at CpG sites annotated to the HOOK2 gene was associated with the presence of T2D. Interestingly, when these results were analyzed by sex, female T2D samples were found hypermethylated at the cg04657146-region and the cg11738485-region of the *HOOK2* gene, whilst male samples were found hypomethylated in this latter region only[45]. Tang et al. reported a significant association only in males when investigating the overall *BCL11A* methylation in T2D patients[27].

While in another study among the same population, significantly elevated methylation levels of *GCK* CpG4 were observed in T2D patients than in the healthy controls. Also, this association was characteristic to males only[33]. Further, serum IGFBP-7 protein levels were similar among newly diagnosed and treated T2D patients and were not correlated with *IGFBP7* DNAm overall, but solely in males[47].

The Role of Sex-Specific DNAm in CVD

1. *Coronary Heart Disease*

Eight articles[48-55] investigated the associations between DNAm and CHD and MI. All studies applied a candidate-gene approach and included a total of 2,353 participants (1,010 women and 1,343 men) from China, Italy and the Netherlands. The summary of sex-specific findings can be found in **Supplemental figure 2**.

One study reported that a higher DNAm at the imprinted loci of *INS* and *GNASAS* was associated with the incidence of MI in women (INS: +2.5%, P=0.002; GNASAS: +4.2%, P=0.001)[48]. Hypermethylation at one locus and at both loci was associated with odds ratios (ORs) of 2.8 and 8.6, respectively (Ptrend=3.0×10−4) while no association was observed among men. Similarly, one study revealed a female-specific significant association between methylation at *PLA2G7* promoter and risk of CHD[49] and another reported a female specific association of *CDKN2B* methylation with CHD [women with CHD (mean, SD: 7.21±2.40%) compared with women without CHD. In contrast, four studies reported men-specific associations between DNAm of various genes and CHD[43, 50, 51]. Peng et al, reported significant associations of the methylated promoter of the *ABCG1* and *GALNT2* genes with an increased risk of CHD overall and among men only[50]. Also, CHD patients had significantly lower levels of *APOE* methylation than non-CHD controls. In addition, rs7412-T and rs7259620-A were protective factors for CHD in males (rs7412-T: OR=0.527, allele P=0.004; rs7259620-A: OR=0.743, allele P=0.029)[54]. Giannakopoulou et al reported a sex specific increased methylation in the *CTH* promoter gene in 34 patients who had coronary artery bypass surgery (CABG) (19.1%) compared to 16 control subjects (10.3%). Increased methylation levels were observed in male CABG patients compared to male control subjects while in females this was not observed[51]. Furthermore, Xu at al., showed that CHD cases had a significantly lower methylation level (mean, SD: 49.77 ± 6.43%) compared with controls, while there was no difference of *GCK* methylation level between males and females and no significant interaction between gender and disease[52]. However, a significant difference of the CpG2 methylation level with CHD was observed in males only[52]. On the other hand, one study evaluated the association between the DNA methylation profiles of genes involved in One-Carbon Metabolism (OCM) and the homocysteine (Hcy) pathway, with the myocardial infraction risk due to the low B-vitamins intake[53], based in

the rationale that B-vitamins and folates pathway may modulate DNA methylation[53]. The results from this study showed an inverse association between B-vitamins intake and the hypermethylation in three genes (*TCN2* promoter, *CBS* 50UTR, *AMT* gene-body) in male cases, as well as two genes (*PON1* gene-body, *CBS* 50UTR) in female cases[53].

2. *Stroke*

Four articles[15, 56-58] reported sex-specific associations between DNAm and stroke. Among these, three articles[56-58] used data from the same population for their analyses. Two studies applied global and two others candidate gene approaches. These studies applied a cross-sectional design, used blood samples and included a total of 1,045 non-overlapping participants (459 women and 586 men) from diverse ethnic backgrounds, such as Chinese-Taiwanese[56-58] and Spanish[15]. The summary of their sex-specific findings is shown in **Supplemental figure 2**.

Two of the studies used a candidate gene approach and performed pyrosequencing to assess methylation of the targeted regions in the gene promoter[57, 58], while the two other studies investigated the global DNAm of the LINE-1 elements using pyrosequencing[56] or luminometric methylation assay (LUMA)[15]. Further, two studies found a significant decrease in LINE-1 methylation in men compared to women in cases of stroke[15, 56]. One of them also reported that cases of ischemic stroke presented a lower methylation level compared to controls. In addition, this hypomethylation of LINE-1 in men was associated with an increased stroke risk by 1.2-fold after adjusting for risk factors, while no significant association was observed in women[56]. On the other hand, among the two studies investigating the estrogen receptor alpha *ERα*[57] and the matrix metalloproteinase-2 (*MMP-2*)[58] respectively, the methylation levels were lower in individuals with stroke compared to controls, in all the CpG sites analyzed in both studies. Further, when exploring the sex-specific associations, the two studies obtained contrary results. One study found a significant difference in methylation levels in all the investigated CpGs annotated to the gene *ERα* only between female cases and controls[57]. Whereas the other study reported a significant difference between the methylation levels in one out of eight CpGs annotated to *MMP-2* only between male cases and controls[58]. None of these studies investigated the difference between male and female cases.

Genes, pathways and cardiovascular disease

Studies included in this systematic review report that methylated CpG sites annotated to *KDM6A, PLA2G7, CETP, ABCG1, LIPC, BCL11A, ADD1, CNN1B, HOOK2, PLTP, GALNT2, PON1, TCN2, CBS, AMT, CTH, INS, GNAS-AS1, MMP2, CCDC8, MAP3K13, FTO, ESR1, CDKN2B, APOE, IGFBP7, PTPN1, GCK* and *PTX3* were differently methylated for men

and women. An overview of these genes, function and their sex specific methylations is provided in **Table 1** and **Supplemental table 2**. In addition, **Figure 2** shows the prioritized pathway connectivity between cardio-metabolic genes that were found to be differentially methylated in men and women. The most significant pathways, that in **Figure 2** are shown with darker red nodes with more representative enrichment include the Vitamin B12 Metabolism, Statin Pathway, Plasma lipoprotein, Plasma lipoprotein assembly, remodeling and clearance and Cholesterol metabolism. Hence, the most important genes connecting these pathways that merit further consideration were: *ABCG1, APOE, PLTP, LIPC, CETP, CTH* and *INS*. Overall, the majority of the genes reported in this review were previously known to be associated with CVD risk factors or CVD outcomes.

Discussion

In this review, we systematically summarized the current evidence on sex differences in DNA methylation in relation to cardiometabolic diseases. We included 30 unique studies that had either stratified their analyses by sex and/or specified that their results did not differ among sexes by testing for statistical sex-interaction. Overall, our findings indicate that global DNAm might influence cardiometabolic risk in a sex-specific manner, and that DNAm at 31 gene sites could be differentially associated with various cardiometabolic traits in men and women.

1. Global DNA methylation

We identified four studies suggesting that altered LINE-1 DNAm may play a role in CVD risk in a sex-specific manner: (i) DNAm measured in LINE-1 repeats was inversely associated with different serum lipids in men and women[14, 25], (ii) decreased LINE-1 was associated with higher stroke risk in the overall sample and in men[56] and (iii) DNAm measured using genomic 5-methyl cytosine content and LUMA indicated hypomethylation in male as compared to female stroke cases[15].

In the current review, global DNA hypomethylation was associated with poorer outcomes. In particular, global DNA hypomethylation was associated with higher LDL and lower HDL levels in the overall sample and in Samoan men, but not in women[14] and increased stroke risk in Chinese men but not in women[53]. These findings are in line with observations in healthy Caucasian men where subjects with decreased LINE-1 methylation were more likely to develop ischemic heart disease and stroke (women were not included in this study)[59]. Global DNA hypomethylation has been previously reported in stroke patients as compared with healthy[59] and in a large study with participants from European ancestry, decreased global DNAm was observed in male as compared to

female stroke cases[56]. In experimental studies, global DNA hypomethylation has been shown to precede the formation of atherosclerosis in Apoe-/- mice, and has been associated with hyperhomocysteinemia and aortic lipid deposition in mutant mice deficient in methylenetetrahydrofolate reductase[60]. While in humans global loss of DNAm has been previously associated with atherosclerosis in both, atherosclerotic lesions[61] and peripheral blood leukocytes[62] but also with blood lipids, inflammation and blood pressure[10] implying that LINE-1 hypomethylation could be associated with an unfavorable cardiovascular risk profile. Global DNAm is considered a robust measurement of the overall genomic methylation which is reported to be one of the earliest molecular changes in the transition of a cell from a normal to a diseased state[63]. Blood DNA hypomethylation might be easily measured and could be used to identify people at risk of cardiovascular events. Our findings emphasize the need of sex-specific approaches when further exploring the possible role of global DNAm as a biomarker and potential intervention target in cardiometabolic diseases.

2. *Epigenome wide-association studies and candidate gene approach*

We identified only six studies[12, 31, 41, 42, 44, 46] that investigated differentially methylated regions in the genome with cardiometabolic diseases in a hypothesis-free approach. Among these, three EWAS[12, 31, 44] reported sex-specific associations, and *KDMA6A, FTO, MAP3K13* and *CCDC8* were some of the important genes that were found to be methylated in a sex-specific manner with blood lipids and glycaemia traits. Among 25 candidate gene studies, 22 studies reported sex-specific associations between DNAm and cardiometabolic diseases at the following genes *PLA2G7, BCL11A, KDM6A, LIPC, ABCG1, PLTP, CETP, ADD1, CNN1B, HOOK2, GFBP-7,PTPN1, GCK, PTX3, ABCG1, GALNT2, CDKN2B, APOE, CTH, GNASAS, INS, PON1, TCN2, CBS, AMT, MMP-2* and *ER-α*. Based on the prioritized pathway connectivity analysis, although, limited, the evidence suggests an involvement of biological pathways related to vitamin B12 metabolism, statin pathway, plasma lipoprotein, plasma lipoprotein assembly, remodeling and clearance and cholesterol metabolism, with sex differences in cardiometabolic diseases (**Figure 2**). Some of the most relevant genes from the pathway analysis were *ABCG1, APOE, PLTP, LIPC, CETP, CTH* and *INS*. Overall, these genes have been associated to cardiometabolic outcomes, however little evidence links them to epigenetics and cardiometabolic diseases and even less to sex differences in cardiometabolic diseases.

ABCG1 gene is a transmembrane cholesterol transporter that effluxes cellular cholesterol from macrophages by delivering cholesterol to mature high-density lipoprotein particles. Beyond a role in cellular lipid homeostasis, ABCG1 equally participates to glucose and lipid metabolism by controlling the secretion and activity of insulin and

lipoprotein lipase. Moreover, there is a growing body of evidence suggesting that modulation of *ABCG1* expression might contribute to the development of diabetes and obesity[64], which are major risk factors of CVD. The *ABCG1, GALNT2* and *HMGCR* genes have been previously associated with pathogenesis and progression of CHD through manipulating the various lipid pathways[65, 66]. In the current review, hypermethylation of these three genes was associated with higher levels of total cholesterol and LDL , and increased CHD risk in men[50], while it was linked to higher levels of TG in women but not with risk of CHD[7]. The expression of *ABCG1* gene reduces cholesterol accumulation in macrophages by promoting the transfer of intracellular cholesterol into HDL pathway[67]. In contrast to this, hypermethylation at *PLA2G7* was associated with levels of total cholesterol, triglycerides and ApoB in females but not in males, and also only female CHD cases were hypermethylated as compared to controls[5]. *PLA2G7* is the coding gene for Lp-PLA2 whose abnormal activity can cause high risk of CHD and may serve as a diagnostic marker for CHD[68]. Therefore, the sex disparities in the *ABCG1* and *PLA2G7* methylation may have an effect in the molecular mechanisms underlying the sex-specific pathophysiology of CHD and may provide epigenetic clues to explain the inconsistency in the epidemiological studies. However, both studies were done in Han Chinese population, and sample size was rather small (only 85 CHD patients and 54 participants without CHD[50] and 36 CHD cases and 36 controls). Hence, further replication studies with larger sample size and in different ethnic populations are required to confirm these findings.

The *APOE* gene encodes apolipoprotein ε (ApoE), a protein that associates with lipids to form lipoproteins that package and traffic cholesterol and lipids through the bloodstream and has been linked with numerous physiological conditions, including healthy aging[69], cardiovascular disease[70], diabetes[71] and cognitive function[72]. One study using samples of 563 blood-bank donors, found that 1% of the inter-individual variation in plasma ApoE levels was attributable to variation of age and sex[73]. Researchers from the ApoEurope project, reported a sex-differential effect of age on mean levels of ApoE[74]. In men, the levels of ApoE levelled off after the age of 45 years, whereas they continued to increase in women[74].

PLTP (phospholipid transfer protein) is essential for the transfer of excess surface lipids from TG-rich lipoproteins to HDL particles. PLTP-mediated phospholipid transport among HDL particles is also known to be associated with HDL particle size and lipid composition. Sex disparities for HDL levels associated with *PLTP* have been previously reported[75]. In the PAGE study, the major allele of rs7679 was associated with higher HDL levels in women only. The locus with the most consistent evidence for sex disparities across the studies is *LPL*, or lipoprotein lipase. Different SNPs in this gene exhibited sex disparities for HDL levels in two prior studies, with a larger effect in

males[76, 77]. In two other studies, *LPL* exhibited sex differences for TG levels, also with a stronger effect in males[75, 78]. LPL is the rate-limiting enzyme for hydrolysis of triglycerides in lipoproteins and polymorphisms and mutations in LPL have been associated with lipid metabolism disorders. Hormone levels have been shown to affect regulation of LPL, including thyroid hormone, estrogen, and testosterone[79], which could possibly and partially explain the observed association in cardiometabolic diseases.

Although these pathways and the respective reported genes need further investigation, confirmation and translational research, the current evidence suggests they could be biologically relevant and could hold the key for future drug discovery, diagnosis and treatment of cardiometabolic diseases overall and in a sex specific manner. Determining the relationships between genes is essential for molecular biology and medicine. These relationships often cluster together into functional and disease pathways, and the characterization of these pathways is necessary to improve disease classification, patient stratification and, ideally, personalized treatment[80].

3. Epigenetic mechanisms in biological processes of sex differences

Sex differences in pathophysiology of cardiometabolic diseases could be attributed to several gender and sex-specific factors[81]. Lifestyle factors (smoking, diet, stress) can determine gender differences by modifying cardiometabolic risk directly, and they can also modify the epigenetic marks in a sex-specific manner leading to sex differences in cardiometabolic diseases[81](**Figure 2**). Sex differences may also be driven by biological dissimilarities rather than different environmental exposures among men and women[81]. In particular, the major mechanisms by which sex might influence cardiometabolic diseases epigenetics may include: (i) the genomic and non-genomic effects of steroid hormones and their receptors on DNAm enzymes, histone modifiers and miRNAs, (ii) genomic imprinting, leading to DNAm of either maternal or paternal alleles and (iii) increased expression of X-chromosomal escape genes in women targeting epigenetic modifications and the expression of non-pseudo-autosomal Y-chromosomal epigenetic modifiers in men[16] .

Sex hormones have been extensively studied in the past decade in regard to cardiometabolic diseases due to the better cardiometabolic profiles in women as compared to their male counterparts. In the current review, we found some implications for the interactions between sex hormones and methylation in modifying sex differences cardiometabolic diseases. Three[15, 57, 58] of the studies included in the current review, investigating epigenetic

changes and stroke and reporting differences between men and women, highlight the importance of sex hormones and their receptors. Using a global DNA methylation approach, Soriano-Tarraga et al[15], found that global hypomethylation was independently associated with stroke subtypes only in females. Moreover, there was an association between lower *ERα* methylation levels and large-artery and cardio-embolic stroke subtypes in women, while in men this association was not observed. It might be that women suffering from a major ischemic stroke may cause a more significant change in *ERα* methylation levels to reduce the brain injury[57]. In line with this, differential DNAm profiles mediated sex differences in the endogenous neuroprotective response to middle cerebral artery occlusion (MCAO) in mice were reported. In female mice, MACO induced selective demethylation of the ERα gene promoter, leading to the increase in ERα expression[82]. Also, sex differences were observed in *MMP-2* methylation, with expression of *MMP-2* being closely related to sex hormones[58]. Males with small-vessel ischemic stroke had lower methylation levels at all *MMP-2* CpG sites, while no association was observed in women[58]. Further exploration of the underlying mechanisms is needed. Sex- and stroke-subtype-specific effects must be taken into consideration when investigating potential strategies to alter the activity of *MMP-2* in patients with ischemic stroke. Steroid hormones can induce, among others, modification of histones. Androgen or estrogen receptors act by binding to hormone response elements in the DNA and attract various cofactors that have inherent histone acetyltransferase or methyl transferase activity. This is particularly known for the *CREB* binding protein and E1A binding protein p300[83]. The histone-modifying enzymes alter the epigenetic state of gene promoters to which the nuclear receptors bind, thereby changing gene expression.

It is known that DNAm contributes to X-chromosome inactivation in females[84], and findings by Garcia-Calzon et al, demonstrated that DNA methylation in the X-chromosome in human liver mirrors the methylome in other human tissues[31]. They reported higher average degree of X-chromosome methylation in females than in males with 37% of the significant sites on the X-chromosome having higher methylation in males[31]. Around 95% of the CpG sites in the X-chromosome that had differential DNAm in human liver between sexes also had different methylation levels between males and females in pancreatic islets and brain independently of the clinical characteristics of the population[12, 85]. Further, they identified four genes on the X-chromosome with large differences in DNA methylation between males and females and being more expressed in liver from females than males: *XIST*, *ARSE*, *RPS4X*, and *KDM6A*[31]. Also, higher *ARSE* and *RPS4X* mRNA expression has been found in pancreatic islets, and higher *XIST* and *RPS4X* mRNA expression was also found in brain from females compared with males[15,][85]. These differences in gene expression in several tissues may explain some metabolic differences

between males and females. Interestingly, these four genes are known to escape X-chromosome inactivation[86-88]. In this study serum HDL levels were positively associated with *KDM6A* mRNA expression in human liver in addition to higher serum HDL levels and higher *KDM6A* expression in females. Also, silencing *KDM6A* in hepatocytes resulted in lower HDL levels and lower expression of key genes encoding proteins that regulate HDL levels, supporting the direct contribution of *KDM6A* in the differences found in HDL levels between males and females[31].

4. Potential clinical implications and recommendations for future research

Although the clinical use of epigenetic marks in the field of cardiometabolic diseases is still in its infancy this is not the case with cancer research. Molecular risk stratification using (epi)genetic marks have been focused on identifying molecular features associated with clinical outcome and have applied them to patients' risk stratification and treatment guidance[89, 90]. Such results indicated that a gene expression score that incorporates prognostic genetic and epigenetic information could be used as a model for treatment response but also for risk stratification and early disease detection. In particular, sex-specific epigenetic marks against or as a supplement to existing risk scores (such as the Framingham Risk Score[91]) may be of added value when predicting the risk of cardiometabolic diseases. This is also supported by our findings suggesting that for cardiometabolic traits epigenetic markers may not be equally good predictors in men and women, emphasizing the role of sex in epigenetic patterns of cardiometabolic diseases. Further, as sex is one of the strongest predictors of treatment response, the epigenetic signatures may be used as markers to indicate the successfulness of pharmacological or dietary/lifestyle interventions in cardiometabolic diseases among sexes. Given the lack of sex-stratification in studies focusing on epigenetic mechanisms and the fact that the majority of the studies were focused to epigenetic changes in autosome chromosomes in regard of cardiometabolic diseases, our review underscores the emerging need for future studies to investigate the influence of sex on epigenetic mechanisms in cardiometabolic diseases. In complex phenotypes such as cardiometabolic diseases, the collection of high-quality blood samples and metabolically active tissues could provide the basis for the creation of large data sets that should accurately incorporate the many sources of variability (age, sex/gender, race/ethnicity). In particular, future prospective observational studies should aim to explore the role of sex when studying the associations between epigenetic marks and mechanistic pathways of cardiometabolic diseases by stratifying their analyses by sex and comparing male and female participants. Second, studying the associations between DNAm and intermediate CVD risk factors

is valuable, however, from the clinical perspective, the value of DNAm as a biomarker of the risk factor is as good as the intermediate risk factor itself. Therefore, as we did not identify studies focusing on outcomes such as stroke or myocardial infarction, it might be of great value for future research to investigate the rol of sex on the epigenetics determinants of stroke and myocardial infarction. Third, potential biological mechanisms underlying sex-specific associations should be further explored in an experimental setting. It is now known that sex differences in morphology and in response to stress exist also in cellular levels[92-94]. Therefore, when translating the observational findings into experimental settings a clear distinction between male and female animal models or cell cultures is of high importance in order to obtain non-biased results on the sex-specific pathophysiology of cardiometabolic diseases.

5. Strengths and limitations

In this systematic review on sexually dimorphic DNAm, we critically appraised the literature following an a priori designed protocol with clearly defined inclusion and exclusion criteria using a comprehensive literature search in five databases. While previous systematic reviews on the topic are limited only to major CVD outcomes[16], our study took in consideration a broad range of cardiometabolic risk factors and diseases. However, the limitations of the findings from this study merit careful consideration. The included studies in this review were limited in sample size and the majority of studies included were cross-sectional assessments, making it difficult to conclude whether DNAm patterns are a cause or consequence of cardiometabolic changes. In addition, the results of some of the studies need cautious interpretation when it comes to the biological or functional relevance of their findings. Even though a study may report a significant difference in DNAm the biological relevance of small differences could be likely minimal and unknown. Studies investigating associations between metabolic syndrome and DNAm also need to be interpreted with caution given the heterogeneity of metabolic syndrome and that the subjects may or may not have dyslipidemia, elevated BP, and hyperglycemia. Therefore interpreting associations between changes in DNAm and subjects classified as having metabolic syndrome is Moreover, although individual studies attempted to adjust for established CVD risk factors, adjustment levels were inconsistent across the studies. Also, DNAm patterns reported in blood samples may not mirror the methylation patterns in the relevant targeted tissues. Further, we did not perform the search for non-coding microRNAs and histone modifications because the scope of our search was DNA methylation. Given the importance of microRNAs and histone modifications as epigenetic mechanisms, future systematic reviews and meta- analyses on microRNAs, histone modifications and

sex differences in different types of cardiovascular tissues would be an added value on the topic. Moreover, we hand searched relevant reviews and references of studies included in the current review in order to minimize the possibility of missing important studies. Also, we cannot exclude the possibility of publication bias from underreporting negative findings. Lastly, a meaningful quantitative pooling of the existing data was unfeasible due to the heterogeneity in the input parameters, assumptions and study design.

6. Conclusions

Although a growing body of evidence suggests biological, genetic and epigenetic sex differences in cardiometabolic diseases, only a small number of studies in the field stratify or present their results by sex. Nevertheless, the cumulative evidence from the studies that reported sex-based results, suggest that epigenetic changes in specific individual genes might be differently associated with cardiometabolic traits in males and females, encouraging further and larger-scale investigation. Robust, replicable results from carefully designed studies have the potential to uncover the molecular biological processes involved in disease onset and progression. In addition, future studies should help characterize gene regulatory effects of non-coding genetic variations, and, hopefully, give indications into disease-relevant biological pathways which could be addressed by preventive or therapeutic interventions. Clearly, a considerable amount of functional work is required in the future to expand our field of view beyond the classic biological mechanisms involved in sex differences of cardiometabolic diseases, and that could be important to design new drugs that target sex-specific mechanisms and permit more precise and efficient care.

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Author's contribution

Study concept and design: TM, MG and OHF; Data search strategy: WMB; Literature search, acquisition, collection, interpretation of data and drafting the manuscript: EA, COR, XZ, JN, EPF, KVEB, VGJ, TM and MG. Critical revision of the manuscript for important intellectual content: M. Ghanbari, TV, WA, VH and OHF. Study supervision: TM and MG; All authors approved the final version of the manuscript.

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