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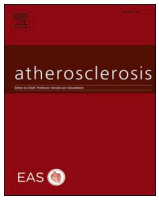

Review article

Sex differences in the genetic and molecular mechanisms of coronary artery disease

Tim R. Sakkers [a], Michal Mokry [a,b], Mete Civelek [c,d], Jeanette Erdmann [e], Gerard Pasterkamp [b], Ernest Diez Benavente [a], Hester M. den Ruijter [a,*]

[a] Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht University, Heidelberglaan 100, 3508, GA, Utrecht, the Netherlands
[b] Central Diagnostic Laboratory, University Medical Center Utrecht, Utrecht University, Heidelberglaan 100, 3508, GA, Utrecht, the Netherlands
[c] Center for Public Health Genomics, University of Virginia, 1335 Lee St, Charlottesville, VA, 22908, USA
[d] Department of Biomedical Engineering, University of Virginia, 351 McCormick Road, Charlottesville, VA, 22904, USA
[e] Institute for Cardiogenetics, University of Lübeck, Ratzeburger Allee 160, 23562, Lübeck, Germany

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ABSTRACT

Sex differences in coronary artery disease (CAD) presentation, risk factors and prognosis have been widely studied. Similarly, studies on atherosclerosis have shown prominent sex differences in plaque biology. Our understanding of the underlying genetic and molecular mechanisms that drive these differences remains fragmented and largely understudied. Through reviewing genetic and epigenetic studies, we identified more than 40 sex-differential candidate genes (13 within known CAD loci) that may explain, at least in part, sex differences in vascular remodeling, lipid metabolism and endothelial dysfunction. Studies with transcriptomic and single-cell RNA sequencing data from atherosclerotic plaques highlight potential sex differences in smooth muscle cell and endothelial cell biology. Especially, phenotypic switching of smooth muscle cells seems to play a crucial role in female atherosclerosis. This matches the known sex differences in atherosclerotic phenotypes, with men being more prone to lipid-rich plaques, while women are more likely to develop fibrous plaques with endothelial dysfunction. To unravel the complex mechanisms that drive sex differences in CAD, increased statistical power and adjustments to study designs and analysis strategies are required. This entails increasing inclusion rates of women, performing well-defined sex-stratified analyses and the integration of multi-omics data.

1. Introduction

Sex differences in the clinical presentation of coronary artery disease (CAD) and its associated risk factors have been known for many decades, and sex differences on CAD incidence, prevalence and mortality are well documented [1,2]. Generally, women present with CAD 10 years later than men, leading to an increased prevalence of comorbidities and risk factors at the time of diagnosis [3,4]. Although most traditional risk factors are shared between both sexes, smoking, diabetes and psychosocial factors tend to have a stronger association with CAD in women [5–8]. Furthermore, while men typically experience chest pain as a primary symptom, women present with a broader range of symptoms, including pain between the shoulder blades [9].

More recently, (semi)quantitative histological assessments of atherosclerotic plaques have offered important insights into sex differences in plaque phenotype and composition [10–14]. Typically, atherosclerotic plaques found in women are fibrous, whereas those in men tend to be more atheromatous. To what extent these phenotypic differences are driven by (epi)genetic and transcriptional regulation remains to be defined. We hypothesize that sex differences in genetic and molecular mechanisms are part of specific biological processes acting in different cell types which relate to atherosclerosis. In this review, we describe sex-specific mechanisms in atherosclerosis and CAD using different layers of omics data. We highlight sex differences in genetics using genome-wide association studies, elaborate on the fast-evolving field of epigenetics and explore sex-differences in plaque biology through transcriptome analyses and state-of-the-art single-cell RNA data. Identifying these genetic and molecular mechanisms would provide valuable targets for future research and inspire new therapies that benefit both men and women.

* Corresponding author. Division of Heart & Lungs Department of Cardiology Laboratory of Experimental Cardiology, Huispostnummer G.02.5.23, P.O. Box 85500, 3508, GA, Utrecht, the Netherlands.
E-mail address: H.M.denRuijter-2@umcutrecht.nl (H.M. den Ruijter).

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2. Sex differences in CAD and atherosclerosis

2.1. Presentation, risk factors and prognosis

Cardiovascular disease (CVD) is the leading cause of mortality worldwide among men and women reaching 18.6 million deaths in 2019. Among all cardiovascular disorders, coronary artery disease (CAD) is the most prevalent [1,15,16]. In European Society of Cardiology (ESC) member countries, CAD accounts for 45% of these deaths in women and 39% in men [17]. Symptoms of CAD have been described to differ between sexes. Men typically present with chest pain, while women have more symptoms overall. Although chest pain remains the most prevalent symptom in women, they also often present with pain between the shoulder blades, neck and jaw [9]. Nausea, vomiting, and fatigue are also more common in women. Generally, CAD develops 5–10 years later in women than in men, resulting in more comorbidities and cardiovascular risk factors at the moment of presentation [3,4]. Although most traditional risk factors are shared between sexes, smoking, diabetes and psychosocial factors are more strongly associated with CAD-related adverse events in women [5–8]. In addition, there are sex-specific risk factors such as hypertensive pregnancy disorders (pre-eclampsia and gestational hypertension) and menopause, which are unique to women [18–22]. Despite an encouraging trend of decreasing CAD death rates in the past decades, women suffer from worse short- and long-term prognoses, such as higher mortality rates after myocardial infarction (MI) than men (adjusted mortality: ~24% in women vs. ~21% in men (1992), ~19% in women vs. ~16% in men (2010)) [1,23–27]. This is likely explained, at least in part, by substantial differences found in total time to treatment for women with CAD, in which delayed seeking for medical care plays an important role [28]. These topics are discussed also in 2 reviews of this special issue [29,30]. Many academic papers, including those cited in this review, misuse the term ‘gender’ when referring to ‘sex’ differences. We would like to emphasize the importance of using accurate terminology in literature when discussing sex and gender differences (Box 1).

2.2. Atherosclerotic plaque biology

CAD is most often caused by atherosclerosis, the buildup of plaques in the vessel wall, characterized by chronic inflammation and accumulation of lipids. Sex differences in atherosclerosis is a rapidly evolving field, with growing interest in unraveling and characterizing the exact composition of the female and male atherosclerotic plaques [30,38]. In general, women have smaller and more fibrous atherosclerotic plaques, while plaques in men are found to be more atheromatous. Plaques in women are described as stable plaques which are fibrous plaques with high collagen and smooth muscle cell (SMC) content, in which phenotypic switching of SMCs plays an essential role [10–14]. Yet, stable plaques can become symptomatic by mechanisms that promote plaque erosion, and thrombi are formed on intact stable plaques (referred to as white thrombi [39]) [24,25]. On the other hand, atheromatous plaques present with more inflammatory cells, calcification, lipids and hemorrhage, making them more likely to rupture [10–14]. This difference in plaque composition facilitates a distinct pathophysiological process in thrombosis, where thrombus formation mainly happens on ruptured plaques (referred to as red thrombi [39]) [13,40,41]. Disparities in time to treatment might be related to plaque morphology. Analyses of coronary plaques retrieved from sudden death victims highlighted that blood vessels containing ruptured plaque are typically more severely narrowed than eroded plaques, which suggests that these patients are more likely to confer with early presentation of CAD-related events [42].

2.3. Sex chromosomes and hormones

In human biology, sex differences arise from a complex interplay between sex chromosomes, sex hormones and environmental factors, which cause differential activation of molecular mechanisms and therefore, can influence the phenotypic presentation of CAD and atherosclerosis. An extensive review on the biological significance of sex chromosomes and the mechanistic effect of sex hormones is beyond the scope of this review and has been comprehensively described elsewhere [43–45]. This topic is also discussed in a review of this special issue [46].

In brief, sex is determined by the combination of sex chromosomes (X and Y), where females have two X chromosomes and males have one copy of both. However, other combinations are possible, like in Turner syndrome (45,X) and Klinefelter syndrome (47,XXY), although these syndromes are rare [43]. These chromosome X aneuploidies have been strongly linked to an impaired cardiometabolic profile [43]. The Y chromosome contains the sex-determining region (SRY gene), which encodes the testis determining factor, a transcriptional regulator important for initiating male sex determination and development. Both X and Y chromosomes contain various protein-coding genes, which are known to be important regulators across many biological processes. To compensate for the unequal expression of X-linked genes between women (XX) and men (XY), one copy of the X chromosome is silenced through X-inactivation, which starts in female embryogenesis. However, about 15% of X-linked protein coding genes escape inactivation and are, therefore, overexpressed in women, making these genes important targets to consider when studying sex differences [43,44]. These gene escapees have been implicated in the prevalence of autoimmune diseases among women with female-to-male ratios of 9:1 in systemic lupus erythematosus (SLE) [47]. Studies on the Y chromosome have mostly

Box 1
Differences between sex and gender in cardiovascular research.

When addressing disease prevalence, presentation, risk factors, diagnostic evaluation and overall health outcomes, it is important to consider both sex and gender [31]. Sex and gender are distinct but interrelated concepts in biology and disease [32,33]. Sex is a biological and physiological variable that is genetically determined. Gender, on the other hand, is a multidimensional cultural concept that refers to socially constructed norms, behaviors, and expectations. Sex and gender play a pivotal role in cardiovascular research. Firstly, women are underrepresented in most cardiovascular studies and (pre)clinical trials, including diagnostic research, which might be caused by gender differences in health-seeking behavior and their perception of complaints [34,35]. In addition, differences in risk factor profiles related to gender (e.g. smoking) may have epigenetic effects that induce changes in gene expression, a mechanism usually associated with sex [36,37]. At the same time, we describe distinct plaque phenotypes and thrombus etiology between men and women. These are biological differences related to sex. This suggests that men and women should not only be represented equally but also studied separately by sex in cardiovascular research.

Thus, when designing studies in the cardiovascular field, or any other field for that matter, researchers should be intentional in the inclusion of both sex and gender. This review focuses on differences in genetics and will therefore refer to ‘sex’ as a core variable. Nevertheless, we acknowledge the interplay with gender when we discuss epigenetics and transcriptomics.

focused on Loss of chromosome Y (or LOY), a type of mosaic aneuploidy. This describes a phenomenon where cells possess an abnormal number of chromosomes, losing the Y chromosome, which was first identified several decades ago. LOY increases with age and is the most commonly occurring mutation in a male's genome. With the possibilities of using genetic data to determine LOY in DNA from blood, several groups have published its importance for many diseases such as Alzheimer's and cardiovascular diseases [48,49]. Recent studies have identified an immune regulatory function for the Y chromosome [50]. Given the involvement of immune cells in atherosclerosis, LOY was assessed in patients undergoing carotid endarterectomy. Out of 366 men with severe atherosclerosis, 61 exhibited some degree of LOY in blood, which was shown to predict secondary major cardiovascular events (MACE) [51].

As for the sex hormones, estrogens (e.g. estradiol) are responsible for regulating the female reproductive system and secondary characteristics, while androgens (e.g. testosterone) are responsible for male sex development. Their regulation happens through estrogen and androgen receptors, which are expressed in both sexes and typically in all cardiovascular tissues [43]. Estradiol reduces SMC proliferation and oxidative stress, while estradiol and testosterone inhibit vasoconstriction and inflammation, by independent mechanisms [43–45]. The effects of sex hormones on endothelium are discussed also in [52]. Furthermore, estradiol and testosterone have been associated with improved blood lipid profile [43,53]. Excessive testosterone levels, however, seem to have the opposite effect. On the other hand, a low testosterone/estradiol (T/E2) ratio was found to associate with increased plaque and systemic inflammation, and future outcome in men with severe atherosclerosis [54]. However, low T/E2 ratio showed a limited effect on gene expression of late-stage atherosclerotic plaques in the same patients [55]. Overall, estrogens have a protective effect on atherosclerosis, which partially explains the lower incidence of CVD observed in premenopausal women. Conversely, the increased risk of CVD during menopause might be attributed, in part, to a sudden drop in estrogen levels [56]. Although estrogen replacement therapy may have a beneficial effect on CAD risk in postmenopausal women [57], contradictions are found related to thrombosis risk and unimproved lipid composition [43]. Therefore, timing of postmenopausal estrogen therapy is crucial [58].

3. Genetics

3.1. Exclusion of sex from GWAS

In the past fifteen years, genome-wide association studies (GWAS) of CAD have substantially increased our knowledge about the genetic landscape underpinning this complex disease. The goal of GWAS is to identify genetic variants, or single nucleotide polymorphisms (SNPs), that associate with a particular trait or risk of disease. Two recent large CAD GWAS by Tcheandjieu et al. [59] and Aragam et al. [60] included over a million participants each. These large cohorts have enabled the identification of more than 300 genetic risk loci for CAD [61]. This has, in turn, allowed the identification of hundreds of candidate genes involved in atherosclerotic pathophysiology. These genes are involved in biological pathways such as vascular remodeling, thrombosis, inflammation, lipid metabolism, neovascularization, NO-signaling and blood pressure, among others [61]. Sex differences and genes influencing the lipidome and metabolome are discussed in [62].

Beyond insights into disease etiology and mechanisms, the utility of GWAS relies on risk stratification, prevention, and even the discovery of novel therapeutic targets [63]. At the same time, more CAD loci are being discovered by large ongoing biobanks (e.g. UK Biobank [60] and the Million Veteran Program [59]), with an increasing number of participants and inclusion of populations with diverse genetic ancestries. Most GWAS report their identified SNPs and corresponding candidate gene as: *SNP ID number (candidate gene)*, for example, *rs6883598 (FBN2)*. Prioritization of candidate genes can be done through multiple approaches, including, among others, linkage disequilibrium, expression quantitative trait locus (eQTL) colocalization, advanced bioinformatics analyses (gene ontology, protein-protein interaction networks, pathway analysis etc.) and known functional annotations [64]. It is common to use a combination of approaches and assign a score to each method. This combined scoring approach justifies linking an SNP to a candidate gene. However, it is critical to note that candidate gene prioritization is not always definitive and requires additional experimental studies to establish causal relationships.

Women are often underrepresented in CAD GWAS, which also repeatedly fail to account for sex as a biological variable in their analyses. If at all considered, the analyses are mostly adjusted for sex [65–68]. Sex-combined analyses assume homogeneity of the effect found for genetic polymorphisms in men and women [69]. This phenomenon hampers discovering new relationships between disease/traits and SNPs that may be specific to one sex and are currently missed [70,71]. Sex-specific SNP-disease association might, in turn, provide new insights into the mechanisms that drive sexual dimorphism in CAD.

To achieve this, sex stratification and SNP-sex interactions should become common practices in all GWAS analyses. Even in the instances where sex stratification is performed due to differences in the prevalence of the disease between males and females, the female GWAS often suffers from weaker statistical power and, therefore, smaller effect sizes [60]. Another possible explanation for these smaller effect sizes could be the potential misclassification of predominantly women in CAD GWAS (Box 2). Furthermore, GWAS often exclude sex chromosomes from their 'genome-wide' analyses, especially the X chromosome [44,72,73]. The

Box 2
Misclassification of CAD in women.

Patients presenting with symptoms of MI often undergo coronary angiography to determine the location and severity of stenosis. However, many of these patients undergoing coronary angiography do not show obstructive coronary lesions and are, therefore, diagnosed with ischemia with non-obstructive coronary artery disease (INOCA) [75]. This is in most cases due to abnormalities in the coronary microvasculature disturbing the coronary blood flow and oxygen uptake within the cardiac tissue. This impairment of the microcirculation is called coronary microvascular dysfunction (CMD). Another cause of INOCA is vasospastic angina, which presents with epicardial and/or microvascular spasms. However, most patients present with a combination of two manifestations (e.g. CMD with microvascular spasm). This relatively understudied form of CAD causes selection bias in many current studies. Women with CAD more often present with non-obstructive CAD than men, which would classify them as controls in studies with obstructive CAD as an outcome. Even though little is known on the mechanisms of coronary dysfunction, it has been suggested to overlap with atherosclerosis [76]. This may explain the smaller effect sizes found in women, as seen in large CAD GWAS. Conversely, spontaneous coronary artery dissection (SCAD) is significantly more prevalent in younger women than men and accounts for a considerable proportion of acute coronary syndromes (ACS) at a younger age. It is often treated similarly to CAD and may imply that SCAD patients have contributed to the CAD cases, although pathophysiology is different.

main reason for this is the analytical challenges the X chromosome presents, including the bias in disease associations found for X-linked variants, caused by random X-inactivation (and gene escapees). Another reason is simply that women have two copies of the X chromosome and men only one, consistently obtaining lower signals for X-linked variants in men. In 2009, GWAS helped identify over 2800 significant variants for 300 different traits [72,73]. Although the X chromosome represents 5% of the human genome and carries over 800 protein-coding genes, only 15 SNPs (~0.5%) were linked to chromosome X (NHGRI GWAS Catalog). The underrepresentation of XWAS (X-chromosomal association studies) in GWAS is often defended by arguing that the statistical methods are not available to account for XX and X-inactivation. However, since 2014 a "how to" guide for analyzing X chromosomal data within a standard GWAS is available [74].

The following sections summarize the sex-specific associations for CAD and atherosclerosis in GWAS and XWAS. Additionally, we briefly recapitulate the results from candidate gene studies.

3.2. Candidate gene studies on CAD

Genome-wide analyses of large study cohorts became feasible quickly after the advancement of sequencing and genotyping technologies. Before the GWAS era, candidate gene studies were performed to overcome cost issues, while at the same time providing sufficient statistical power [77]. These candidate gene studies of CAD provided the first sex-specific association between genetic polymorphisms and disease. The CAD risk variants rs3742264 (*CPB2*), rs2295752 (*F13A1*), rs2774279 (*USF1*) [78] and rs5888 (*SCARB1*) [79] showed a sex-specific effect in women. No associations were found for these variants in the joint analysis of men and women. CPB2 and F13A1 are both involved in the coagulation cascade, stabilizing fibrin clots, while USF1 is a cellular transcription factor, exhibiting inconclusive effects on atherosclerotic plaque formation [80]. *SCARB1* is a HDL receptor facilitating reverse cholesterol transport from peripheral tissues to the liver, which is hypothesized to be protective for atherosclerosis [79]. Candidate gene studies, however, are limited to studying only a pre-selected group of genes. GWAS consider the entire genome which allows for identification of risk variants that may not have been previously suspected or known. In this way, a GWAS provides a more comprehensive and hypothesis-free view of the genetic architecture underlying CAD pathophysiology [81,82]. It is not surprising therefore that the four sex-specific variants described here were not replicated in subsequent GWAS.

3.3. GWAS on CAD

Early CAD GWAS that performed sex stratification or SNP-sex interaction are limited. These studies showed rs7865618 (*CDKN2B-AS1*) [83], rs16986953 (*AK097927*, near *APOB*) [84] and rs28451064 (*KCNE2*, *MRPS6*, *SLC5A3* and *AP000318.2*)[85] as potential CAD risk variants in men, and not women. CDKN2B-AS1 and APOB are involved in lipid metabolism (including LDL cholesterol) and are highly expressed in atherosclerosis, promoting lipid uptake and intracellular lipid accumulation [86–88]. CDKN2B-AS1 is also involved in vascular remodeling [89] (e.g. SMC proliferation and apoptosis) and pathways related to non-coding RNAs [61]. Although the role of candidate genes *KCNE2*, *MRPS6*, *SLC5A3* and *AP000318.2* is unknown, the nearest locus has been associated with traits that are known to differ between women and men, like bone mineral density, waist-to-hip ratio and pulse pressure [85,90]. Only one variant, rs715 (*CPS1*), was identified specifically for women, indicating a protective effect for CAD [91]. CPS1 is a mitochondrial enzyme involved in producing urea and glycine metabolism. Of these three sex-stratified GWAS, two had an overall underrepresentation of females in their population (~39%) [83,84], while one study showed proper female representation (~53%) [85]. However, female contribution drops significantly in all studies when only CAD cases are assessed (down to 22.2% [83]). Although showing sex-specific associations, these analyses were still largely underpowered. The new generation of GWAS comprise over a million participants and offer adequate statistical power, which has led to proper identification of sex-differential [60] and X-chromosomal [59] variants. The CAD GWAS by Aragam et al. [60] analyzed ten de novo studies combined with previously published summary statistics using meta-analysis methodologies and included 181.522 CAD cases (on average 32.4% women) and 1.165.690 controls (on average 53.1% women). They were only able to perform a sex-stratified GWAS on a smaller subset of studies comprising 77.080 cases (29.8% women) and 550.952 controls (56.4% women). Ten variants were discovered that had a differential effect between the sexes (referred to in the study as sex-heterogeneous), of which five were found to be sex-specific. Nine had a greater effect in men, whereas one had a stronger effect in women. The male-biased variants include rs149722146 (*EDNRA*), rs4977574 (*CDKN2B-AS1*, described before), rs11225975 (*MIR4693*, near *PDGFD*), rs9521672 (*COL4A1*), rs12740374 (*CELSR2*), rs112422902 (*PVRL2*), rs186399184 (*ICA1L*), rs190352900 (*RAPH1*), and rs28451064 (*NCRNA00310*).

Of these, *EDNRA*, *CDKN2B-AS1*, *MIR4693*, *PDGFD* and *COL4A1* are previously identified CAD loci, and their functions have been described [60,61,77]. EDNRA, a receptor for the potent vasoconstrictor endothelin-1, is involved in endothelial dysfunction through disrupted NO-signaling and is a potential diagnostic and therapeutic target for the early progression of atherosclerosis [92–94]. PDGFD and COL4A1 are involved in vascular remodeling and have been shown to play a role in atherosclerosis [61,86,95,96]. The exact biological mechanism of *MIR4693* remains to be elucidated. *CELSR2* is located on the *SORT1* CAD locus and its gene expression in liver cells was shown to correlate with lower LDL cholesterol and CAD risk [97]. *PVRL2* (now known as *NECTIN2*) is a cholesterol-responsive gene located on the *APOE* CAD locus and has been implicated in transendothelial migration of leukocytes into the early plaque [98]. *ICA1L* is located within the *CARF* CAD locus and its brain protein levels have been associated with small vessel strokes and non-lobar intracerebral hemorrhages [99]. A potential sex-specific function for *RAPH1* and *NCRNA00310*, or their relation to CAD remains unknown.

Only one variant, rs7696877 (*MYOZ2*), showed a stronger effect in women compared to men [60]. *MYOZ2* is expressed in human cardiac muscle and was found to be protective for cardiac hypertrophy in mice, although sex-specific effects of this gene are unknown [100,101].

Despite previous effort [102], the first X-chromosomal genome-wide significant loci for CAD were only discovered recently. The CAD GWAS by Tcheandjieu et al. [59], including 243.392 cases and 849.686 controls (<10% women), identified nine genetic risk variants on chromosome X: rs147967693 (*DNASE1L1*), rs5975828 (*RBMX*), rs5929743 (*MAP7D3*), rs7884019 (*TDGF1P3*), rs2342572 (*SETP4*), rs2066280 (*VDAC1P1*), rs398484 (*CYSLTR1*), rs1410127 (*OPHN1*) and rs5934659 (*TBL1X*). While these X-linked loci are per definition potentially related to sex differences (i.e. present in the sex-chromosomes), it could be argued that these signals only reflect male biology as ~91% of the study population consists of men. Their relevance for female biology remains to be clarified. This overrepresentation of one sex, however, allowed the researchers to identify the first genome-wide significant X-chromosomal

contribution to CAD.

3.4. GWAS on CAD mortality

The CAD GWAS mentioned earlier have provided interesting targets that could potentially explain sex differences in disease onset. However, rather than solely analyzing CAD incidence, assessing disease progression among CAD patients could yield valuable insights into the genetic factors that underlie sex differences in major events, such as mortality. A sex-stratified GWAS on incident mortality in patients with CAD (25.4% women) identified 15 variants conferring sex-specific risk in women [103]. Of these SNPs, rs7217169 (*RAP1GAP2*), rs8133010 (*PDE9A*), rs8021816 (*PRKD1*) and rs12145981 (*LPGAT1*) were annotated to biologically relevant genes in cardiovascular disease. *RAP1GAP2* and *PDE9A* are involved in platelet activation, an initial thrombosis stage [104,105]. *PDE9A* is also upregulated in human heart failure with preserved ejection fraction (HFpEF) myocardium and its inhibition in mice even shows therapeutic potential by improving diastolic dysfunction, an early stage of heart failure more prevalent in women [106]. *PRDK1* is important in cell adhesion, migration and differentiation, and has been linked to epithelial-to-mesenchymal transition (EMT) [107]. EMT closely mimics endothelial-to-mesenchymal transition (EndMT), a fundamental process in atherosclerosis pathophysiology [108]. *LPGAT1* is involved in hepatic lipid synthesis and atherosclerosis [109]. Of the 8 variants that showed increased mortality risk in men, rs9932462 (*EMP2/TEKT5*) and rs2835913 (*KCNJ6*) could be linked to relevant candidate genes [103]. *EMP2* is important for cell adhesion, contraction and proliferation, and has been shown to regulate vasculogenesis and angiogenesis [110]. *KCNJ6* is part of a G protein-coupled inwardly rectifying potassium channel (*GIRK*) family, which is expressed in, among others, cardiac cells and has been suggested to play a role in hypertension by modulating potassium secretion into the bloodstream [103].

3.5. Genetic variants associated with CIMT

Non-invasive imaging of carotid intima-media thickness (CIMT) is an important measure for arterial injury and has been shown to correlate with clinical cardiovascular events with pronounced sexual dimorphisms [111,112]. Four genetic variants from candidate gene studies have been associated with CIMT in a sex-stratified manner, as reviewed by Winham et al. [44]. Two of these SNPs were specific to women and annotated to *IL6* and *OLR1*. IL-6 is a well-known inflammatory cytokine and increased plasma levels have been associated with endothelial dysfunction, atherosclerosis and myocardial infarction [113]. Similarly, OLR1, the main receptor for ox-LDL, has proven to be one of the key players in atherosclerotic plaque development, expressed on endothelial cells, SMCs and monocytes/macrophages [114–116]. Interestingly, an important stimulus for OLR1 receptor expression is IL-6. The two male-specific variants for CIMT were found in genes *PDE4D* and *CDKN2A*. PDE4D degrades cyclic AMP, a key signaling molecule in many biological processes, and has been implicated in stroke pathology through atherosclerosis [117,118]. *CDKN2A* has been associated with monocyte/macrophage proliferation and accelerated atherosclerosis [119].

One GWAS found an SNP-by-sex interaction for rs7616559 (*LEKR1*) and rs2081015 (*GALNT10*) in men [120], while two additional GWAS have reported sex stratified analyses [121,122]. The first sex-specific GWAS was performed on a population with European ancestry (UKB), which identified a female-specific SNP rs309563, with *VCAN* as the most probable gene candidate. VCAN is an essential proteoglycan expressed in the vascular wall of healthy and diseased vessels, which has been shown to accumulate in atherosclerotic plaque progression [123]. Furthermore, three male-specific SNPs were identified: rs35099106 (*LOC401324*), rs2912063 (*MCPH1*) and rs1065853 (*BCAM*). The role of these candidate genes remains unknown. More recently, a sex-stratified GWAS on sub-Saharan African populations (AWI-Gen) discovered two new female-specific SNPs, rs150840489 (*LARP6*) and rs115473055 (*PROK1*) [122]. LARP6 is involved in collagen regulation targeting type I collagen mRNA and has therefore, been implicated in plaque instability [124,125]. Its locus has also been associated with CAD before in a Taiwanese population [126]. PROK1 stimulates angiogenesis in endocrine glands, including placenta, and its expression has been strongly linked to VEGF [127]. One male-specific variant rs190770959 was found and located in the *SNX29* gene. Not much is known about *SNX29* in plaque pathology [128]. The locus is, however, located near sex-dependent differentially methylated regions that are found to be modulated by testosterone in mouse livers, which might partially explain the male-specific signal [129].

3.6. Concluding remarks on genetic studies of CAD

GWAS have become a widely used method for discovering genetic variants associated with different diseases and traits. These studies have provided the first genetic framework for identifying individuals at higher risk for developing CAD. However, most CAD GWAS suffer from a low representation and/or inadequate classification of CAD in women, leading to weak statistical power and smaller effect sizes in women. On top of that, these studies often fail to consider sex as a biological variable in their analyses. Addressing this issue by increasing female inclusion rates and performing proper classification and sex stratification could identify targets that help elucidate the sex-specific disease mechanisms underlying CAD. Hence, providing more effective therapeutic targets.

Currently, a little more than 40 sex-differential candidate genes have been identified for CAD, CAD mortality and CIMT using candidate gene studies and GWAS (Table 1). These findings suggest sex-specific effects in lipid metabolism, thrombosis, endothelial dysfunction, and vascular remodeling. Genes that could be mapped to CAD risk loci were mainly involved in lipid metabolism and vascular remodeling (Fig. 1). While these loci can provide valuable insights into the genetic architecture behind CAD and atherosclerosis, the clinical relevance of these SNPs should be carefully interpreted. Having a disease-associated SNP does not necessarily mean an individual develops the disease and similarly, disease-associated SNPs can contribute to different outcomes. Additionally, there is great variation in the combination of SNPs an individual can possess, leading to potentially different clinical outcomes. Currently, studies are being undertaken that combine the information of multiple SNPs into polygenic risk scores (PRS) which could potentially identify patients at risk for polygenic diseases like CAD [130]. Recently, it has been shown that for traits with known sex disparities, PRSs have higher prediction accuracy when calculated using sex-specific GWAS data. However, this requires large and balanced sample sizes for both sexes, which is still challenging [131].

CAD develops through a complex interplay of genetics plus additional molecular mechanisms such as epigenetic regulation and changes in gene expression patterns in the context of different environmental exposures. These mechanisms differ across cell types and allow for tissue-specific analyses of, for example, atherosclerotic plaque. Integration of additional -omics layers like epigenetics and transcriptomics offers a more complete understanding of the atherosclerotic processes

Table 1
Studies reporting sex-specific or sex interaction effects for genetic (SNPs) or epigenetic (CpGs) associations with CAD, CAD mortality, carotid intima-media thickness (CIMT) and myocardial infarction (MI). X-linked variants we describe as 'potentially sex-specific'. *originally reported as 'sex interaction' [78,132], but defined as 'sex-specific' effect because there was only a significant difference found in one sex. HR = hazard ratio; OR = odds ratio; β = Beta; ns = non-significant.

Phenotype	Type of analysis	Sample size	Females	Cases *vs.* Control (% females)	Candidate gene	SNP	CAD loci	Female effect	Male effect	Type of effect	Reference
CAD	Candidate gene study	1023	277 (27.1%)	400 (25,8%) *vs.* 623 (27,9%)	CPB2	rs3742264	–	HR 0.3	ns	Sex specific*	Silander et al. [78]
CAD	Candidate gene study				F13A1	rs2295752	–	HR 2.2	ns	Sex specific*	Silander et al. [78]
CAD	Candidate gene study				USF1	rs2774279	–	HR 1.9	ns	Sex specific*	Silander et al. [78]
CAD	Candidate gene study	1051	574 (54.6%)	505 (53,7%) *vs.* 546 (55,5%)	SCARB1	rs5888	Lipid metabolism	OR 1.3	ns	Sex specific	Goodarzynejad et al. [79]
CAD	GWAS	4738	1880 (39.7%)	1758 (22.2%) *vs.* 2980 (50%)	CDKN2B-AS1	rs7865618	Vascular remodeling	ns	OR 1.38	Sex specific	Liu et al. [83]
CAD	GWAS	194.427	(~38.5%)	63.746 *vs.* 130,681	AK097927 (near APOB)	rs16986953	(Lipid metabolism)	ns	OR 1.11	Sex specific	Deloukas et al. [84]
CAD	GWAS	317.509	171.263 (53.9%)	9847 cases (31.2%)	KCNE2	rs28451064	Unknown	ns	HR 1.18	Sex specific	Huang et al. (2021) [85]
CAD	GWAS	628.032	333.496 (53.1%)	77.080 (29.8%) *vs.* 550.952 (56.4%)	EDNRA	rs149722146	NO-signaling	ns	OR 1.23	Sex specific	Aragam et al. [60]
CAD	GWAS				CDKN2B-AS1	rs4977574	Vascular remodeling/ Pathways related to ncRNAs	OR 1.16	OR 1.21	Sex interaction	Aragam et al. [60]
CAD	GWAS				MIR4693 (near PDGFD)	rs11225975	Unknown (Vascular remodeling)	ns	OR 1.06	Sex specific	Aragam et al. [60]
CAD	GWAS				COL4A1	rs9521672	Vascular remodeling	ns	OR 1.06	Sex specific	Aragam et al. [60]
CAD	GWAS				CELSR2	rs12740374	Lipid metabolism	OR 0.94	OR 0.89	Sex interaction	Aragam et al. [60]
CAD	GWAS				PVRL2 (known as NECTIN2)	rs112422902	Lipid metabolism	ns	OR 0.86	Sex specific	Aragam et al. [60]
CAD	GWAS				ICA1L	rs186399184	Proliferation and transcriptional regulation	OR 1.05	OR 1.13	Sex interaction	Aragam et al. [60]
CAD	GWAS				RAPH1	rs190352900	–	ns	OR 1.09	Sex specific	Aragam et al. [60]
CAD	GWAS				NCRNA00310	rs28451064	–	OR 1.05	OR 1.15	Sex interaction	Aragam et al. [60]
CAD	GWAS				MYOZ2	rs7696877	–	OR 0.94	OR 0.98	Sex interaction	Aragam et al. [60]
CAD	GWAS	1.093.078	~100.563 (~9.2%)	243.392 *vs.* 849.686	DNASE1L1	rs147967693	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				RBMX	rs5975828	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				MAP7D3	rs5929743	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				TDGF1P3	rs7884019	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				SETP4	rs2342572	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				VDAC1P1	rs2066280	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				CYSLTR1	rs398484	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				OPHN1	rs1410127	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD	GWAS				TBL1X	rs5934659	–	–	–	X-linked (potentially sex-specific)	Tcheandjieu et al. [59]
CAD mortality	GWAS	684	174 (25.4%)	–	RAP1GAP2	rs7217169	–	HR 4.06	ns	Sex specific	Dungan et al. [103]
CAD mortality	GWAS			–	PDE9A	rs8133010	–	HR 3.22	ns	Sex specific	Dungan et al. [103]

(*continued on next page*)

Table 1 (*continued*)

Phenotype	Type of analysis	Sample size	Females	Cases *vs.* Control (% females)	Candidate gene	SNP	CAD loci	Female effect	Male effect	Type of effect	Reference
CAD mortality	GWAS			–	PRKD1	rs8021816	–	HR 5.86	ns	Sex specific	Dungan et al. [103]
CAD mortality	GWAS			–	LPGAT1	rs12145981	–	HR 3.40	ns	Sex specific	Dungan et al. [103]
CAD mortality	GWAS			–	EMP2/TEKT5	rs9932462	–	ns	HR 4.92	Sex specific	Dungan et al. [103]
CAD mortality	GWAS			–	KCNJ6	rs2835913	–	ns	HR 3.46	Sex specific	Dungan et al. [103]
CIMT	Candidate gene study	2421	1259 (52%)	–	IL-6	–	–		ns	Sex specific	Tanaka et al. [113]
CIMT	Candidate gene study	1013	513 (52.4%)	–	PDE4D	rs702553	–	ns	OR 2.16	Sex specific	Liao et al. [117]
CIMT	Candidate gene study	–	–	–	OLR1	–	–		ns	Sex specific	Winham et al. [44]
CIMT	Candidate gene study	–	–	–	CDKN2A	–	–	ns		Sex specific	Winham et al. [44]
CIMT	GWAS	1.341	807 (60.2%)	–	LEKR1	rs7616559	–		β 0.064	Sex interaction	Dong et al. [120]
CIMT	GWAS			–	GALNT10	rs2081015	–		β 0.051	Sex interaction	Dong et al. [120]
CIMT	GWAS	22.179	11.471 (51.7%)	–	VCAN	rs309563	–	β 0.01	ns	Sex specific	Strawbridge et al. [121]
CIMT	GWAS			–	LOC401324	rs35099106	–	ns	β 0.016	Sex specific	Strawbridge et al. [121]
CIMT	GWAS			–	MCPH1	rs2912063	–	ns	β 0.017	Sex specific	Strawbridge et al. [121]
CIMT	GWAS			–	BCAM	rs1065853	–	ns	β 0.027	Sex specific	Strawbridge et al. [121]
CIMT	GWAS	7894	3963 (50.2%)	–	LARP6	rs150840489	–	β −0.051	ns	Sex specific	Boua et al. [122]
CIMT	GWAS			–	PROK1	rs115473055	–	β −0.026	ns	Sex specific	Boua et al. [122]
CIMT	GWAS			–	SNX29	rs190770959	–	ns	β −0.056	Sex specific	Boua et al. [122]

Phenotype	Type of analysis	Sample size	Females	Cases *vs.* Control (% females)	Candidate gene	CpG	CAD loci	Female effect	Male effect	Type of effect	Reference
Incident MI	Candidate gene study	248	119 (48%)	122 (46.7%) *vs.* 126 (50.8%)	INS	–	–	Hyper methylation	ns	Sex specific	Talens et al. () [133]
Incident MI	Candidate gene study				GNASAS	–	–	Hyper methylation	ns	Sex specific	Talens et al. [133]
CAD	Candidate gene study	72	36 (50%)	36 (50%) *vs.* 36 (50%)	PLAGG7	–	–	Hyper methylation	ns	Sex specific	Jiang et al. [134]
CAD	Candidate gene study	334	102 (30.5%)	178 (24.1%) *vs.* 156 (37.8%)	CTH	–	–	ns	Hyper methylation	Sex specific	Giannakopoulou et al. [132]
CAD	Candidate gene study	72	36 (50%)	36 (50%) *vs.* 36 (50%)	CDKN2B	–	Vascular remodeling	Hyper methylation	ns	Sex specific	Chen et al. [61]
CAD	Candidate gene study	1076	533 (49.5%)	640 (48.9%) *vs.* 436 (50.5%)	APOE	–	Lipid metabolism	ns	Hypo methylation	Sex specific	Ji et al. [135]

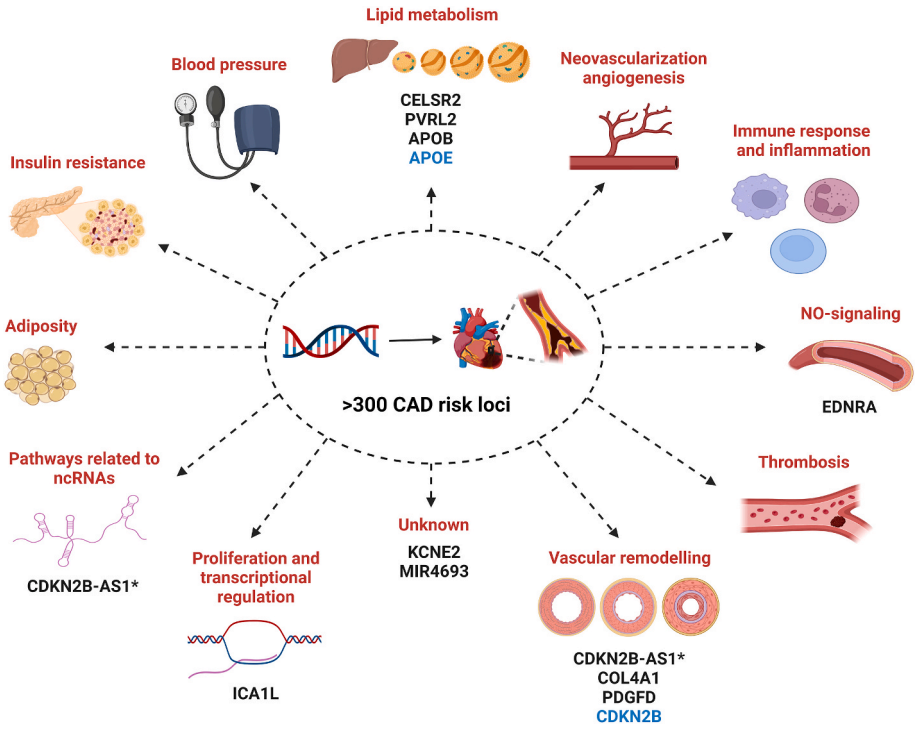

Fig. 1. Identified candidate genes from CAD loci that show sex-differential effect in genetic (black) or epigenetic (blue) studies.
*Genes that were identified by two independent studies. Figure adapted from Chen et al. [61], originally published by Erdmann et al. [77]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

underlying CAD and will be discussed in the following sections.

4. Epigenetics

4.1. The role of epigenetics in CAD

To fully grasp the complexity of CAD as a multifactorial disease, we here review the evidence on sex differences in the epigenetic regulation of CAD pathology. Epigenetics are changes of DNA which, unlike genetic mutations, are reversible and do not affect the underlying DNA sequence. These epigenetic changes dynamically regulate gene expression and consist of DNA methylation, histone post-translational modifications and non-coding RNAs [137]. The last two forms of regulation are beyond the scope of this review and are summarized elsewhere [138]. In relation to CAD, DNA methylation has been studied the most because it is relatively stable and easily detected with micro-arrays and bisulfite sequencing after biochemical conversion [139]. DNA methylation is adding a methyl group to the C5 position of cytosine residues. In mammals, this almost exclusively happens when the cytosine (C) is located next to a guanine (G) in the DNA sequence and is referred to as a CpG site. DNA with a high density of CpG sites adjacent to each other is called a CpG island. DNA methylation is involved in developmental processes such as genomic imprinting, X-inactivation, cell differentiation and aging [140], but also plays an important role in how risk factors affect cardiovascular diseases, including CAD [137]. Examples are obesity and smoking which have been associated with changes in DNA methylation patterns and are known risk factors for CAD [37,141]. In the following chapter, we will discuss the recent developments in studying DNA methylation patterns in tissues important for CAD, highlighting studies that report sex differences.

4.2. DNA methylation in CAD and atherosclerosis

Initially, the association between DNA methylation and CAD was studied by exploring changes in global methylation levels. Most of these studies used methylation levels of repetitive elements (e.g. LINE-1 and ALU) as a proxy for global methylation. Their findings, however, are inconsistent, showing associations with CAD for both hyper-and hypomethylated repetitive elements [142–145]. Greater resolution of DNA methylation was required in the form of individual CpG sites at a gene-specific level instead of global methylation. Similar to the field of genetics, first candidate gene studies and later epigenome-wide association studies (EWAS) became common practice to assess the epigenetic role of DNA methylation in CAD. Of the 34 candidate gene studies on CAD and atherosclerosis, as reviewed by Sanlés et al. [142], only four genes showed consistent findings. These associations with CAD include hypermethylation in *ESRα*, *ABCG1* and *FOXP3*, and hypomethylation in *IL6*. EWAS offers a more comprehensive view of DNA methylation in CAD and, similarly to GWAS, does not consider all possible CpGs in the human genome. So far, most studies have applied microarrays, Infinium HumanMethylation450 (~450.000 CpGs array) and MethylationEPIC (~850.000 CpGs array) BeadChip, which provide a preselected subset of CpGs primarily located in CpG islands, genes and enhancers [146]. Like GWAS, sex chromosomes are frequently removed from EWAS analyses. Most EWAS measured DNA methylation in blood, which is justified when studying the cardiometabolic component of CAD. However, given that DNA methylation is highly tissue-specific, measuring DNA methylation directly from the affected vascular tissue offers a more accurate view of the atherosclerotic processes that contribute to CAD.

Based on 11 EWAS performed until 2017, as reviewed by Sanlés et al. [142], blood DNA methylation in 1540 genes was associated with CAD and atherosclerosis. Among these, 52 genes were reported methylated in

the same direction by at least two EWAS, independent of the CpG site. When considering identified CpGs, only eight genes remained [142,147–150]. Of the eight aforementioned genes, CpG methylation of six of those genes was identified from plaque tissue: cg09414535 (*GRIP1*), cg10222534 (*KCNJ14*), cg26215428 (*PKD2*), cg14345676 (*HRH2*), cg19485804 (*NGEF*) and cg18752854 (*TNS1*) [147,148]. These results were obtained from donor-matched healthy and atherosclerotic tissues (coronary and aortic plaques), and all showed hypermethylation in plaque tissue. A more recent EWAS on atherosclerotic plaque [151] found five more CpGs meeting the same criteria: cg15648389 (*HOXC4*), cg17466857 (*HOXA11-HOXA11-AS*), cg15700739 (*HOXC4/HOXC5*), cg02384661 (*HOXC11*) and cg03217995 (*HOXA9*) [147,148]. However, these CpGs were linked to the HOX family genes, which have been extensively studied in location-mediated development [152]. This means that the observed differences could also be attributed to distinct origins of the obtained samples rather than actual differences between atherosclerotic and healthy tissue.

Almost all EWAS were conducted in a case-control study design, studying patients having CAD versus healthy controls. However, unlike disease-associated SNPs, EWAS CpGs are not necessarily causal to disease, as CpG methylation can also be affected by CAD pathology itself [139]. Therefore, more recent EWAS aim to apply a prospective approach where DNA methylation is associated with incident CAD. From four EWAS, a total of 66 CpGs from blood were found to associate with incident CAD [145,153–155]. However, no overlapping CpGs were found between the different EWAS, probably due to population and phenotype heterogeneity [139]. Two of these studies performed Mendelian randomization for CpGs that contained meQTLs (SNPs that affect CpG methylation), which supported causal associations between DNA methylation and incident CAD for cg22304262 (*SLC1A5*), cg26470101 (*ITGA6*, near *DLX2*) and cg07289306 (*MIR138-1*) [153,154]. It is worth noting that these prospective EWAS use blood samples instead of plaque.

4.3. Sex differences in DNA methylation

Epigenetics can be tissue-specific and be directly linked to changes in gene expression. However, sex differences in epigenetics remain largely unstudied. In 2018, a systematic review by Hartman et al. [156] found that of the 75 studies that covered sex differences in cardiovascular epigenetics, only 13 stratified some of their data by sex. All studies looked at DNA methylation in blood, particularly focusing on CAD risk factors. Two candidate gene studies found sex differences in methylation patterns that associated with CAD. Jiang et al. [134] found that hypermethylation of the *PLA2G7* promoter associated with CAD in women, but not in men. *PLA2G7* encodes lipoprotein-associated phospholipase A2 (Lp-PLA2) which plays a central role in atherosclerosis and is involved in both immune response and lipid metabolism. Lp-PLA2 is abundantly expressed in the necrotic core of atherosclerotic plaques where it stimulates the formation of proinflammatory and cytotoxic products through the hydrolysis of oxidized phospholipids [134,157]. Therapeutic inhibition of Lp-PLA2 reduced necrotic core expansion in CAD patients [158]. Second, Talens et al. [133] demonstrated that hypermethylation of the *INS* and *GNASAS* loci is associated with incident MI among women. Hypermethylation of *INS* has been associated with lower expression of insulin in pancreatic β-cells, while hypermethylation of *GNASAS* associates with higher expression of Gas in adipose tissue, a key component of the cAMP-dependent pathway [133,159–161]. Both play an important role in the development of obesity, an important risk factor for CAD [162]. More recently, three additional candidate genes studies were performed that showed sex bias in CAD-associated DNA methylation patterns for *APOE* [135], *CTH* [132] and *CDKN2B* [163]. Hypo-and hypermethylation of *APOE* and *CTH* respectively, was associated with CAD only in men. *APOE* is a known CAD locus involved in lipid metabolism. Apolipoprotein E has repeatedly been shown to possess atheroprotective capabilities, beyond regulating plasma lipid levels [164,165]. *CTH* encodes cystathionine γ-lyase (CSE) which produces hydrogen sulfide (H2S) in the endothelium [132]. Altered in H2S bioavailability has been suggested as novel biomarker for endothelial dysfunction [166]. An association between hypermethylation of *CDKN2B* and CAD was only found in women [163]. CDKN2B is a cyclin dependent kinase inhibitor that has been implicated in the atherosclerotic plaque development through VSMC proliferation [167]. A critical region of the *CDKN2B* gene overlaps with *CDKN2B-AS1* [168], a well-known CAD candidate gene, which showed a sex-biased association towards men as we previously discussed (see section *Genetics*). This suggests some CAD loci might reveal sex-biased effects across different biological layers.

The aforementioned studies reporting sexually dimorphic DNA methylation patterns in CAD contain some critical limitations. They all focused on the methylation of preselected genes and failed to mention the identified CpG site. Moreover, all CpGs were identified in blood, which hampers the interpretation from an atherosclerosis perspective. We recently performed sex-differential methylation analysis of atherosclerotic plaques and identified 4848 autosomal CpG sites, most of which were hypermethylated in female atherosclerotic tissue compared to male tissue [169]. This considerable amount of sex-differential CpGs might represent the known sex differences in plaque biology between men and women. Based on cell-type deconvolution of the DNA methylation data, we were able to show that SMC-like cells are more prevalent in plaques from women and immune-like cells are more prevalent in plaques from men, which is in line with histology findings and underscore the cell-specificity of DNA methylation [10–14]. To further underscore the relevance of sex stratification in epigenetics, we conducted a brief analysis of the previously identified plaque CpGs (Box 3).

4.4. Concluding remarks on epigenetic studies of CAD

The field of epigenetics offers an additional biological layer of information on top of the already widely studied genetic variations in CAD. These reversible changes in DNA define cell identity and allow for tissue-specific analyses. Like GWAS in genetics, EWAS gives the most comprehensive view of changes in DNA methylation patterns related to atherosclerosis and CAD. When applying strict selection criteria, as shown in Sanles et al. [142], only six CpGs remained that were associated with atherosclerotic plaque tissue. None of them were considered for proper sex stratification (Box 3). In addition, EWAS sample sizes are still small, which means there is limited statistical power to detect sex differences and, if present, display small effect sizes.

To the best of our knowledge, only five candidate gene studies found sex differences in DNA methylation in relation to CAD [132–135,163]. DNA methylation at these genes suggests sex-specific effects in lipid metabolism, endothelial dysfunction and vascular remodeling. All these studies, however, analyzed blood methylation and failed to describe methylation at single CpG context. We suggest that to study the role of atherosclerosis in CAD, epigenome-wide analyses of atherosclerotic plaques offer a more direct translation towards atherosclerosis, as conducted by Hartman et al. [169]. These findings indicate some clear sex differences in plaque DNA methylation patterns while at the same time hinting towards distinct cell biology. Integration of gene expression data

Box 3
Sex stratification of identified plaque CpGs.

Six CpGs (HOX-gene related CpGs excluded) were previously identified as being indicative for plaque biology in atherosclerosis patients, all showing hypermethylation in plaque tissue compared to donor-matched healthy tissue (See section *Epigenetics*). However, sex differences were not accounted for. To show how these CpGs would differ between sexes and the potential impact of stratifying such analysis, we aimed to define their methylation status in plaques. For this, we used the plaque methylation data from Siemelink et al. [37] of 492 carotid endarterectomy patients. The methylation values of the selected CpGs were compared between female (n = 148) and male (n = 344) plaques (Fig. 2). All six CpGs were significantly higher methylated in plaques from men, of which five remained after multiple testing. Although interpretation of this result should be done carefully, it demonstrates that sex differences are important to consider when analyzing DNA methylation data in atherosclerosis.

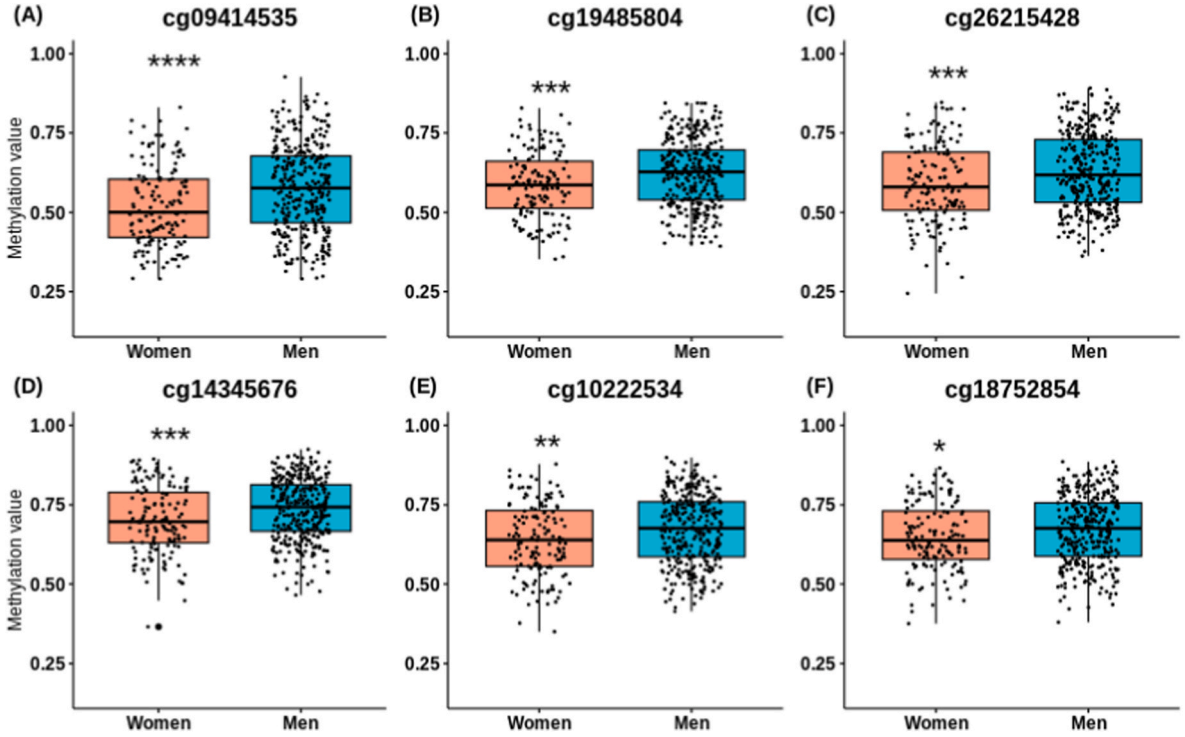

Fig. 2. Boxplots representing sex-stratified methylation of CpGs that were associated with atherosclerotic plaque tissue.
(A–F) All six CpGs were significantly higher methylated in plaques from men [37]. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

could help confirm these findings and provide more insights into the pathobiology of atherosclerosis and its sex differences in CAD.

5. Transcriptomics

5.1. Gene expression and gene regulatory networks

GWAS and EWAS have provided many potential targets for CAD risk stratification, diagnosis, prevention and treatment. However, our ability to translate this (epi)genetic framework into CAD etiology and mechanism remains limited due to an incomplete understanding of its molecular basis. Efforts to decode GWAS and EWAS signals into relevant disease pathways have been made through analysis of plaque gene expression and gene regulatory networks. One of these transcriptomic-based studies revealed the existence of different plaque sub-phenotypes on top of the classical concept of having either 'fibrous' or 'atheromatous' plaques [170]. This supports the diversity and complexity found in the histopathological assessment of atherosclerotic lesions. Five different plaque clusters were identified, including the fibro-collagenous, intermediate, lipomatous, fibro-inflammatory and fibro-cellular plaque phenotypes. The fibro-cellular phenotype showed the highest expression of CAD GWAS candidate genes and patients with this sub-phenotype had the highest polygenic risk score for CAD, suggesting that the previously described genetics play a more prominent role in this plaque phenotype underlying symptomatic disease [170]. Genome-wide analysis of gene expression in atherosclerosis, comparing plaques (aortic, carotid and femoral) with healthy vascular tissue, identified differentially expressed genes (DEG) related to vascular remodeling (matrix metalloproteinases), lipid metabolism (apolipoproteins and cholesterol-related receptors), immune response (inflammatory markers and chemotactic cytokines) and calcification (osteopontin) [171]. More recently, a transcriptome-wide association study (TWAS) was performed that integrated gene expression from CAD-relevant tissues (n = 9) with CAD GWAS data (37.997 cases vs. 42.854 controls) to identify gene-disease associations and prioritize candidate genes [172]. Of the 114 genes that were identified, 96 were located in known CAD loci, including *ENDRA*, *CDKN2B-AS1*, *CELSR2*, *ICA1L* and *KCNE2*, for which we show a sex differential effect (see section *Genetics*). Although these gene expression analyses provide valuable insights into atherosclerosis pathophysiology, findings are solely based on the description of

individual genes. Instead, gene connectivity analysis allows for the identification of groups of genes that are organized in gene regulatory networks (GRN) [173]. These GRN better define the regulation of CAD candidate genes. Through integration of GWAS data, Talukdar et al. [174] was able to identify 30 GRNs causal to CAD, which were interconnected across seven vascular and metabolic tissues [175,176]. This study population, however, mainly consisted of males (>90%) and therefore, hardly encompasses molecular mechanisms in female CAD patients. We investigated this principle by comparing different compositions of the same study population, varying the proportion of males and females [173]. Here, we focused on gene activity in aortic plaques from patients with severe CAD. It was found that connectivity patterns generated from 90% males closely mimics those generated from a 100% male network, while gene connectivity from 100% females does remarkably different patterns. Thus, plaques from women and men show clear differences in gene connectivity and networks, which indicate the importance of sex stratification in transcriptome analyses. Performing sex-stratified network analysis, we prioritized three clinically relevant GRNs as being indicative for female plaque biology in women [173]. Using single-cell RNA data from plaque, we showed that one GRN, enriched for EMT and myogenesis, showed the highest expression in plaque SMCs, which was significantly higher in female SMCs. Further characterization of this GRN confirmed its relevance in SMC differentiation and suggested that sex differences in plaque biology involved phenotypic switching of plaque SMCs. More recently, we identified two SMC GRNs in female carotid plaques that strongly overlaps this GRN found in female aortic plaques [177]. Differential gene expression analyses between male and female carotid plaques showed that female-biased genes were enriched for terms associated with SMC biology, like ECM organization and TGF-β signaling [169]. Moreover, promoter methylation of these female-biased genes was lower in deconvoluted cell types related to SMCs.

5.2. Single-cell transcriptomics

Despite initial attempts to define sex differences at the transcriptomics level, the exact molecular pathways that drive these differences remain unknown. The phenotypic switching of SMCs as a potential mechanism for female atherosclerosis inspires gene expression studies on at individual cell level. Single-cell RNA sequencing (scRNA-seq) of atherosclerotic plaques has increased our understanding of this transcriptome-based cellular landscape [136,178,179]. Cell-type-specific expression patterns reveal plaque heterogeneity by identifying more than 15 distinct cell populations, including endothelial cells, SMCs, myeloid cells, NK-cells, lymphocytes and interconversions of these cell-types. The composition of these cell populations did not differ between sexes [136].

Single-cell analysis of coronary plaques reveals the transition of SMCs into a modulated cell state ('fibromyocytes') that can differentiate into macrophage-like cells and ECM-producing 'synthetic' SMCs [180]. Similarly, single-cell analysis of carotid plaques identified an intermediate SMC state ('SEM' cells) that can differentiate into macrophage-like and fibrochondrocyte-like cells, or return to its original SMC phenotype [181]. Integrative analyses by Pan et al. [181] suggest that the modulated fibromyocytes defined by Wirka et al. [180], consist of both SEM and fibrochondrocyte-like cells. Although the transition of SMCs towards a modulated cell state has been shown to be an essential process in atherosclerosis, its exact role and, therefore, the therapeutic value remains elusive. It is also worth mentioning the underrepresentation of females in this single-cell transcriptomic studies.

Furthermore, scRNA-seq allows the integration of CAD GWAS results through cell-specific enrichment of candidate genes, which show most prominent expression in plaque SMCs and endothelial cells [182]. Similarly, we aimed to define cell-specific expression of our identified candidate genes that show sex differences in genetics or epigenetics (Box 4).

Box 4
Single-cell analysis of sex-differential candidate genes.

Using scRNA-seq plaque data from Depuydt et al. [136], we explore cell-specific expression patterns of our previously identified sex-differential candidate genes (see sections *Genetics & Epigenetics*). We show that most dominant expression can be found in SMCs, endothelial cells and different forms of macrophages, suggesting that the sex differences found for CAD might be explained by biological processes related to these cell-types (Fig. 3). Interestingly, these same cell types were identified as the main players in the female GRNs from carotid and aortic plaques [173,177].

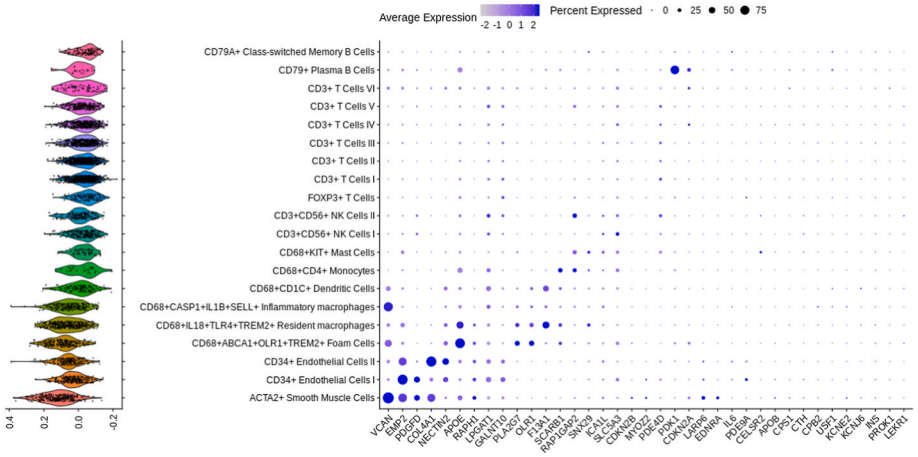

Fig. 3. Dot plot depicting the expression of sex-differential candidate genes in single cells from carotid plaques (46 patients, 26 males and 20 females). Violin plot represents the corresponding module score expression per carotid plaque cell-type, showing highest expression in SMCs, endothelial cells and macrophages. Not all sex-differential genes could be retrieved in scRNA-seq data [136].

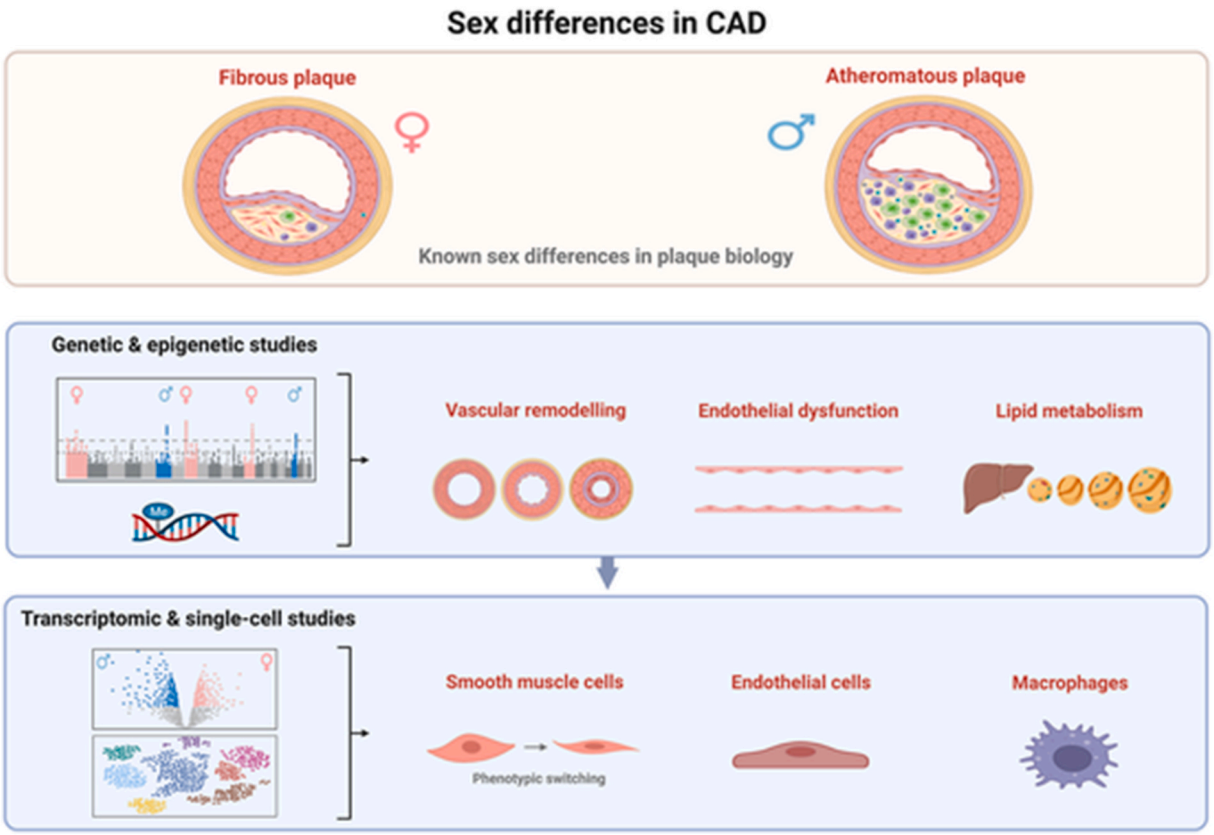

Fig. 4. Overview of the identified genetic, molecular and cellular mechanisms that potentially drive known sex differences in CAD.

6. Conclusion and considerations

Extensive research has established that there are differences between men and women in CAD presentation, risk factors and prognosis [1]. Additionally, research on atherosclerosis has demonstrated significant sex differences in plaque phenotype and composition [10–14]. The underlying genetic and molecular mechanisms that drive these differences remain largely understudied and are therefore poorly understood. In this review, we used different layers of omics data to describe sex differences in genetics, epigenetics and transcriptomics (Graphical abstract). Although many studies provided valuable insights into the genetic architecture underlying CAD, most fail to consider sex as a biological variable and hamper the discovery of new disease-SNP associations that may be specific to one sex. Studies that performed sex analyses [44,59,60,78,79,83–85,103,113,117,121,122] identified more than 40 genetic variants (11 within known CAD loci, Table 1). This suggests sex-specific effects in lipid metabolism, thrombosis, endothelial dysfunction and vascular remodeling. In epigenetics, six regions (two within known CAD loci) were found in the blood that showed sex-specific methylation in CAD patients [132–135,163]. DNA methylation at these genes suggests sex-specific effects in lipid metabolism, endothelial dysfunction and vascular remodeling. It is worth noting that none of the targets identified through genetic and epigenetic analyses were found to be directly involved in estrogen signaling pathways. Our ability to translate this (epi)genetic framework into CAD etiology and mechanisms, however, remains limited due to an incomplete understanding of its molecular basis. Efforts to decode GWAS and EWAS signals into relevant disease pathways have been made through analysis of plaque gene expression, gene regulatory networks and single-cell transcriptomics. Plaques from women and men show clear differences in gene network activity [173]. We highlight one female GRN, enriched for EMT and myogenesis, that showed highest expression in sex-biased expression in plaque SMCs (higher in females). Further characterization of this GRN confirmed its relevance in SMC differentiation. Single-cell analyses of plaques confirmed that SMCs possess remarkably phenotypic plasticity [180,181]. Furthermore, scRNA-seq allows the integration of CAD GWAS results through cell-specific enrichment of candidate genes, which show most prominent expression in plaque SMCs and endothelial cells [182]. Similar expression patterns can be found for the sex-differential genes that were identified in genetics and epigenetics, which show highest expression in SMCs, endothelial cells and different forms of macrophages (Box 4). All these findings suggest that sex differences in plaque biology involve phenotypic switching of plaque SMCs. Our CAD targets (Table 1) present a limited indication of inflammation being a sex-biased process in atherosclerosis. Only IL-6 has shown a sex-specific effect in its association with carotid intima media thickness. However, there is extensive literature on the sex-bias nature of many immune processes [183] and its relation to lipid metabolism [184]. Furthermore, some of the included studies in this review exploring plaque transcriptomics and single-cell data suggest the presence of sex disparities in immune-related processes, involving mainly macrophages (Box 4) [173,174]. Further research is required to comprehend the mechanisms by which sex differences manifest in the context of inflammation and atherosclerosis. Through reviewing genetic and epigenetic studies, we collated evidence that sex differences are primarily present in pathophysiological processes that pertain to vascular remodeling, lipid metabolism and endothelial dysfunction. Additionally, based on transcriptomics studies, we highlight the crucial role of SMCs in female atherosclerosis through phenotypic switching, a process that potentially contributes to vascular remodeling. Our findings also hint towards sex differences in endothelial cell biology. In conclusion, our research indicates that the identified pathophysiological processes potentially act through SMCs, endothelial cells, and potentially, macrophages. These may contribute to the established sex disparities in atherosclerosis, where lipid-rich and inflammatory plaques are more prevalent in men, while fibrous plaques and endothelial dysfunction are more common in women [10–14].

Another overall trend that covers all three layers of omics data is the underrepresentation of women and the scarcity of proper sex stratification in studies on CAD. Even in the GWAS studies that account for sex, there is low participation of women, especially in the group of CAD cases [59,60,83,84,103]. In combination with the still unrecognized misclassification of women in CAD (Box 2), this phenomenon hampers the discovery of female-specific CAD genetic variants. DNA methylation offers the advantage of performing tissue-specific analyses, comparing atherosclerotic plaques with donor-matched healthy artery tissue. Based on strict selection criteria, six CpGs were identified as important markers for atherosclerosis [142,147,148]. However, these EWAS failed to properly incorporate sex as a variable and therefore missed potential sex-specific plaque methylation patterns (Box 3). Similarly, a recent TWAS was performed that integrated gene expression from nine CAD-relevant tissues with CAD GWAS data and identified 114 genes susceptible for CAD (96 within known CAD loci) [172]. Although this included five of our sex-differential genes (Table 1), stratification might have provided some insights into sex-specific gene expression patterns. Finally, there is a lot to be gained in the field of single-cell transcriptomics. By including more patients and implementing more efficient cell isolation protocols, scRNA-seq could provide a reliable transcriptome–based cellular landscape of male and female plaques.

The number of large-scale studies that have investigated sex differences in CAD on (epi)genetic and transcriptional level is very limited. To gain a more comprehensive understanding of the complex mechanisms that drive sex differences in CAD, increased statistical power and appropriate adjustments to study designs and analyses are required. An equal distribution of male and female participants with adequate sample sizes (in most cases by increasing female inclusion rates) and performing well-defined sex stratification and analysis would have to become the norm. Integrating different layers of omics data could, in turn, give us a complete understanding of the sex-specific disease mechanisms in CAD and provide new therapeutic targets that benefit both men and women.

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Declaration of competing interest

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

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