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Sex-dimorphic gene effects on survival outcomes in people with coronary artery disease

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ahjo.2022.100152.

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Abstract

Background: Ischemic coronary heart disease (IHD) is the leading cause of death worldwide. Genetic variation is presumed to be a major factor underlying sex differences for IHD events, including mortality. The purpose of this study was to identify sex-specific candidate genes associated with all-cause mortality among people diagnosed with coronary artery disease (CAD).

Methods: We performed a sex-stratified, exploratory genome-wide association (GWAS) screen using existing data from CAD-diagnosed males ($n = 510$) and females ($n = 174$) who reported European ancestry from the Duke Catheterization Genetics biorepository. Extant genotype data for 785,945 autosomal SNPs generated with the Human Omni1-Quad BeadChip (Illumina, CA, USA) were analyzed using an additive inheritance model. We estimated instantaneous risk of all-cause mortality by genotype groups across the 11-year follow-up using Cox multivariate regression, covarying for age and genomic ancestry.

Results: The top GWAS hits associated with all-cause mortality among people with CAD included 8 SNPs among males and 15 among females ($p = 1 \times 10^{-6}$ or 10^{-7}), adjusted for covariates. Cross-sex comparisons revealed distinct candidate genes. Biologically relevant candidates included rs9932462 (*EMP2/TEKT5)* and rs2835913 (*KCNJ6)* among males and rs7217169 (*RAP1GAP2*), rs8021816 (*PRKD1*), rs8133010 (*PDE9A*), and rs12145981 (*LPGAT1*) among females.

Conclusions: We report 20 sex-specific candidate genes having suggestive association with all-cause mortality among CAD-diagnosed subjects. Findings demonstrate proof of principle for identifying sex-associated genetic factors that may help explain differential mortality risk in people with CAD. Replication and meta-analyses in larger studies with more diverse samples will strengthen future work in this area.

Keywords

Sex differences; Sex dimorphism; Coronary artery disease; Survival analysis; Genome-wide association study

1. Introduction

Coronary artery disease (CAD) and the related conditions of acute coronary syndrome (ACS) and myocardial infarction (MI)—collectively known as ischemic coronary heart disease (IHD)—kill more people worldwide than any other disease [1]. Sex differences in IHD are well established in the literature with respect to biology (physiology, pathophysiology, biomarkers), epidemiology (age of onset, risk factors, symptomatology),

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and clinical phenotypes (angina etiology, ischemic events, and mortality) [2,3]. For example, males tend to experience IHD events and death at earlier ages than women; however, after age 65, the female mortality rate from IHD rises steeply in comparison to that of males [4]. Women in the U.S. are at 50 % greater risk of a mis- or undiagnosed heart attack and 2–3 times greater risk for IHD-related mortality than men despite shared risk factors and similar access to care [2,5,6]. After MI, 35 % of women have another heart attack within 6 years, nearly double the incidence in males [7]. And one in five women assessed as being at moderate or intermediate risk of MI at initial evaluation will die within 30 days of seeking emergency care [7].

The known sex differences in biologic and epidemiologic risk factors, however, fail to fully explain the disparities in cardiac outcomes and mortality between men and women [2,3,8]. Genetic variation has been implicated as a major risk factor for IHD, as it precedes all other known factors. Yet sex differences in gene variation and the related mechanisms that contribute to IHD remain poorly understood. Observations of genetic effects in risk for IHD that are shared between the sexes or unique to them have been limited to candidate gene explorations using a case-control gene association design [9–13] or focused on genomic risk score prediction of CAD risk among the sexes [60]. While such studies have provided proof of principle for sex-dimorphic gene effects in IHD, the search for genetic associations for sex differences in IHD *event outcomes* has lagged.

We previously identified associations between single nucleotide polymorphisms (SNPs) within CAD candidate genes and longitudinal survivorship among people with prevalent CAD. These findings, however, replicated only among males [14]. In our recent genome-wide association study (GWAS) to identify novel candidate genes for survivorship outcomes among people with CAD (sex-combined analyses) [15], we noted evidence in the literature of dimorphic sex effects on lethal cardiovascular phenotypes for two of our top candidates. Our prior work revealed improved survival time among rs587936 C allele carriers with CAD [15]. This SNP annotated to *DAB2IP* (disabled homolog 2 interacting protein), a ras/GAP tumor suppressor gene which is highly expressed in vascular tissue and has multiple lines of evidence in the literature for relation to atherosclerosis, as previously reviewed [15]. In the sex-associated literature, allelic variation in *DAB2IP* (rs7025486, A allele) was associated with faster time to rupture of abdominal aortic aneurysm in women compared to men [16]. Our second top hit was rs13007553, with T allele carriers conferring higher risk for all-cause mortality [15]. This SNPresides between *MYT1L* (Myelin Transcription Factor 1 Like) and *EIPR1* (EARP Complex And GARP Complex Interacting Protein 1; *alias*, *TSSC1*) on Chromosome 2; it is part of a female-specific linkage peak (2p25.3) associated with higher mitochondrial DNA levels among families with idiopathic thrombophilia cases (logarithm of the odds [LOD] score = 3.09) [17]. Of note, mitochondrial variation (inherited through maternal lineage) has been implicated in sexual dimorphism of cardiovascular diseases [18], and thrombophilia of any type is associated with increased risk of CAD and major adverse cardiac events (MACE). In our GWAS models that identified *DAB2IP* and *MYT1L/EIPR1* as candidate genes for CAD survivorship, we controlled for sex as a covariate. In the present work, we employ a sex-stratified GWAS to screen for sex-specific candidate genes associated with all-cause mortality among people with CAD.

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2. Methods

2.1. Design

To identify sex-specific gene associations with survivorship in people with CAD, we performed a sex-stratified, exploratory GWAS screen using existing GWAS data from the Duke Catheterization Genetics (CATHGEN) biorepository.

2.2. Participants/study population

The Institutional Review Board for an academic medical center in the southeastern U.S. approved the primary CATHGEN cohort biorepository (N = 9334) [19], the GWAS substudy of the first 2203 participants >18 years of age enrolled in CATHGEN [20,21], and the present sex-stratified GWAS screen (N = 684). Briefly, the CATHGEN biorepository recruited participants who were undergoing evaluation for ischemic heart disease via cardiac catheterization at the academic medical center. Patients were ineligible for CATHGEN participation if valvular heart disease was either a primary or secondary indication for their coronary heart catheterization or if they had a pre-enrollment history of pulmonary hypertension, transplant, right heart catheterization, congenital heart disease, severe congestive heart failure (New York Heart Association [NYHA] class IV at baseline), or peripheral arterial disease intervention. Data available in the biorepository include genome-wide genotype data, demographic and clinical data abstracted from medical records, and follow-up data of mortality events at 6 months and then annually. A de-identified, anonymous data set containing the variables for our secondary analysis was curated for this project.

2.2.1. Inclusion criteria—We compiled the sample for the present analysis by selecting CAD cases from the CATHGEN GWAS substudy, resulting in 684 participants of self-reported White/European ancestry. To define positive CAD status, we followed the primary CATHGEN study's criterion of a Duke CAD index ≥32 (at least one vessel having at least 75 % stenosis), as determined by clinical coronary heart catheterization [19]. Of note, the Duke CAD index reflects both the extent and location of stenosis. It is used as an indicator of disease severity and includes a score for the presence of left main coronary artery disease.

2.2.2. Exclusion criteria—Our primary analysis excluded the non-CAD control participants due to our focus on all-cause mortality events among people with CAD (survivorship with CAD phenotype). We excluded participants with CAD from the survival analyses if they died within 14 days of their initial catheterization in order to mitigate any undue influence on the time-to-event results involving mortality due to procedural intervention. The limited number of Black/African American, Hispanic, Asian and Pacific Islanders present in the GWAS substudy sample provided inadequate power to include in the analyses, particularly as each group would need to be further stratified by sex for the present exploratory GWAS screen.

2.3. Data sources and variables

2.3.1. Sample collection and genotyping—All sample collection, processing, genotyping and quality control (QC) were performed for the primary CATHGEN study

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in same lab at the Molecular Genomic Core at the Duke Molecular Physiology Institute using the same protocols [20,21]. Following informed consent, blood was obtained from the femoral artery, immediately processed to separate plasma, and frozen at −80 °C. Genomic DNA was extracted from blood using the Puregene system (Gentra Systems, Minneapolis, MN, USA). Genotyping was performed using 200 ng of DNA with the Illumina Human Omni1-Quad BeadChip (Illumina, San Diego, CA, USA) following the manufacturer's protocol. This BeadChip is designed to capture 95 % of genomic variation among people of European ancestry. After genotyping, BeadChips were imaged using the Illumina iScan system. Genotypes were called using Illumina's GenomeStudio V2010.2 software (version 1.7.4, Genotyping module). SNPs with <98 % call frequency or minor allele frequency (MAF) < 0.01 in all races or that were out of Hardy-Weinberg equilibrium ($p < 5 \times 10^{-6}$) were excluded, resulting in 785,945 autosomal SNPs for analysis. Samples with <98 % call frequency for all SNPs, mismatch between subject gender selfidentification and sex chromosome makeup, or cryptic relatedness were excluded (172 samples) [20,21]. Post-QC CATHGEN genotypes are stored in the Duke PEDIGENE® biorepository database.

2.3.2. Variables and outcomes—We defined the time-to-event outcome variable as number of days from study enrollment (baseline: time at coronary catheterization and blood collection) to death from any cause (event). Time-to-event for surviving individuals was censored on the date of last follow-up (censor), consistent with our previously defined "survivorship in CAD" phenotype [14,22]. Clinical and medical history data came from the Duke Databank for Cardiovascular Disease, the data repository for the CATHGEN study. All patients in CATHGEN had one 6-month follow-up, then annual follow-up for all-cause mortality for a maximum of nearly 12 years. CATHGEN study staff adjudicated death events via vitalrecords searches (National Death Index and Social Security Death Index) [21].

Biological sex was determined using a standard genomic approach for gender-mismatch analysis via X-Chromosome zygosity status, as previously reported for the GWAS substudy [20]. Only data from gender matches with at least 98 % concordance were included. Age at time of event or censor was calculated based on date of birth.

2.4. Statistical analysis

Statistical analyses were performed using the R survival package [23]. We calculated means and frequencies for baseline demographic variables, diagnoses, and events. Individuals with CAD were first stratified by biological sex. Each SNP was analyzed individually, using an additive genetic inheritance model as informed by our prior work with survivorship with CAD [14]. The additive genetic model applies a value of zero to wild-type genotype carriers, a value of one to heterozygous genotype carriers, and a value of two to homozygous minor allele genotype carriers [24]. We employed Cox multivariate regression models to estimate instantaneous risk (hazard) of all-cause mortality by genotype groups. We fit a minimally adjusted model covarying age and four principal components of global genomic ancestry to account for this sample's European population admixture (PLINK program, V1.9) [25,26]. Our base model included age and genomic ancestry principle components as covariates. We also tested the base model with the following additional covariates (adjusted model): body mass index (BMI) and history at baseline enrollment of the following:

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smoking, hypertension, diabetes mellitus, hyperlipidemia, and aspirin use. We constructed Kaplan-Meier curves to show survival probabilities by genotype. Post-hoc, we explored the sex-stratified base model in gene-centric analyses (all genotyped SNPs in the top candidate genes) and also tested the base model in non-CAD controls.

2.4.1. Statistical screening thresholds—Our target association level was the standard GWAS threshold, $p = 1 \times 10^{-8}$. Where this stringent threshold was not met, we accepted variants meeting the threshold of $p = 1 \times 10^{-5}$, indicating suggestive associations for candidate gene identification.

3. Results

3.1. Demographic characteristics

We present demographic and clinical characteristics in Table 1. Compared to males, on average, females tended to be older yet have slightly lower CAD severity (CAD index) with better ejection fraction and were more likely to have type 2 diabetes but less likely to be smokers. Comparing females younger than age 65 with those aged 65 and older, we observed very similar CAD severity and prevalence of risk factors, with the exception of lower prevalence of diabetes, hyperlipidemia, and smoking history among the older female group.

3.2. Events

We present the follow-up times and events in Table 2. The median and maximum follow-up times were 5.5 years and 10.8 years, respectively. A total of 159 all-cause mortality events were observed in the sample, representing a 23.3 % mortality rate. Males and females had similar median and maximum follow-up days; males had a slightly higher mortality rate (23.9 %) compared to females (21.3 %).

3.3. Genome-wide screen of sex-associated survivorship with CAD

Quantile-quantile (Q-Q) plots by sex strata indicate that our observed genomic signals were consistent with the expected distribution under the null hypotheses (Fig. S1). The Manhattan plots (Fig. S2) show the negative log10(*p*-value) for each SNP by chromosome. Results of the sex-stratified models are presented in Tables 3 (male) and 4 (female).

3.3.1. Males—We identified seven candidate loci (among eight SNPs) for all-cause mortality in our sample of males with European ancestry and a diagnosis of CAD. All SNPs identified in males had $p \leq 1 \times 10^{-6}$, but none met the $p = 1 \times 10^{-8}$ GWAS threshold. All SNP effects remained significant after controlling for additional covariates (BMI and history of the following: smoking, hypertension, diabetes mellitus, hyperlipidemia, and aspirin use), as shown in Table 3 (*p*-adj). We observed a negative hazard ratio (HR = 0.51) in one SNP (rs12150051), indicating a potential protective effect. The average per-allele risk effect (HR) among the SNPs with HR > 1.00 was 3.10 (range 2.35–4.92). Kaplan-Meier survival curves for male-associated exemplar SNPs are presented in Fig. 1 (*EMP2/TEKT5 and KCNJ6*). These exemplars were selected because of their biological relevance identified via literature review and bioinformatic investigation of NCBI Entrez Gene [27], Weizmann Institute's

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GeneCards [28], and the UniProtKB/Swiss-Prot [29] databases during the gene annotation phase.

3.3.2. Females—In the analysis of our sample of females with European ancestry who had been diagnosed with CAD, we identified 14 candidate loci (among 15 SNPs) meeting $p \leq 1 \times 10^{-6}$, but none met the $p = 1 \times 10^{-8}$ GWAS threshold. All female SNP effects remained significant after controlling for additional covariates (BMI and history of the following: smoking, hypertension, diabetes mellitus, hyperlipidemia, and aspirin use), as shown in Table 4 (*p-adj*). All SNPs identified in females were associated with increased risk of all-cause mortality, with per-allele risk effect ranging from 3.22 to 19.61. Kaplan-Meier survival curves for female-associated exemplar SNPs are presented in Fig. 2 (*RAP1GAP2*, *PRKD1*, *PDE9A*, and *LPGAT1*). These exemplars were selected because of their biological relevance identified during the gene annotation phase.

3.3.3. Cross-sex SNP comparisons—To explore potential for shared gene effects between sexes, we crosschecked all sex-associated SNPs in the alternate sex category (i.e., checked top male GWA hits in females and vice-versa). We summarize these comparisons in the last two columns of Tables 3 and 4 and in Fig. 3. One SNP identified in males (rs17103766; *BRMS1L/ LINC00609*) was detected at $p < .05$ among females. Conversely, none of the top SNPs identified in females were significant in males (*p*-value range .09–.91).

3.3.4. Exploration of gene-centric SNPs—After identifying the top sex-specific candidate genes for all-cause mortality in males and females with CAD, we evaluated the total number of SNPs in each candidate gene that met $p < .05$ in Cox multivariate association with all-cause mortality, controlling for age and four principle components of global genomic ancestry. In Supplemental Tables S1 and S2, we note the additional SNPs for each candidate gene that met QC metrics and had $p < .05$. This analysis revealed additional signals among all candidate genes in both males and females.

3.3.5. Exploration of non-CAD controls—In a post-hoc analysis, we explored the top sex-associated SNPs among non-CAD control groups (stratified by sex, base model) to affirm lack of association with all-cause mortality in those without CAD. Non-CAD controls were defined as having no clinically appreciable CAD (Duke CAD index <23 and number of significantly obstructed vessels = 0), corresponding to no major epicardial vessel with >74 % occlusion as demonstrated by coronary angiography at enrollment, and no documented history of cerebrovascular or peripheral vascular disease, myocardial infarction, organ transplant, or interventional or surgical coronary revascularization (coronary artery bypass graft, stent, or intracoronary procedures) at enrollment [19]. Results are presented in Tables S3 and S4. None of the male-associated SNPs were associated with survival among male non-CAD controls; only one female SNP was marginally associated ($p = .02$) with all-cause mortality among female non-CAD controls (intergenic rs9599764 annotated to LINC00457/*NBEA*).

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4. Discussion

Our sex-stratified, exploratory GWAS screen identified suggestive associations with 20 potential sex-specific candidate genes for SNP-wise sex effects on all-cause mortality with clinically defined CAD (Fig. 4). Prior researchers have reported sex-specific candidate genes for CAD-associated outcomes using sex-stratified analyses with a priori candidate genes. *SCARB1*, a known quantitative trait locus for HDL-cholesterol level (HDL-QTL6) [30,31], was associated with increased risk for premature CAD among females ($n = 574$) but not males ($n = 477$) [11]. *CPS1* (2q34) is a GWAS candidate validated in the CARDIo-GRAM study as strongly associated with protection from CAD among females but not males [12]. The well-established 9p21 CAD risk locus demonstrated stronger effects in males than females in a GWAS reanalysis [13]. A genomic risk score approach also demonstrated sexual dimorphic effects on prediction of incident and prevalent CAD, and identified a novel gene-sex interaction at locus 21q22.11 [32]. The present GWAS scan of sex-associated gene effects on the longitudinal endpoint of all-cause mortality in CAD cases is a novel addition to the literature. That our candidate genes were different from those reported as sex-dimorphic in the literature reflects the distinct differences in candidate-gene versus GWAS approaches and dichotomous versus longitudinal event outcomes.

Our only evidence of shared genetic association between men and women involved a single SNP (rs1703766) identified in males that also showed moderate significance among females. This variant is located on Chromosome 14q13.2 between *BRMS1L* (BRMS1 like transcriptional repressor) and *LINC00609* [33]. *BRMS1L* is part of p53 cell-cycle arrest, apoptosis, and senescence functions. Variations in *BRMS1L* mRNA gene expression have been associated with invasion, migration, and poor patient outcomes in breast cancer [34]. In addition, we found eight SNPs across seven candidate genes or loci for all-cause mortality among males with CAD and 15 SNPs across 13 candidate genes or loci among females with CAD.

4.1. Candidates in males

All eight of the SNPs we identified in males were associated with increased risk of all-cause mortality (HR > 1; Table 3) and remained significant in models adjusted for multiple cardiovascular risk covariates. SNP-wise effects for two of these markers mapped to the intergenic region *UNC13C/LOC105370829* (15q21.3). In a recent study, the nearby 15q21.1 region was identified as one of five major susceptibility loci for spontaneous coronary artery dissection (SCAD; *OR* = 1.75, 95 % *CI* [1.40–2.18], $p = 7.23e{-}7$); however, the investigators conducted their two-phase GWAS analysis in women only ($n = 667$), while we identified this association exclusively in males [35].

Among the SNPs we identified in males, the most biologically plausible candidates include those annotated to the genes *EMP2/TEKT5* and *KCNJ6*. Presence of the heterozygous genotype of *EMP2/TEKT5* SNP rs9932462 was associated with a 4.42-fold increased risk of all-cause mortality (95 % *CI* [2.57, 9.40], $p = 1.4 \times 10^{-6}$). As shown in Fig. 1a, the lack of GG homozygous risk carriers for rs9932462 (*EMP2/TEKT5*) indicates that the hazard estimate is primarily informing on the presence of a single risk allele among heterozygous genotype carriers. Notably, *EMP2* is implicated in a wide array of

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atherosclerosis endophenotypes, as it has been shown to regulate migration of blood vessel endothelial cells [36], cell contraction [37,38], focal adhesion density, F-actin conformation and cell adhesion capacity [39], and cellular proliferation [40]. *EMP2* also promotes angiogenesis and vasculogenesis [41] and is involved in cell death and cell blebbing [42]. Interestingly, rs9932462, an intergenic SNP, is located adjacent to *TEKT5*, which encodes a protein suspected to be a structural component of the sperm flagellum [29]. *EMP2* rs9932462 variation was not associated with survival among women with CAD ($p = 1.00$) [29].

Meanwhile, for each copy of the rs2835913 G risk allele within *KCNJ6*, we observed an approximately 3.5-fold increased risk of all-cause mortality among males (*HR* = 3.45, 95 % *CI* [2.03, 5.90], $p = 4.8 \times 10^{-6}$) compared to non-G carriers. The very limited representation of the risk homozygous genotype for rs2835913 (*KCNJ6*) again indicates that the hazard estimate is primarily informing on the presence of a single risk allele among heterozygous genotype carriers. Variation in rs2835913 was not associated with the outcome event among women ($p = .30$). *KCNJ6* encodes a member of the G protein-coupled inwardlyrectifying potassium channel (GIRK) family [33]. The gene is expressed in cardiac and neuronal cells where it modulates heart rate and neuronal circuit activity, respectively [33]. An increase in intracellular potassium has homeostatic and physiologic effects that signal catecholamine release, which stimulates alpha 1 adrenergic receptor to cause potassium to shift out of the cells and into the blood. An increase in extracellular potassium induces arterial vasodilation in normal blood vessels, thereby increasing skeletal blood flow. Within endothelial cells, neurohumoral mediators and physical forces (such as vascular sheer stress) can cause potassium ions to be released. It may be that SNP variation in *KCNJ6* leads to alterations of intra- and extracellular potassium balance and results in hypertension, a major risk factor for CAD and mortality. *KCNJ6* polymorphisms have been positively associated with blood pressure response to variations in dietary sodium intake among 1906 participants of the GenSalt study [43]. Given that potassium channel activity is physiologically correlated with cardiac rhythm, *KCNJ6* polymorphism may influence mortality risk via arrhythmias. In the literature, *KCNJ6* variants were associated with long QT syndrome among a large Australian family [44]. *KCNJ6* variation has also been explored for relation to various pain phenotypes, including pain tolerance and pain outcomes, with promising but largely inconclusive findings (as reviewed by Matic et al.) [45] Replication and further research are needed to better understand the influence of *EMP2* and *KCNJ6* SNP variation on survival outcomes in males with CAD.

4.2. Candidates in females

None of the 15 candidate SNPs identified in females with CAD met $p < .05$ among the male sample (Table 4). All of these SNPs conferred increased risk of death (*HR* range 3.2–19.6), even after controlling for multiple cardiac risk covariates. Of the top female candidate SNPs, four annotate to biologically relevant genes: rs7217169 (*RAP1GAP2*, Chr 17p13.3), rs8021816 (*PRKD1*, Chr 14q12), rs8133010 (*PDE9A*, Chr 21q22.3) and rs12145981 (*LPGAT1*, Chr 1q13.3). Among women with CAD, each copy of the G risk allele was associated with a 4.06-fold (rs7217169; *RAP1GAP2*), 3.22-fold (rs813133010; *PDE9A*), and 3.4-fold (rs12145981; *LPGAT1*) increased risk of all-cause mortality compared to non-

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carriers. Similarly, each copy of the C risk allele for rs8021816 (*PRKD1*) was associated with a 5.86-fold increased risk of all-cause mortality compared to non-C carriers. Fig. 2 shows a limited frequency of risk homozygous genotype for *RAP1GAP2* (frequency of 0.005) and *PRDK1* SNPs (frequency of 0.002).

RAP1GAP2 (RAP1 GTPase activating protein 2) encodes a GTPase-activating protein that activates the small guanine-nucleotide-binding protein rap1 in platelets [46]. The protein complex RAP1GAP2 is expressed in platelets and activates both rap1 protein and glycoprotein receptors GPIIb/IIIa to elicit maximum platelet aggregation responses [46]. Endothelial damage due to IHD thus activates *RAP1GAP2* and causes the release of dense granules from platelets, leading to thrombosis, inflammation, and aggregation [47].

PDE9A (phosphodiesterase 9A) catalyzes the hydrolysis of cAMP and cGMP to their corresponding monophosphates [33]. *PDE9A* is linked to pathways related to platelet homeostasis and response to elevated platelet cytosolic calcium [48]. Variations in the expression of this gene have been reported in mice with diastolic dysfunction and in humans with heart failure with preserved ejection fraction (HFpEF) [49]. More recently, *PDE9A* inhibition was shown to improve diastolic dysfunction in murine models [50].

Located on Chromosome 14q12, rs8021816 maps to protein kinase D1 (*PRDK1*) and was present in 5 % of our sample of females of European ancestry with CAD. *PRDK1* has many roles in various cellular processes, including cell migration, differentiation, and survival as well as regulation of cell shape and adhesion [51]. It has been associated with congenital heart defects and is part of the beta-adrenergic signaling pathway [52].

The candidate SNP rs12145981 annotates to *LPGAT1*, or lysophosphatidylglycerol acyltransferase 1, whose protein product is an important precursor for the synthesis of cardiolipin. Cardiolipin is a phospholipid exclusive to the inner membrane of the mitochondria and constitutes 20 % of the mitochondria's total lipids [53]. This phospholipid is essential to proper enzymatic function during mitochondrial energy metabolism [53]. Extracellular cardiolipin transfer has been implicated in apoptosis and cardiolipin may also serve as a proton trap for oxidative phosphorylation [54]. In a unique prothrombotic condition, anti-cardiolipin antibodies are associated with risk for recurrent thrombotic events that can occur as early as the teen years [55]. These autoantibodies have been detected among young women experiencing repeated spontaneous abortions [55]. Cardiolipin has also been associated with dilated cardiomyopathy and progressive familial heart block [56]. *LPGAT1* itself has been implicated in cholesterol secretion and atherosclerosis [57].

4.3. Robustness and effect sizes of associations

Because authors have asserted that female-associated findings tend to be less robust in the literature of sex-dimorphic gene associations, we evaluated the robustness of statistical findings and effect sizes of the top GWAS hits among the male and female groups [13]. Comparing effect sizes of genetic associations in the male and female groups, the mean *HR* for males was 3.10 with an average 95 % *CI* width of 3.20, whereas among females the mean *HR* was 5.54 and average 95 % *CI* width was 10.38. The *p*-values were comparable between the sexes. The 95 % *CI* range for the tested SNPs was wider for females than males

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despite the fact that minor allele frequencies were similar between sexes (Fig. 4). These results are most likely due to the smaller sample size for females compared to males.

We report multiple additional SNPs meeting nominal significance ($p < .05$) for each sex-associated candidate gene in Tables S1–S2. These results strengthen the lines of genomic evidence for our candidates and support future meta-analyses and study comparisons.

4.4. Strengths and limitations

Our results are strengthened by additional lines of evidence from significant covariate-adjusted models and from demonstrated lack of significance among non-CAD control groups (with the exception of a single female SNP, rs9599764 having a marginal p-value). However, we caution inferences about the lack of association in controls, as it may be an artefact of limited mortality endpoints among the non-CAD control sample. The CATHGEN clinical cardiovascular biorepository provided exquisite phenotyping of clinically defined CAD along with 11-year longitudinal data on annual follow-up and mortality events to support the testing of our hypothesis of the existence of male and female sex-associated genetic effects in people with CAD. The repository's inclusion of death events adjudicated via national vital records is a strength. However, because the primary study's data on cause of death were too limited for use as an endpoint and causes of death are often inaccurate on death certificates [58], we were confined to the outcome of all-cause mortality within a subset of CAD cases as a proxy for CAD-related death. This study only examined sex as a biological variable of self-reported males and females. We were unable to evaluate other gender designations. Both the male and female groups had insufficient power, thus our GWAS screen should be considered exploratory and our results interpreted carefully. SNP-wise association with survival endpoints requires a minimum of 299 events to achieve 80 % power ($\alpha = 0.05$), assuming a 20 % event rate [59]. In the present sample, we observed 159 total events. The small sizes of the sex-stratified group increases the likelihood of Type I error. For some top SNPs, the frequency of risk homozygous genotype was low (<1 %), therefore the additive genetic models were informing largely on the presence of the risk allele among heterozygous genotype carriers. Generalizability of our results is also limited because our sample of CAD-diagnosed individuals was confined to self-identified White individuals with European ancestry in the southeastern U.S. Despite retaining significance in models adjusted for multiple cardiovascular risk factors including aspirin use, the effect of medication use on survival presents a particular concern for confounding. Namely, statins, beta-blockers, and antiplatelet agents are wellestablished, independent predictors of survival and MACE among people with diagnosed CAD [60–62], and thus, are considered first-line therapy for prevention of MACE among people with CAD (including during the CATHGEN recruitment phase). However, detailed medication phenotyping and medical record adjudication for these additional drugs were not part of the initial study design and data collection, therefore, the observed SNP effects on survival may be influenced by the use of MACE prevention medications. Relatedly, sex differences in treatment, adherence, and response for MACE prevention medications represent a potential confounding concern; however, meta-analyses examining sex differences in efficacy of antiplatelet [63], antihypertensive [64], and statin therapies [65] revealed no major differences in outcomes between men and women. Regarding aspirin use (which we

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were able to control for in the fully adjusted model), a meta-analysis of aspirin efficacy by sex revealed reductions in composite MACE among both sexes; however, the effect in females was attributed to reductions in stroke, while the effect in males was attributed to reductions in MI [[66]]. Addressing the influence of cardiac medications and their sex differences are future priorities in order to refine the sex-associated genetic contribution to CAD-related mortality. The present study does not include replication analyses, and all results should be interpreted with caution.

5. Conclusion

In the present study, we have identified numerous sex-specific GWA candidates having suggestive association with all-cause mortality risk among people with clinically diagnosed CAD. Our hypothesis that there are candidate genes for CAD unique to each biological sex was further supported by our demonstration of: minimal overlap in candidate genes between males and females, retained significance in models adjusted for cardiovascular risk factors, and lack of SNP associations among non-CAD control groups (save for one female SNP). This study demonstrates proof of principle for identifying sex-associated genetic factors that may help to explain differential mortality risk in people with CAD. Replication and meta-analyses will strengthen future work in this area.

Together with evidence from the literature, our sex-dimorphic candidate gene findings support the need for the expanded use of sex as a biological variable in research examining cardiovascular health, as supported by the NIH [67]. The present exploratory results require replication in larger studies with more diverse samples. The burden of CAD-related mortality falls heavily on Black and Brown people, particularly women [6,8,68]. Yet their limited representation in genomics research continues to deny them potential benefits of such research [69,70]. Furthermore, it diminishes our ability to study whether and how population genomic effects interact with social determinants of health and discrimination as contributors to disparities in mortality [71,72]. While the present study has limitations related to inclusion and representation, we are investigating approaches to adequately power research among trans-ethnic cohorts as next steps. The present and future investigations of the effects of sex-associated genomic variants in CAD will improve our understanding of sex-based disparities in CAD symptoms and outcomes and may lead to the development of personalized CAD therapeutics in males and females.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

IHD	ischemic heart disease
CAD	coronary artery disease
GWAS	genome-wide association study
ACS	acute coronary syndrome
MI	myocardial infarction
MACE	major adverse cardiac event
SNP	single nucleotide polymorphism

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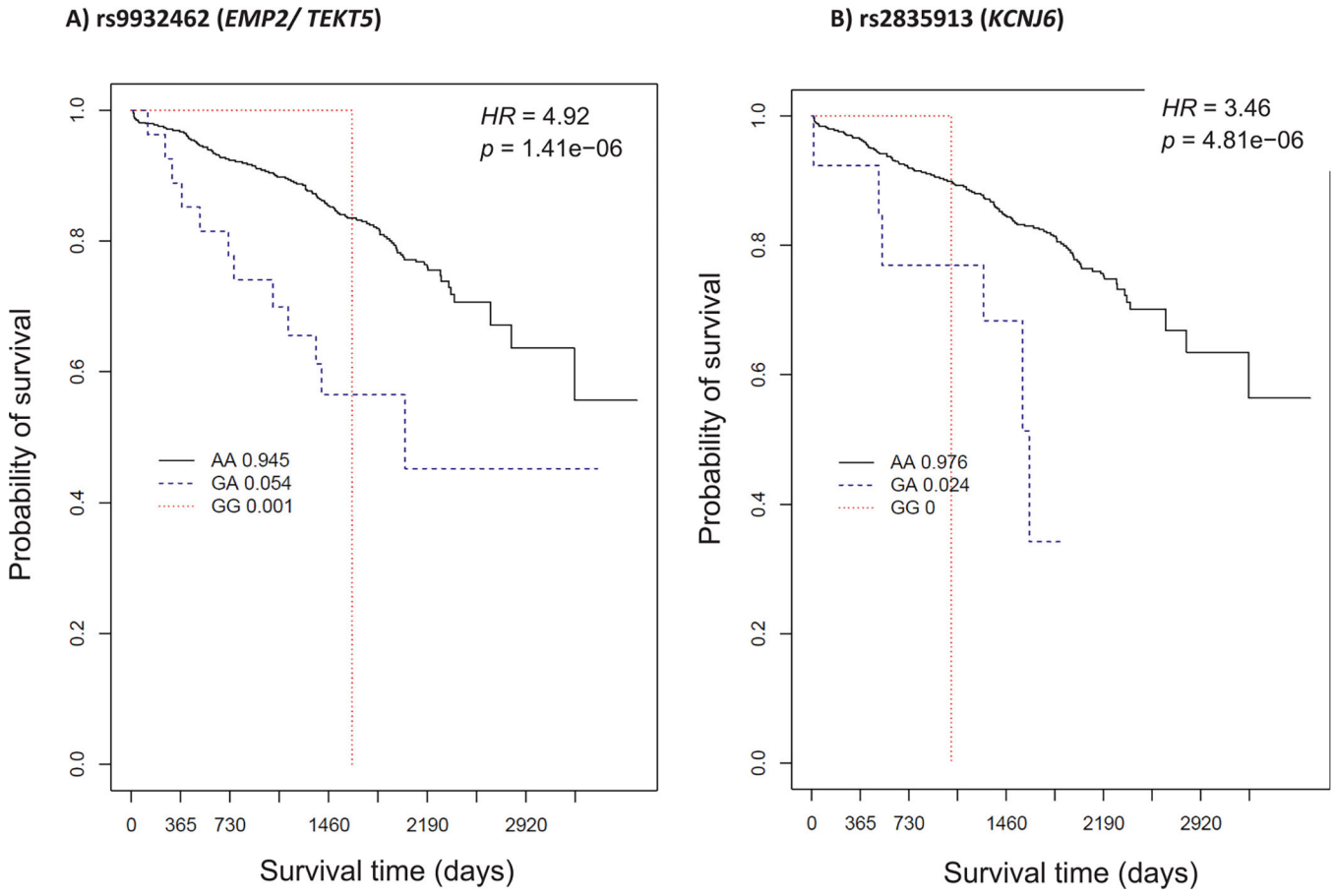

Fig. 1.

Exemplar Kaplan-Meier curves of survival time in days (x-axis) and all-cause survival probability (y-axis) by genotype category among males with CAD. The solid black line represents wild-type homozygous (AA) genotype carriers, the blue dashed line represents heterozygous (GA) genotype carriers, and the red dotted line represents carriers with two copies of the minor ("risk") allele (GG; risk homozygous genotype). A) The frequency of rs9932462 risk homozygous genotype was extremely low in this group. Males with CAD having the heterozygous genotype had a 4.92-fold increased risk of all-cause mortality compared to males having the wild-type homozygous genotype (95 % *CI* [2.57, 9.40], $p = 1.41e{-}06$). B) The frequency of rs2835913 risk homozygous carriers was also very low; males with CAD having the heterozygous genotype had a 3.5-fold increase in risk of all-cause mortality compared to wild-type homozygous genotype carriers (*HR* = 3.46, 95 % CI [2.03, 5.90], $p = 4.81e{-}06$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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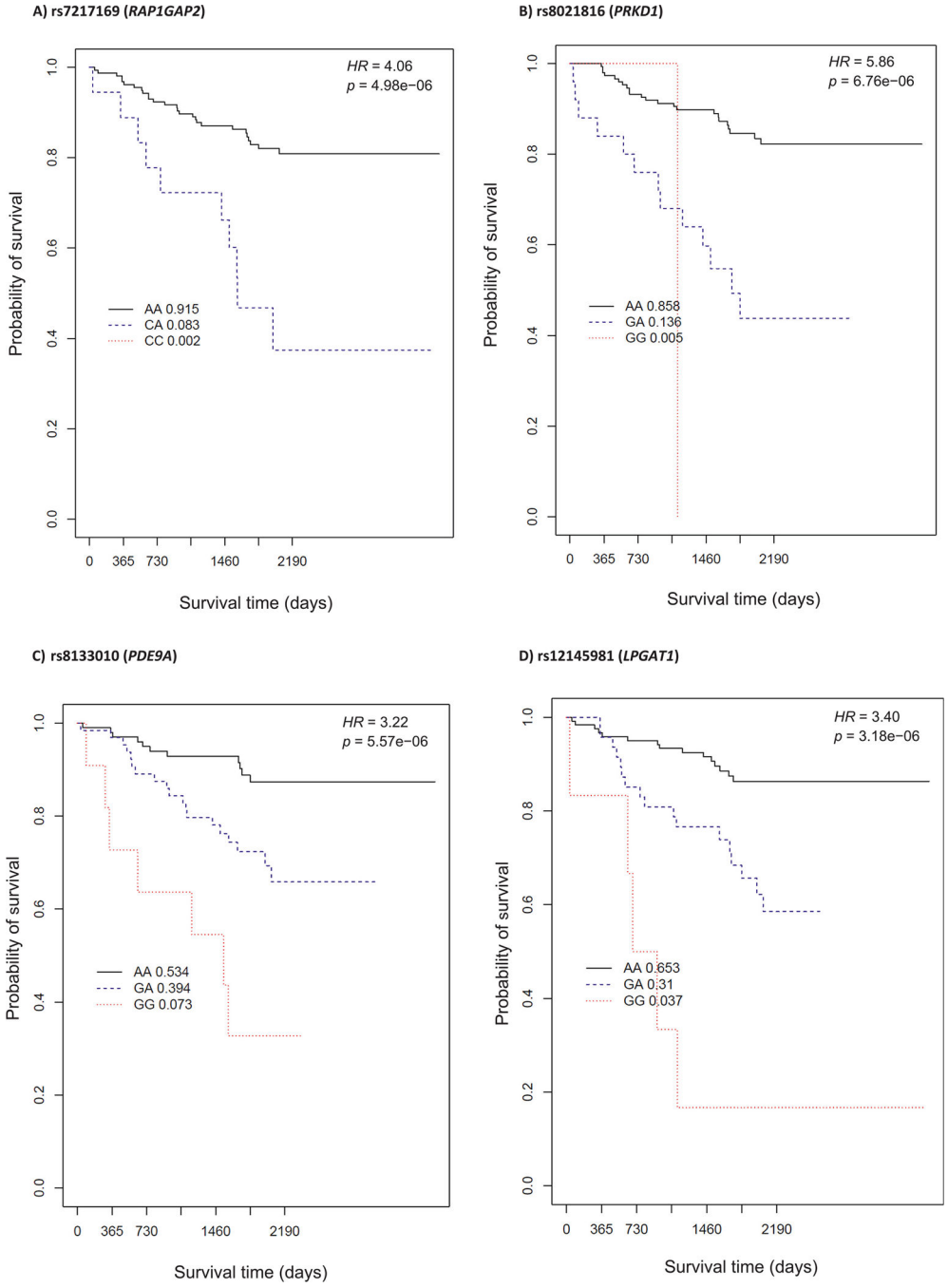

Fig. 2.

Exemplar Kaplan-Meier curves of survival time in days (x-axis) and all-cause survival probability (y-axis) by genotype category among females with CAD. A) rs7217169 (*RAP1GAP2*); B) rs8021816 (*PRKD1*); C) rs8133010 (*PDE9A*); and D) rs12145981 (*LPGAT1*). The black solid line represents wild-type homozygous genotype carriers, the blue dashed line represents heterozygous genotype carriers, and the red dotted line represents carriers with two copies of the minor (“risk”) allele (risk homozygous genotype). The frequency of rs7217169 and rs8021816 risk homozygous genotype was low. Compared

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to wild-type homozygous genotype carriers, A) each copy of the *RAP1GAP2* rs7217169 G (risk) allele was associated with a 4.06-fold increased risk of all-cause mortality among females with CAD (95 % *CI* [2.22, 7.41], $p = 4.98e{-}06$); B) each copy of the *PRKD1* rs8021816 C (risk) allele was associated with a 5.86-fold increased risk of all-cause mortality (95 % *CI* [2.71, 12.65], $p = 6.76e{-}06$); C) each copy of the *PDE9A* rs8133010 G (risk) allele was associated with a 3.22-fold increased event risk (95 % *CI* [1.94, 5.33], $p = 5.57e{-}06$); and D) each copy of the *LPGAT1* rs12145981 G (risk) allele was associated with a 3.40-fold increased event risk (95 % *CI* [2.03, 5.68], $p = 3.18e{-}06$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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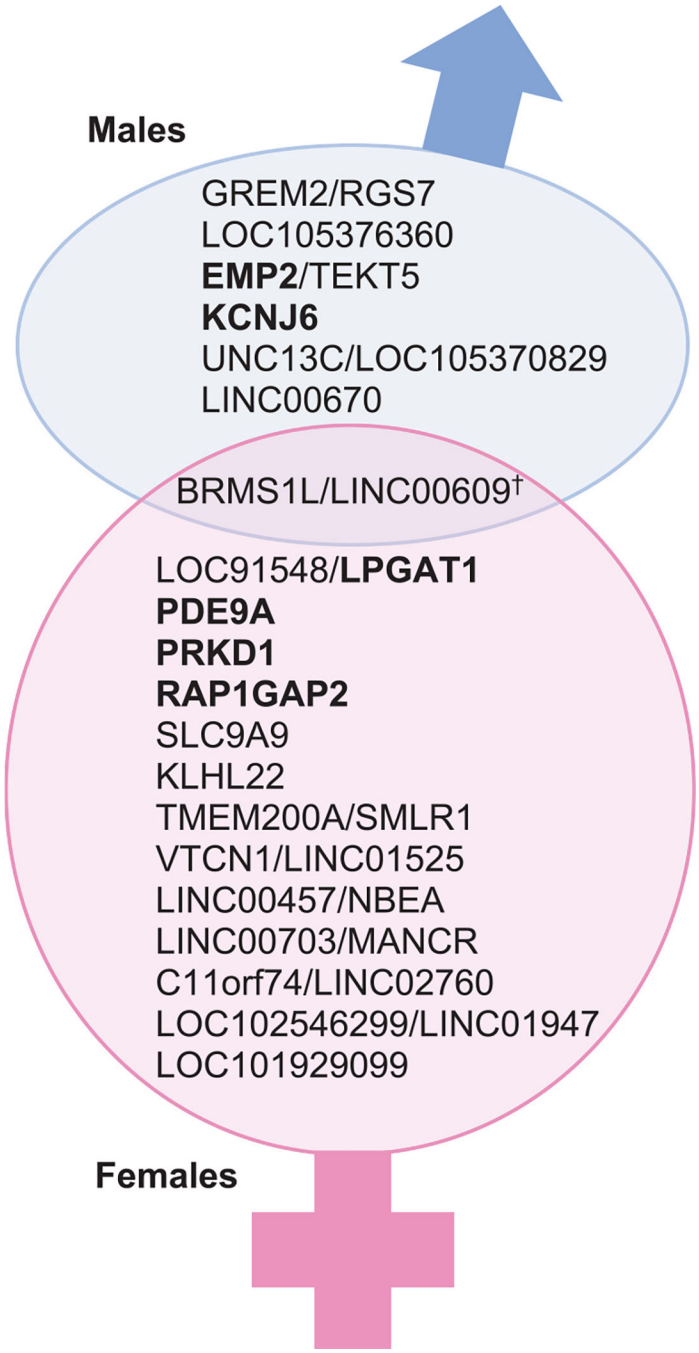

Fig. 3.

Venn diagram of top candidate genes and shared genetic variation, by sex. Genes in bold have biological relevance to cardiovascular disease or survival. †Top male SNP that also shows $p < .05$ among females (does not appear in list of top candidates for females).

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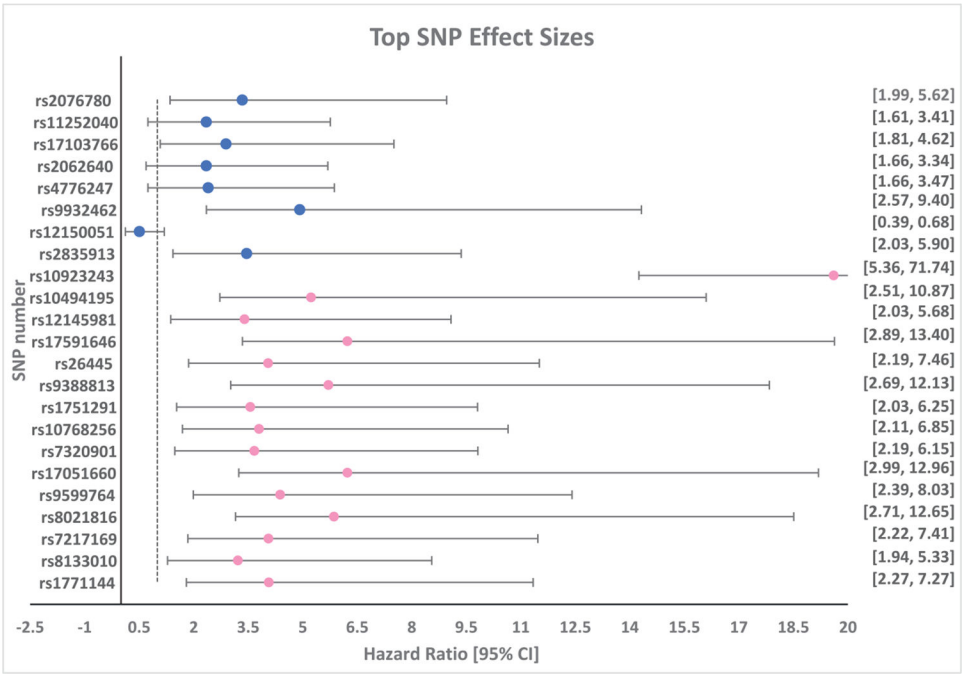

Fig. 4.

Forest plot of top SNP effect sizes. Circles indicate hazard ratio (HR), by male (blue) and female (pink). Horizontal lines indicate the 95 % confidence interval (CI), also provided in brackets, far right. Vertical dotted line indicates HR threshold value of 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 1

Demographic and clinical characteristics ($N = 684$).

Characteristic	Male ($n = 510$)	Female ($n = 174$)	p	Female Age < 65 ($n = 105$)	Female Age ≥ 65 ($n = 69$)
Age (years), mean ± SD	63.9 ± 10.99	67.3 ± 10.78	0.0006	56.17 ± 5.50	74.5 ± 6.22
CAD index, mean ± SD	54.1 ± 18.8	49.5 ± 17.4	0.003	48.48 ± 17.2	50.10 ± 17.4
BMI (kg/m^2), mean ± SD	29.1 ± 5.8	30.8 ± 7.9	0.11	32.26 ± 9.69	28.85 ± 7.18
History of hypertension, %	66.7 %	64.9 %	0.06	71.0 %	77.1 %
History of type 2 diabetes mellitus, %	30.2 %	32.2 %	0.69	44.9 %	23.8 %
History of dyslipidemia, %	68.8 %	64.9 %	0.39	75.4 %	58.1 %
History of smoking, %	55.3 %	43.1 %	0.007	52.2 %	37.1 %
Ejection fraction (%), mean ± SD	54.0 ± 13.3	58.4 ± 12.8	0.0001	58.46 ± 12.48	58.36 ± 13.07
Creatinine (mg/dL), mean ± SD	1.3 ± 0.87	1.1 ± 0.73	0.002	0.98 ± 0.89	1.09 ± 0.60
History of myocardial infarct (MI), %	39.0 %	30.5 %	0.05	34.8 %	27.6 %
History of stroke, %	8.4 %	13.8 %	0.06	15.9 %	12.3 %
Aspirin use, %	84.7 %	86.2 %	0.72	87.0 %	85.7 %

BMI = body mass index; CAD = coronary artery disease; SD = standard deviation. Histories of hypertension, type 2 diabetes mellitus, dyslipidemia, smoking, myocardial infarct (MI), stroke, and aspirin use defined elsewhere [21].

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Table 2

Follow-up and events.

Follow-up and events	**Total (*N* = 684)**	**Male (*n* = 510)**	**Female (*n* = 174)**
Median follow-up, days (years)	2004 (5.5)	2002 (5.5)	2028 (5.6)
Max. follow-up, days (years)	3953 (10.8)	3953 (10.8)	3875 (10.6)
Death events, *n* (%)	159 (23.3)	122 (23.9)	37 (21.3)

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Table 3

Single nucleotide polymorphisms (SNPs) significantly associated with survival in males (n = 510) with coronary artery disease and European ancestry.

SNP	Type	Gene	Chr	Minor Allele	*MAF*	*p*	*p*-adj	*HR*	95 % *CI*	MAF	Female *p*
rs2076780	Intergenic	GREM2/RGS7	1q43	A	0.03	5.19e–06	1.61e–05	3.34	[1.99, 5.62]	0.03	0.49
rs11252040	ncRNA intronic	LOC105376360	10p15.2	G	0.07	7.82e–06	1.22e–05	2.35	[1.61, 3.41]	0.09	0.79
rs17103766	Intergenic	BRMS1L/LINC00609	14q13.2	G	0.04	8.33e–06	2.83e–05	2.89	[1.81, 4.62]	0.02	**0.04**
rs2062640	Intergenic	UNC13C/LOC105370829	15q21.3	G	0.11	1.79e–06	4.49e–07	2.35	[1.66, 3.34]	0.11	0.62
rs4776247	Intergenic	UNC13C/LOC105370829	15q21.3	A	0.10	3.44e–06	2.38e–06	2.40	[1.66, 3.47]	0.10	0.48
rs9932462	Intergenic	EMP2/TEKT5	16p13.13	G	0.01	1.41e–06	1.55e–06	4.92	[2.57, 9.40]	0.01	1.00
rs12150051	ncRNA intronic	LINC00670	17p12	C	0.41	3.12e–06	1.38e–06	0.51	[0.39, 0.68]	0.40	0.49
rs2835913	Intronic	KCNJ6	21q22.13	G	0.03	4.81e–06	1.66e–06	3.46	[2.03, 5.90]	0.03	0.30

Bold *p*-value indicates $p < .05$ in females. Chr = chromosome; *CI* = confidence interval; *HR* = hazard ratio; *MAF* = minor allele frequency; ncRNA = noncoding RNA. Full gene names provided in Table S5 of supplemental materials.

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Table 4

Single nucleotide polymorphisms (SNPs) significantly associated with survival in females (n = 174) with coronary artery disease and European ancestry.

SNP	Type	Gene	Chr	Minor Allele	MAF	*p*	*p*-adj	*HR*	95 % *CI*	Male	
										MAF	*p*
rs10923243	Intergenic	VTCN1/LINC01525	1p13.1	G	0.01	6.91e–06	2.49e–06	19.61	[5.36, 71.74]	0.02	0.53
rs10494195	ncRNA intronic	LOC101929099	1p13.1	A	0.06	9.55e–06	8.83e–06	5.23	[2.51, 10.87]	0.08	0.75
rs12145981	Intergenic	LOC91548/LPGAT1	1q32.3	G	0.17	3.18e–06	2.41e–06	3.40	[2.03, 5.68]	0.18	0.91
rs17591646	Intronic	SLC9A9	3q24	G	0.05	2.88e–06	1.28e–04	6.23	[2.89, 13.40]	0.08	0.80
rs26445	Intergenic	LOC102546299/LINC01947	5q34	A	0.07	7.75e–06	6.20e–05	4.05	[2.19, 7.46]	0.08	0.09
rs9388813	Intergenic	TMEM200A/SMLR1	6q23.1	A	0.04	5.85e–06	3.70e–06	5.71	[2.69, 12.13]	0.05	0.09
rs1751291	Intergenic	LINC00703/MANCR	10p15.1	G	0.18	9.70e–06	1.05e–05	3.56	[2.03, 6.25]	0.15	0.51
rs10768256	Intergenic	C11orf74/LINC02760	11p12	A	0.09	9.28e–06	2.95e–05	3.80	[2.11, 6.85]	0.09	0.24
rs7320901	Intergenic	LINC00457/NBEA	13q13.3	A	0.15	7.88e–07	3.10e–06	3.67	[2.19, 6.15]	0.13	0.24
rs17051660	Intergenic	LINC00457/NBEA	13q13.3	A	0.05	1.02e–06	2.37e–07	6.23	[2.99, 12.96]	0.06	0.67
rs9599764	Intergenic	LINC00457/NBEA	13q13.3	G	0.14	1.72e–06	5.45e–06	4.38	[2.39, 8.03]	0.14	0.44
rs8021816	Intronic	PRKD1	14q12	C	0.05	6.76e–06	1.27e–06	5.86	[2.71, 12.65]	0.07	0.60
rs7217169	Intronic	RAP1GAP2	17p13.3	G	0.08	4.98e–06	9.51e–06	4.06	[2.22, 7.41]	0.08	0.47
rs8133010	Intronic	PDE9A	21q22.3	G	0.25	5.57e–06	2.02e–06	3.22	[1.94, 5.33]	0.27	0.64
rs1771144	Intronic	KLHL22	22q11.21	A	0.15	2.26e–06	4.15e–06	4.07	[2.27, 7.27]	0.19	0.72

Note. Chr = chromosome; *CI* = confidence interval; *HR* = hazard ratio; MAF = minor allele frequency; ncRNA = noncoding RNA. Full gene names provided in Table S5 of supplemental materials.

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