Post-Genomic Update on a Classical Candidate Gene for Coronary Artery Disease: *ESR1*

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Background—After age, sex is the most important risk factor for coronary artery disease (CAD). The mechanism through which women are protected from CAD is still largely unknown, but the observed sex difference suggests the involvement of the reproductive steroid hormone signaling system. Genetic association studies of the gene-encoding Estrogen Receptor α (*ESR1*) have shown conflicting results, although only a limited range of variation in the gene has been investigated.

Methods and Results—We exploited information made available by advanced new methods and resources in complex disease genetics to revisit the question of *ESR1*'s role in risk of CAD. We performed a meta-analysis of 14 genome-wide association studies (CARDIoGRAM discovery analysis, N=≈87 000) to search for population-wide and sex-specific associations between CAD risk and common genetic variants throughout the coding, noncoding, and flanking regions of *ESR1*. In addition to samples from the MIGen (N=≈6000), WTCCC (N=≈7400), and Framingham (N=≈3700) studies, we extended this search to a larger number of common and uncommon variants by imputation into a panel of haplotypes constructed using data from the 1000 Genomes Project. Despite the widespread expression of ERα in vascular tissues, we found no evidence for involvement of common or low-frequency genetic variation throughout the *ESR1* gene in modifying risk of CAD, either in the general population or as a function of sex.

Conclusions—We suggest that future research on the genetic basis of sex-related differences in CAD risk should initially prioritize other genes in the reproductive steroid hormone biosynthesis system. **(*Circ Cardiovasc Genet*. 2011;4:647-654.)**

Key Words: coronary artery disease ■ estrogen receptor alpha ■ menopause ■ polymorphism ■ single nucleotide ■ genetic association studies ■ meta-analysis

After age, sex is the most important risk factor for coronary artery disease (CAD), with women aged 35 to 74 years having 2 to 3 times lower myocardial infarction (MI) incidence than age-matched men.[1] The mechanism through which women are protected from MI/CAD is still largely unknown, but the observed sex difference and the fact that CAD risk in postmenopausal women approaches that of males suggests the involvement of the sex steroid hormone system. This hypothesis was initially supported by the results of observational studies that showed lower CAD risk among postmenopausal women undergoing hormone replacement therapy[2,3]; however, initial clinical trials of hormone replacement therapy have shown unexpected negative results,[4,5] even unanticipated harm, al-

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URLs: Ensembl genome browser, www.ensembl.org; IMPUTE2 software, mathgen.stats.ox.ac.uk/impute/impute_v2.html; 1000 Genomes Project, www.1000genomes.org; HapMap, hapmap.ncbi.nlm.nih.gov/; dbSNP database, www.ncbi.nlm.nih.gov/snp/.

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though the timing of initiation of therapy may explain these conflicting results.[6–8]

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The fact that CAD clusters in families[9] (estimated heritability 38% to 57%[10]), coupled with the observation of sex- and menopause-related differences in risk, suggests that inter-individual variation in CAD risk may be partly mediated by population-level genetic variation in the genes that encode elements of the sex steroid hormone system. ERα is an important signaling gateway within this system and is expressed in multiple cardiovascular tissues in both males and females.[11] The gene encoding ERα, *ESR1*, has been the subject of several candidate gene association studies in relation to CAD over the past decade, with generally inconsistent results[12–14]; however, only a very limited range of the genetic variation in *ESR1* has been investigated, and the role of this gene in CAD risk remains to be clarified.

The last 5 to 7 years have seen a paradigm shift in our approach to investigating the genetic basis of complex diseases. Advanced new methods, including high-throughput genotyping,[15] genome-wide association studies (GWAS),[16] genotype imputation,[17] second-generation sequencing,[18] along with the availability of resources describing natural human genetic variation (eg, HapMap,[19] 1000 Genomes Project[20]), allow us to explore the effect of genetic variation on phenotype more thoroughly. Also important is the manner and volume in which raw genetic data are now generated and disseminated under a model of cross-study cooperation and public data deposition, which has been key to overcoming many of the problems that limited the success of candidate gene association studies for complex diseases.

While no genome-wide significant evidence for the involvement of *ESR1* variation in CAD risk has been reported in recent GWAS, data from these studies may still provide important information either to support or refute this hypothesis. The fact that many robust new GWAS loci for complex diseases had previously been investigated as candidate genes (eg, *LDLR* in CAD[21] and several recently confirmed loci for low-density lipoprotein, high-density lipoprotein, and triglycerides[22]) highlights the importance of revisiting the role of candidate genes in complex diseases.[23]

Therefore, in this article, we bring these powerful post-genomic methods and resources to bear on a classical CAD candidate gene to resolve a long-running unanswered question in cardiovascular genetics. For common variation in a genomic region centered on *ESR1*, we report the results of a large meta-analysis of GWAS of MI and CAD and explore possible sex-specific differences. We also investigate the effect on CAD risk of low-frequency variation in this region.

Materials and Methods

Coronary Artery Disease GWAS Meta-Analysis

The Coronary ARtery DIsease Genome-wide Replication And Meta-analysis (CARDIoGRAM) Consortium was formed with the purpose of identifying novel susceptibility loci for CAD. Briefly, the CARDIoGRAM discovery analysis combined data from 14 published and unpublished primary GWAS in individuals of European ancestry, including 22 233 cases with CAD (stable or unstable coronary events) (30.9% of which were females) and 64 762 controls[21] (58.1% of which were females).

Each primary GWAS performed a logistic regression analysis to test for association between genotyped and imputed (using the HapMap Phase II reference panel[19]) single-nucleotide polymorphisms (SNPs) and risk of CAD under an additive disease model adjusted for age and sex. (See the online-only Supplemental Methods for a more detailed summary of the genotyping and quality control methods used.)

In this study, we meta-analyzed these study-level results using inverse-variance weighting under a fixed-effects model. We performed a random-effects meta-analysis for SNPs with significant between-study heterogeneity (P-heterogeneity <0.01), on the basis of Cochran's Q statistic. These analyses were carried out for each of 535 SNPs in a genomic region containing the entire coding and noncoding region of *ESR1* (see the online-only Supplemental Table 1) and a 50kb region upstream and downstream of the gene (≈547kb; Chr6, 151927808 to 152474406, GRCh37.p1).

Sex-Stratified Analysis

An equivalent analysis to that described above was performed separately for females and males in 13 of the 14 contributing studies (data for the Cohorts for Heart and Aging Research in Genomic Epidemiology [CHARGE] Consortium not available), and the results were meta-analyzed in a similar way. We also formally tested for interaction between each SNP and sex by using the sex-specific effects and variances within each study to estimate those of the SNP-sex interaction term (online-only Supplemental Methods). We then meta-analyzed the results as described for the unstratified analysis.

Fine-Mapping Analysis

Public GWAS Data Sources

To perform fine-mapping studies in the region of interest, we used publicly available genotype and phenotype data from 3 large published GWAS: (1) The Myocardial Infarction Genetics Consortium (MIGen[24]) is a case-control GWAS consisting of 2967 cases of early-onset MI and 3075 age- and sex-matched controls from 6 international sites in the United States and Europe; (2) The Wellcome Trust Case-Control Consortium (WTCCC[25]) is a case-control GWAS of CAD consisting of 1988 cases and 5380 controls from the United Kingdom; (3) The Framingham Share Initiative dataset includes genetic data and longitudinal phenotype data, such as incidence of major cardiovascular events, for ≈9000 individuals from the Framingham Heart Study (http://www.framinghamheartstudy.org), of which we have included 3717 in the present study (selected to maximize the number of subjects free from cardiovascular disease at baseline who had genetic data and complete follow-up data; 464 events [see below for phenotype definition]; mean follow-up, 13.5 years; online-only Supplementary Appendix 1; Lluís-Ganella et al, unpublished data, 2011).

The phenotypic characteristics of these studies were as follows: MIGen cases were males aged <50 years or females aged <60 years who were diagnosed with MI on the basis of autopsy evidence, a combination of chest pain and electrocardiographic evidence, or elevation of cardiac biomarkers; WTCCC cases had a validated history of either MI or coronary revascularization (coronary artery bypass surgery or percutaneous coronary angioplasty) before their 66th birthday; in the Framingham sample, events included incident cases with MI, angina, coronary revascularization, and death because of CAD.

Of the 6042 individuals in the MIGen sample, 2681 were previously included in the CARDIoGRAM discovery meta-analysis. All of the WTCCC cases (N=≈1988) and approximately half of the controls (N=≈2938) were also included in the CARDIoGRAM meta-analysis, as were many of the individuals in the Framingham sample, as part of the CHARGE Consortium.[26]

Genome-wide genotype data and associated phenotype data for the MIGen and Framingham samples were obtained via the database of Genotypes And Phenotypes (dbgap.ncbi.nlm.nih.gov; project num-

ber 2392). Data for the WTCCC sample were obtained from the European Genotype Archive (www.ebi.ac.uk/ega) with permission from the WTCCC Data Access Committee (www.wtccc.org.uk).

1000 Genomes Imputation in MIGen, WTCCC, and Framingham

See the online-only Supplemental Figure 1 for a summary of the quality control steps, imputation process, association analyses, and meta-analyses performed for this analysis.

Imputation of untyped genetic variants in individuals from the MIGen, WTCCC, and Framingham samples was performed using IMPUTE2.[17] Imputation was performed for SNPs in the region of interest using a reference panel of phased haplotypes (available from mathgen.stats.ox.ac.uk/impute/impute_v2.html), based on the August 2010 data release from the 1000 Genomes Project[20] (1kG; 566 haplotypes from populations of European ancestry, EUR: CEU, TSI, GBR, FIN, and IBS). As input for this process, we included only directly genotyped SNPs with high call rate (≥95%) and whose genotype frequencies were in Hardy-Weinberg equilibrium ($P \geq 10^{-6}$). We carried forward to the analysis stage only those SNPs imputed with high quality (IMPUTE2 *INFO* metric ≥0.5).

Association and Meta-Analysis of Genotyped and Imputed SNPs in MIGen, WTCCC, and Framingham

A logistic regression analysis of association between allele dosage of imputed and genotyped SNPs and MI/CAD was performed separately in the MIGen, WTCCC, and Framingham samples, with adjustment for sex. Adjustment for age or other clinical covariates was not possible because no further phenotype data were available in all studies; however, the association results in the Framingham and MIGen samples were very similar after additional adjustment for age at event (data not shown), and both the MIGen and WTCCC studies were age- and sex-matched by design. To account for interrelatedness, the analysis of the Framingham sample was also adjusted for the first 2 genetic principal components.[27] The results from these 3 studies were meta-analyzed as described above for the CARDIoGRAM analysis.

Statistical Analysis

Apart from imputation, all analyses were performed using R version 2.11 (packages and functions indicated below by *<package>::<function>*). Fixed- and random-effects meta-analyses were performed using *rmeta::meta.DSL*. Association testing was performed using *stats::glm* for the case-control studies and *survival::coxph* for the cohort study.

To account for multiple testing, we used a Bonferroni correction based on the effective number of independent tests in the region of interest to set the threshold for declaring statistical significance (regional significance level). Since many SNPs in the region of interest were not independent, we used the technique proposed by Cheverud[28] to estimate the effective number of independent tests (n^{eff}; separately for the CARDIoGRAM and fine-mapping results); for this estimation, we computed pairwise linkage disequilibrium (LD) (r^2) between all pairs of SNPs in the region of interest using genotype data from the HapMapII+III CEU (344 haplotypes) or 1000 Genomes project EUR (566 haplotypes) reference panels of phased haplotypes for the CARDIoGRAM and fine-mapping analyses, respectively. LD calculations in the region of interest were performed using *SNPassoc::LD*.[29]

We computed the power of each analysis to detect significant associations (online-only Supplemental Methods). Briefly, for each SNP, we computed the power of our analysis to exceed the threshold for declaring statistical significance after adjustment for multiple testing and expressed this power in 2 ways: the minimum odds ratio (OR) the analysis had high or moderate power to detect (Type II error=20% or 50%, respectively); and the power of the analysis to detect each of a series of ORs (eg, 1.05, 1.1, etc.). We computed these values for each SNP and took the mean for all SNPs within each of a series of subranges of minor allele frequency (MAF= [0, 0.01], [0.01, 0.02], etc).

Results

Meta-Analysis of Genome-Wide Association Studies of CAD (CARDIoGRAM)

Figure A shows a regional plot of global *P*-values from the CARDIoGRAM meta-analysis for 535 genotyped and imputed (HapMap II) SNPs in the region of interest. Considering a threshold for declaring statistically significant association of $P \approx 1.02 \times 10^{-4}$ ($n^{eff} \approx 503$), we observed no significant association between common SNPs in this gene and risk of CAD. This analysis had high power (≈80%) to detect ORs of ≥1.10, ≥1.28, and ≥1.33 and moderate power (≈50%) to detect ORs of ≥1.08, ≥1.23, and ≥1.26 for SNPs with MAF ≥0.15, ≥0.05, and ≥0.01, respectively (online-only Supplemental Table 2).

The strongest association in this region was observed for a series of 18 SNPs lying within a ≈24 kb region of strong LD between noncoding exons E1 and T1[30] at the 5′ end of the gene. The direction of effect on CAD risk of the top SNP in this area (rs7749659, *P*=0.0019; MAF≈0.25) was generally consistent across CARDIoGRAM studies (online-only Supplemental Figure 2; pooled OR [95% CI]: 1.05 [1.02, 1.08] for the G allele; range 0.85 to 1.21; *P*-heterogeneity, 0.28).

Sex-Stratified Analysis

Under the hypothesis that the effect of genetic variation in *ESR1* on CAD risk differs according to sex, we analyzed data from 13 of the 14 CARDIoGRAM discovery cohorts separately in females (n=30 615 [48.8%], of which 6100 [19.9%] were cases) and males (n=32 069 [51.2%], of which 13 846 [43.2%] were cases; online-only Supplemental Figure 3). We used the same criterion for declaring statistical significance as for the unstratified meta-analysis ($p \approx 1.02 \times 10^{-4}$). In females, we had high power (≈80%) to detect ORs of ≥1.18, ≥1.47, and ≥1.58 and moderate power (≈50%) to detect ORs of ≥1.15, ≥1.37, and ≥1.45 for SNPs with MAF ≥0.15, ≥0.05, and ≥0.01, respectively (online-only Supplemental Table 2). In males, we had high power (≈80%) to detect ORs of ≥1.15, ≥1.23, and ≥1.49 and moderate power (≈50%) to detect ORs of ≥1.12, ≥1.18, and ≥1.39 for SNPs with MAF ≥0.15, ≥0.05, and ≥0.01, respectively (online-only Supplemental Table 2).

One SNP, lying ≈35 kb upstream of the most distal noncoding exon (Figure B), exceeded the threshold for regional significance in the test for interaction between sex and genotype as a predictor of CAD risk (rs9479087, MAF: 0.183 in CARDIoGRAM, $P^{int} = 1.2 \times 10^{-5}$; online-only Supplemental Figure 3); however, this variant was not significantly associated with risk in either males (*P*=0.0026; pooled OR [95%CI]: 1.07 [1.03, 1.13]) or females (*P*=0.057; pooled OR [95%CI]: 0.94 [0.89, 1.00]) at the regional significance level. No other regionally significant evidence for association was observed either among females (top result: rs6927072 in intron 3, *P*=0.0081, Figure B) or males (top result: rs9479087, *P*=0.0026; Figure B).

Fine-Mapping Analysis

While the density of SNP data in the HapMap II panel (CARDIoGRAM results) for this region is quite high (mean=1.15 SNPs/kb), it is possible that some stronger true

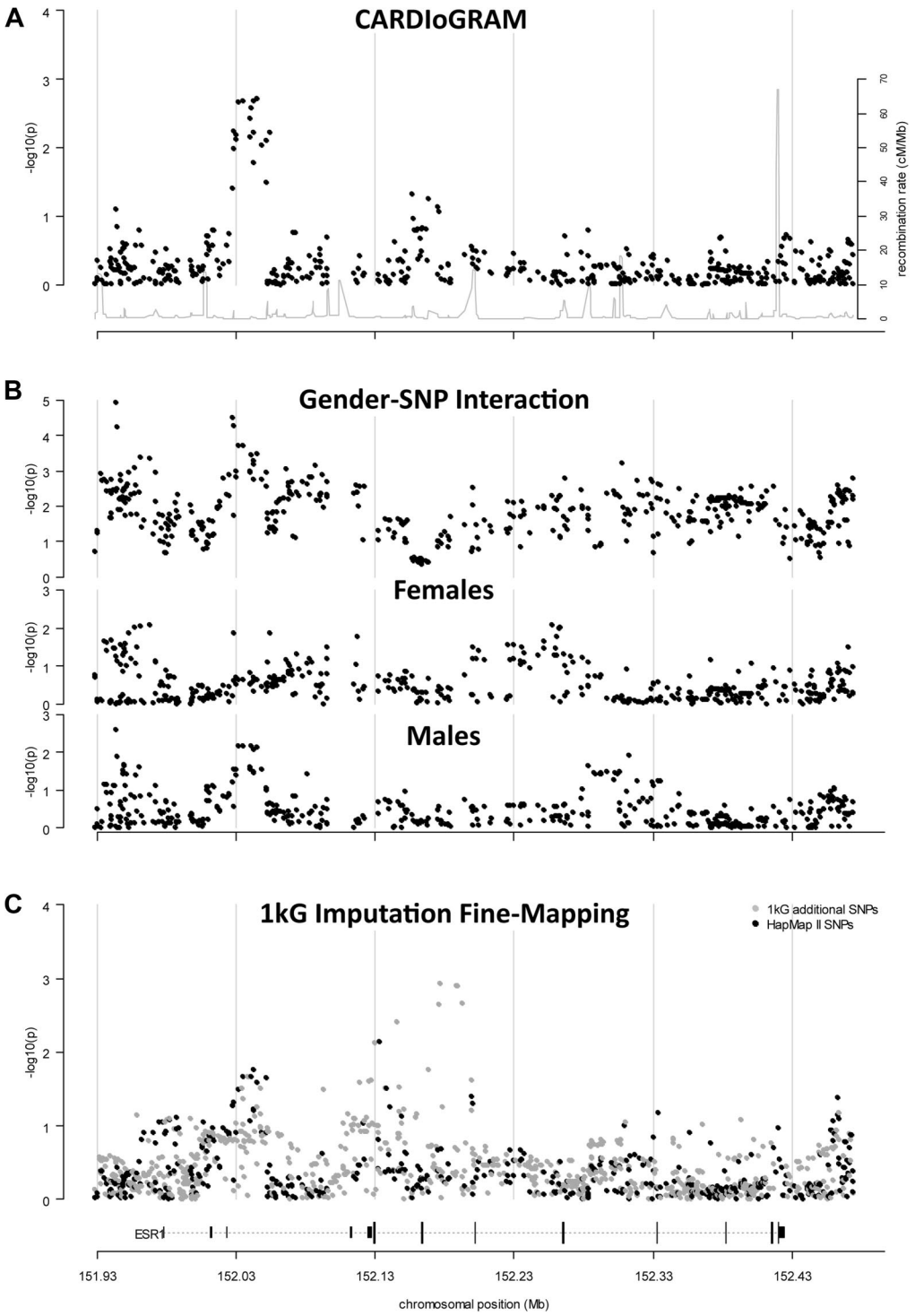

Figure. Regional association results for the *ESR1* gene region. Results of CARDIoGRAM global and sex-stratified meta-analysis and fine mapping analysis for a region of chromosome 6 containing the coding and noncoding exons of *ESR1* and 50 kb of the upstream and downstream flanking regions ($-\log_{10}$(*P*-value) shown as black points). Results shown are for a fixed- or random-effects meta-analysis in the absence or presence of between-study heterogeneity, respectively (see Methods). The position of the *ESR1* gene is shown at the bottom of the plot (dotted line), with coding and noncoding exons shown as long and short vertical bars, respectively. Regional recombination rate (HapMap II) is shown as a gray line in plot A. **A**, Regional association plot of global meta-analysis results from the CARDIoGRAM study. **B**, Regional association plot of sex-stratified meta-analysis results from the CARDIoGRAM study. Results for the test for single-nucleotide polymorphisms (SNP)-sex interaction are shown in the top panel; results for the association test in females and males are shown in the middle and bottom panels, respectively. **C**, Regional association plot of results from fine-mapping meta-analysis (MIGen, WTCCC, and Framingham studies). Results for SNPs that were previously analyzed in the CARDIoGRAM study are shown as black points (ie, SNPs that were directly genotyped or imputed using a reference panel of haplotypes generated from the Phase II HapMap CEU genotypes). Results for additional SNPs that were imputed using a reference panel of haplotypes generated using data from the 1000 Genomes project (August 2010 release) are shown as gray points.

association signals are not captured by these common genotyped and imputed variants. Such signals might be detected by analyzing a higher density map of common and low-frequency SNPs in this region. To explore this possibility, we imputed ≈2500 additional variants from the 1 kG reference panel (≈4.52 SNPs/kb), 1451 of which were imputed with high quality in all 3 samples (≈2.7 SNPs/kb; see the online-only Supplemental Figure 1). Imputation in the 1 kG panel allowed us to test ≈800 additional SNPs within the region of interest that were not included in the CARDIoGRAM meta-analysis. Newly imputed SNPs had a wide range of MAF, although a large proportion had MAF in the range 0.0 to 0.05 (online-only Supplemental Figure 4).

After testing for association between SNPs in the 1 kG panel and CAD in the MIGen, WTCCC, and Framingham samples, meta-analyzing the results and correcting for multiple testing ($n^{eff}\approx1366$; $\alpha_{adj}\approx3.8\times10^{-5}$), we observed no globally significant evidence for association in this region (Figure C). This analysis had high power (≈80%) to detect ORs of ≥1.21, ≥1.44, ≥2.09, and ≥3.14 and moderate power (≈50%) to detect ORs of ≥1.18, ≥1.35, ≥1.85, and ≥2.59 for SNPs with MAF ≥0.15, ≥0.05, ≥0.01, and <0.01, respectively (online-only Supplemental Table 2). The strongest association was observed for variant 6 to 152177055 (Intron 2; $P=0.0012$; pooled OR [95% CI]=1.42 [1.15, 1.76] for the A allele, frequency 0.016; *P*-heterogeneity=0.10). We observed no significant additional sex-specific effects or sex-genotype interactions for these imputed SNPs (data not shown).

Discussion

In this study, we exploited post-genomic tools and resources to expand on previous candidate association studies of *ESR1* in 2 main ways: (1) we analyzed a large number of common and uncommon genetic variants in the coding, noncoding, and flanking regions of the gene, capturing a large proportion of the genetic variation throughout the gene and its regulatory regions; (2) we performed these analyses in large samples of up to ≈85 000 individuals representing multiple populations of European descent, which increases our power to detect subtle risk effects.

Despite this study's power to detect case-control differences in CAD risk of as low as 10% for a broad range of genetic variation throughout this region, we found no evidence of involvement of *ESR1* in modifying CAD risk either at the population level or as a function of sex. We consider these results surprising, given ERα's central role in estrogen and androgen signaling, its widespread expression in vascular tissues, and the importance of sex for CAD risk.

After age, male sex remains the most important independent cardiovascular risk factor (CVRF), and has a far greater impact on total risk than other important risk factors, such as smoking, lipid profile, and diabetes. The physiological basis of this sex difference remains unclear, and limited research into this question has been carried out, compared with that for other risk factors, mainly because sex is nonmodifiable; however, rather than considering male sex as a nonmodifiable cause of increased CAD risk, it is important to remember that sex is a simple mendelian trait determined by the presence or absence of a single gene, *SRY*, which is inherited on the Y chromosome in males. Since, as far as we are aware, no evidence of association between CAD and *SRY* has been reported, it is not appropriate to consider sex as being causally associated with CAD risk. Rather, sex is a trait that is strongly associated with CAD risk via unknown and potentially modifiable factors (eg, physiological, environmental, behavioral factors, etc), whose effects we can partly capture by using sex as a proxy variable. It is important to identify and understand these factors because the ability to modify even a fraction of sex-associated CAD risk might have a marked impact on prevention, possibly more so than by modifying other CVRFs.

All of the loci identified by GWAS to date as being associated with CAD risk are located on autosomes, and it seems likely that most or all of the loci that explain the remaining heritability of CAD risk will also be autosomal. Consequently, these loci are in linkage equilibrium with *SRY* and have equal genotype frequencies in males and females. This leads us to the simple but important conclusion that differences in CAD risk between sex cannot be directly caused by genetic factors but can only arise because of an interaction between sex and other processes associated with risk. Consequently, the present study, like all association studies of primary autosomal genetic variation, does not attempt to explain differences in risk between sex. Instead, we search for population-level differences in CAD risk that are driven by *ESR1* variation and whose effects may or may not be different among females compared with males (ie, that interact with sex).

Over the past decade, candidate-gene association studies[31,13] have reported generally inconsistent results regarding the role of *ESR1* genetic variation in CAD risk. An initial meta-analysis including ≈7000 individuals supported association,[12] but this result was not upheld by 2 subsequent meta-analyses representing ≈16 000[13] and ≈32 000[14] individuals; however, these studies have been restricted to a very limited number of SNPs (especially rs2234693 and rs9340799, previously known as the *PvuII* and *XbaI* variants, which lie in Intron 1) out of the thousands now known to lie within the gene region. We estimate that the 4 most widely studied variants collectively capture (with $r^2\geq0.8$) only ≈2% of the 1450 SNPs tested in our study (data not shown). Therefore, although recent reports have found no evidence of association between *ESR1* variation and CAD risk,[13,14] this question remains unanswered until a more complete survey of the gene is carried out. The potential gain to be made from this is illustrated by recent advances in understanding *ESR1*'s role in modulating bone mineral density and fracture risk, phenotypes that show intriguingly similar patterns of sex-specific and menopause-related risk to those observed for cardiovascular risk. While candidate gene studies of the role of *ESR1* variation in bone mineral density and fracture risk also examined a limited range of genetic variation and obtained similarly inconsistent results,[32,33] a large meta-analysis of several GWAS subsequently confirmed the involvement of *ESR1* variation in modulating these phenotypes,[34] with highly significant evidence for association in the upstream noncoding regulatory region of the gene, in stark

contrast with the lack of association we have observed for CAD.

In the discovery stage of the CARDIoGRAM study, the direction of effect of the lead SNP was largely consistent across the contributing studies (online-only Supplemental Figure 1) but fell well short of the threshold for regional statistical significance. The region of high LD containing this SNP was located within the 5′ regulatory region but did not coincide with the previously reported signal for bone mineral density and fracture risk.[34]

We found no broadly convincing evidence of association between *ESR1* variation and CAD risk as a function of sex. Although the *P*-value of the sex interaction test for 1 SNP exceeded the significance threshold set, with opposing effects observed among males and females, this variant was not significantly associated with CAD risk in either sex considered separately (online-only Supplemental Figure 3). Considering the additional fact that this variant lies at a considerable distance from the regulatory (≈35 kb) and coding (≈186 kb) regions of the gene, we feel that these results do not provide strong evidence of a robust sex-specific association at this locus. In addition to sex, another potential modifier of the putative association between *ESR1* variation and CAD risk is menopausal status among women. Although we were unable to investigate this issue directly, we provide some initial data on this question based on age data from the MIGen study, and we find no evidence of significantly different effects of *ESR1* variation on cardiovascular risk as a function of menopausal status (see the online-only Supplemental Note).

In the fine-mapping analysis, imputation using data from the 1000 Genomes Project allowed us to analyze a much denser map of common variants in the region (online-only Supplemental Figure 4) and especially to explore the role of variants with frequencies below 0.05, which are under-represented in haplotype panels based on data from the HapMap project but which are a potentially important source of risk variance in complex diseases[35,36]; however, we found no additional evidence of association with CAD risk for any of these additional variants.

We highlight the fact that this study is well-powered to detect genetic risk effects with sizes and frequencies that are generally plausible for common complex diseases. For example, in the CARDIoGRAM discovery analysis, we have high power (≈80%) to detect common variants with MAF ≥0.15 that carry risk effects as low as OR ≈1.1 and low-frequency variants (0.01≤MAF ≤0.05) that carry risk effects of OR ≈1.3. In addition, the fine mapping analysis was also powered to detect associations for rare imputed variants with MAF ≤0.01 and effect sizes of approximately OR≈3. Weaker and/or rarer risk effects than these are likely to have limited clinical relevance at the population level. In these power computations, we used stringent statistical significance thresholds that account for multiple testing (see the online-only Supplemental Methods).

The most likely explanation for lack of observable association in this analysis is that no true association exists in this gene, although we note the following limitations in this study's ability to draw this conclusion: First, this study does not address this question in populations with non-European ancestry. Second, some truly associated variants in this gene may not have been detected by this study, although these are unlikely to be simple primary sequence variants with low allelic diversity, such as common or uncommon SNPs, low-copy number polymorphisms, or insertions/deletions. This analysis was also unable to detect very weak or very rare effects (online-only Supplemental Table 2). Third, this study cannot address the role of other potentially relevant forms of variation related to ERα, such as epistasis or epigenetic (eg, promoter methylation), post-transcriptional, or post-translational variation; however, if such variation exists, it is likely to be largely independent of primary sequence variation. Fourth, this study suggests that menopausal status does not modify the effect of *ESR1* variation on female CAD risk but cannot discount this possibility because of the size and imprecise design of that analysis. Fifth, these analyses were not adjusted for classical CVRFs, although a true SNP-CAD association would only be masked by confounding if the SNP had opposing effects on CAD risk and CVRF profile, which seems unlikely. Sixth, most of the studies included in these meta-analyses had a case-control design, which could lead to a bias against the discovery of variants that reduce survival.

Finally, it is important to note that we have analyzed the genetic variation in only 1 of the genes that encode components of the steroid sex hormone system. A more thorough exploration of this system may help to clarify the role of this system in the pathophysiology of coronary risk.

Conclusions

In conclusion, on the basis of data from a large number of subjects representing multiple samples from several populations, we find no evidence for involvement of common or uncommon genetic variation in the coding, noncoding, or flanking regions of the *ESR1* gene in modifying risk of CAD, irrespective of sex; however, data from observational studies and subanalysis of clinical trials continue to support the involvement of the steroid hormone system in modulating CAD risk. Therefore, we consider that the next step in exploring the role of the sex hormone biosynthesis system in modulating CAD risk should initially be to prioritize the investigation of other genes within this system.

Acknowledgments

See the online-only Supplemental Appendix for full list of contributors from the CARDIoGRAM Consortium. We thank all contributing members of the CARDIoGRAM Consortium for the use of cohort-level summary association results. We thank the authors of the MIGen, WTCCC, and Framingham GWAS and 1000 Genomes project for making their data publicly available and the authors of IMPUTE2 for making 1 kG-based phased haplotypes available for public use. A full list of the investigators who contributed to the generation of the WTCCC data are available from http://www.wtccc.org.uk. This manuscript was not prepared in collaboration with investigators of the Framingham Heart Study and does not necessarily reflect the opinions or views of the Framingham Heart Study, Boston University, or the National Heart, Lung, and Blood Institute. We also thank Ana Paula Dantas and Jana Selent for interesting discussions at the design stage.

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CLINICAL PERSPECTIVE

After age, male sex is the most important risk factor for cardiovascular disease; however, little research has been carried out to understand the underlying causes of sex-related risk, in comparison with work on modifiable risk factors. Although the physiological differences between the sexes are evident, the genetic differences are minimal because sex is determined by the presence (in males) of a single gene, *SRY*, on chromosome Y. This leads to the important conclusion that male sex itself is not a cardiovascular risk factor but a proxy variable that captures a large fraction of risk via other unknown, possibly modifiable, metabolic factors that differ between males and females. Sex hormone metabolism is a prime candidate system for explaining sex differences in risk. In this highly powered study, the authors perform an extensive survey of a broad range of genetic variation in the *ESR1* gene, which encodes the principal candidate for explaining sex-related differences in coronary artery disease (CAD) risk, Estrogen Receptor α, ERα. Despite ERα's central role in sex hormone signaling, its widespread expression in vascular tissues, and the importance of sex for CAD risk, the authors find no evidence for the involvement of genetic variation in *ESR1* in modifying CAD risk, either in the general population or separately in males and females. Against the context of a history of inconsistent results regarding this question, this study provides a reasonably conclusive answer and may stimulate a renewed effort to explore other elements of the sex hormone system to explain sex differences in CAD risk.

SUPPLEMENTAL MATERIAL

Post-genomic update on a classical candidate gene for coronary artery disease: *ESR1*

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Supplementary Methods

a. *CARDIoGRAM discovery analysis methods summary*

Genotyping in individual discovery GWA studies was carried out on Affymetrix or Illumina platforms. Approximately 2.3 million imputed genotypes were generated using the MACH, IMPUTE, or BIMBAM imputation algorithms and the HapMap Phase II reference panel[1]). Each primary discovery GWAS performed a logistic regression analysis to test for association between genotyped and imputed SNPs and risk of MI/CAD under an additive disease model adjusted for age and sex and taking into account the uncertainty of imputed genotypes. In every study, the variance inflation factor λ was estimated from genotyped SNPs and also used for adjustment. Quality control of these data was performed centrally according to established criteria including a check of consistency of the given alleles across all studies, quality of the imputation, deviation from Hardy-Weinberg equilibrium in the controls, minor allele frequency, and call rate.

In the present study, a meta-analysis was performed separately for every SNP from every CARDIoGRAM study that passed the quality criteria. The default meta-analysis was a fixed effect model with inverse variance weighting and calculation of two homogeneity statistics: Cochran's Q- and I^2 statistic. When there was no indication of heterogeneity for a SNP (P for Q > 0.01), the fixed effect model was maintained. When heterogeneity was present (P for Q < 0.01), a random effects model (DerSimonian-Laird) was used for that SNP.

b. *Test for interaction between SNP and gender*

To formally test for interaction between each SNP and gender in the CARDIoGRAM and fine mapping analyses (data not shown for the latter), we performed the following steps:

i. Within each CARDIoGRAM study, we computed the beta for the SNP-gender interaction term as the absolute difference between the betas for females and males.
ii. Within each CARDIoGRAM study, we computed the standard error of the SNP-gender interaction term as square root of the sum of the squares of the standard errors of the β from the female and male analysis.
iii. We then used these betas and standard errors to perform fixed or random effects meta-analyses according to the same protocol as that used for the un-stratified analysis.

c. *Power Calculations*

We performed a post-hoc calculation of our analyses' power to detect significant associations. We allowed that power is determined by sample size, the proportion of cases and controls for the case-control studies or the number of events for the cohort study, the effect of a variant on risk

(e.g. OR), and the frequency of the minor allele (MAF) of the associated variant, the p-value threshold required to declare statistical significance, LD between correlated and causal variants, genotyping error, the quality of imputation for imputed variants, between-study heterogeneity in the meta-analysis, and possibly other factors. Of these, MAF is the most important non-modifiable determinant of power, and so we estimated power for a series of representative sub-ranges of MAF. Rather than attempting to parameterize all of the other factors, we captured their effects by using the standard error (SE) from the meta-analysis of all three studies, which is inversely correlated with power. In these power calculations, the variant's effect on disease risk was taken as the beta from the meta-analysis of all studies, and thus represented the odds ratios for the case-control studies and hazard ratio for cohort studies, where applicable; ORs and HRs are considered to be comparable because the prevalence of the phenotype in the cohort studies is relatively low. All power computations were based on an alpha value (Type I error rate) equivalent to the threshold required to declare a statistically significant association after adjustment for multiple testing (see main text). Within each analysis we performed the following steps:

i. For each SNP in the analysis, MAF was taken to be the mean MAF across all studies.
ii. SNPs were binned according to the following sub-ranges of MAF: (0,0.01], (0.01,0.02], (0.02,0.03], (0.03,0.04], (0.04,0.05], (0.05,0.06], (0.06,0.07], (0.07,0.08], (0.08,0.09], (0.09,0.1], (0.1,0.15], (0.15,0.2], (0.2,0.3], (0.3,0.4] and (0.4,0.5].
iii. For each sub-range of MAF the mean of the SE of all SNPs within that sub-range was computed, and used to compute and express the power of the analysis in the following two ways.
iv. The minimum effect size (beta) the analysis had high (~80%) or moderate (~50%) power to detect. The definitions of high and moderate power were selected arbitrarily to indicate where our analysis was well powered to detect risk effects (high power), but also to allow for the fact that, if multiple independent but more subtle effects were present, at least some proportion of these could also be detected (e.g. 50%, moderate power).
v. The power of the analysis to detect each of a series of effect sizes (betas, corresponding to the following odds ratios: 1.05, 1.1, 1.2, 1.3, 1.5, 1.7, 2, 2.5 and 3). These data were computed to help indicate the circumstances under which our study was unable to provide conclusive information, e.g. for rarer variants or for more subtle effect sizes.

The results of these power calculations are shown in Supplementary Table 2

Supplementary Note

Preliminary age-stratified analysis to explore possible menopause-related *ESR1* effects among women

After age and gender, menopausal status among women appears to be one of the strongest determinants of CAD risk. We explored the possibility that the effect of genetic variation in *ESR1* on CAD risk may vary among women according to menopausal status. Although this variable was not available for any of the CARDIoGRAM studies or for the three studies included in the fine mapping analysis, we attempted to capture most of its variation using a proxy variable based on age (<50 years or ≥50 years[2]), and then tested for interaction between this proxy variable and genotype. This analysis was performed only for MIGen owing to the lack of age data for the WTCCC sample, and the low number of events in the Framingham study.

We observed no regionally significant interaction between this proxy variable and genotype for any variant in the region of interest (minimum p-value=0.0012 for rs11968025), although we note the limited sample size of this analysis (number of females aged <50 yrs and ≥50 yrs was 832 (of which 389 were cases) and 582 (of which 274 were cases), respectively).

Supplementary Tables

Supplementary Table 1. Chromosomal locations of coding and non-coding exons in *ESR1*.

Exon Name[*]	Coding[†]	Start[‡]	Stop[‡]	Length (bp)	Position with respect to translation start site[§]	AA length
E2	-	151977808	151977899	91	-151240	
F	-	152011675	152011800	125	-117373	
E1	-	152023011	152023141	130	-106037	
T1	-	152112508	152112595	87	-16540	
T2	-	152112697	152112848	151	-16351	
D	-	152125065	152125160	95	-3983	
C	-	152125748	152126956	1208	-3300	
B	-	152128494	152128645	151	-554	
A	-	152128816	152128978	162	-232	
1	+	152128979[‖]	152129499	521	-70	151
2	+	152163732	152163922	190	34684	64
3	+	152201790	152201906	116	72742	39
4	+	152265308	152265643	335	136260	112
5	+	152332791	152332929	138	203743	46
6	+	152382126	152382259	133	253078	45
7	+	152415520	152415703	183	286472	61
8	+	152419867	152420102	235	290819	77
3' UTR	-	152420103	152424406	4303	291055	

AA: Amino Acid; bp: base pairs. 152177055

* Name assigned by Koš *et al.*[3] to non-coding exons, or sequentially for coding exons

[†] Non-coding, -; coding, +

[‡] Position in GRCh37.p1 determined using information provided by Koš *et al*. for non-coding exons and the Exon 1 start point, and from the Ensembl exon report for the coding exons (ENSG00000091831; www.ensembl.org) otherwise.

[§] Translation start codon begins at 152129048, 70bp downstream of the beginning of Exon 1

[‖] Common splice acceptor site reported by Koš *et al.*

Supplementary Table 2. Power computation – see Supplementary Methods for details

	Minor Allele Frequency range		**(0,0.01]**	**(0.01, 0.02]**	**(0.02, 0.03]**	**(0.03, 0.04]**	**(0.04, 0.05]**	**(0.05, 0.06]**	**(0.06, 0.07]**	**(0.07, 0.08]**	**(0.08, 0.09]**	**(0.09, 0.1]**	**(0.1, 0.15]**	**(0.15, 0.2]**	**(0.2, 0.3]**	**(0.3, 0.4]**	**(0.4, 0.5]**
CARDIoGRAM (*n=86,995*)	Number of SNPs		0	4	13	12	3	15	12	11	13	23	60	79	106	87	97
	OR detectable	high power (0.8)	--	1.329	1.277	1.220	1.200	1.283	1.214	1.203	1.150	1.150	1.121	1.103	1.081	1.074	1.071
		moderate power (0.5)	--	1.263	1.223	1.178	1.161	1.227	1.173	1.164	1.122	1.121	1.098	1.084	1.066	1.060	1.058
	Power (*to detect OR*)	1.05	--	0.001	0.0017	0.0034	0.0047	0.0028	0.0055	0.0086	0.015	0.025	0.048	0.07	0.19	0.27	0.33
		1.1	--	0.011	0.023	0.057	0.085	0.047	0.100	0.15	0.28	0.42	0.62	0.77	0.96	0.98	0.98
		1.2	--	0.2	0.37	0.67	0.79	0.39	0.68	0.71	0.97	0.89	0.96	0.99	1	1	1
		1.3	--	0.68	0.87	0.99	1	0.79	0.98	0.98	1	0.98	1	1	1	1	1
		1.5	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		1.7	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2.5	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		3	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CARDIoGRAM Females (*n=30,615*)	Number of SNPs		0	4	1	8	1	10	8	16	10	19	65	71	102	89	96
	OR detectable	high power (0.8)	--	1.577	1.490	1.400	1.412	1.472	1.365	1.292	1.279	1.222	1.201	1.184	1.162	1.13	1.123
		moderate power (0.5)	--	1.454	1.388	1.318	1.328	1.374	1.291	1.235	1.224	1.18	1.162	1.149	1.132	1.106	1.1
	Power (*to detect OR*)	1.05	--	0.00038	0.00048	0.0007	0.00065	0.00068	0.0012	0.0019	0.0019	0.0033	0.0048	0.0064	0.015	0.024	0.03
		1.1	--	0.0021	0.0029	0.0057	0.0050	0.0051	0.014	0.026	0.026	0.055	0.087	0.12	0.28	0.43	0.5
		1.2	--	0.027	0.042	0.098	0.083	0.1	0.23	0.39	0.39	0.66	0.79	0.89	0.91	0.99	1
		1.3	--	0.14	0.22	0.43	0.39	0.35	0.58	0.79	0.84	0.98	0.99	0.99	0.97	1	1
		1.5	--	0.63	0.82	0.96	0.95	0.77	0.93	0.99	1	1	1	1	1	1	1
		1.7	--	0.93	0.99	1	1	0.97	1	1	1	1	1	1	1	1	1
		2	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		2.5	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
		3	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1

CARDIoGRAM Males (*n=32,069*)	Number of SNPs	0	1	7	6	3	2	3	14	14	23	68	75	100	94	97
	OR detectable: high power (0.8)	--	1.488	1.382	1.304	1.261	1.226	1.336	1.352	1.344	1.202	1.211	1.152	1.123	1.101	1.096
	OR detectable: moderate power (0.5)	--	1.387	1.305	1.244	1.21	1.182	1.269	1.281	1.275	1.164	1.171	1.123	1.1	1.082	1.078
	Power (*to detect OR*): 1.05	--	0.00048	0.0008	0.0014	0.0021	0.0029	0.0015	0.0016	0.0021	0.0068	0.0099	0.017	0.045	0.072	0.09
	Power (*to detect OR*): 1.1	--	0.003	0.0072	0.016	0.029	0.047	0.021	0.024	0.034	0.13	0.19	0.32	0.62	0.79	0.85
	Power (*to detect OR*): 1.2	--	0.043	0.13	0.28	0.45	0.63	0.29	0.23	0.29	0.76	0.75	0.95	0.96	1	1
	Power (*to detect OR*): 1.3	--	0.22	0.49	0.77	0.91	0.99	0.62	0.56	0.58	0.94	0.86	0.97	0.98	1	1
	Power (*to detect OR*): 1.5	--	0.83	0.97	1	1	1	0.98	0.97	0.96	1	0.98	1	1	1	1
	Power (*to detect OR*): 1.7	--	0.99	1	1	1	1	1	1	1	1	1	1	1	1	1
	Power (*to detect OR*): 2	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Power (*to detect OR*): 2.5	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	Power (*to detect OR*): 3	--	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CARDIoGRAM Gender*Genotype Interaction (*n=62,711*)	Number of SNPs	0	0	4	5	3	10	12	11	13	21	60	75	103	85	97
	OR detectable: high power (0.8)	--	--	1.646	1.535	1.525	1.753	1.523	1.345	1.382	1.3	1.265	1.237	1.185	1.17	1.161
	OR detectable: moderate power (0.5)	--	--	1.506	1.422	1.415	1.586	1.413	1.276	1.305	1.24	1.213	1.191	1.15	1.138	1.131
	Power (*to detect OR*): 1.05	--	--	0.00032	0.00042	0.00043	0.00033	0.00053	0.001	0.00085	0.0014	0.002	0.0026	0.0059	0.0081	0.0099
	Power (*to detect OR*): 1.1	--	--	0.0015	0.0024	0.0025	0.0018	0.0039	0.011	0.008	0.017	0.028	0.04	0.11	0.16	0.2
	Power (*to detect OR*): 1.2	--	--	0.017	0.033	0.034	0.025	0.063	0.2	0.14	0.3	0.44	0.57	0.88	0.94	0.96
	Power (*to detect OR*): 1.3	--	--	0.089	0.17	0.18	0.11	0.26	0.65	0.51	0.79	0.9	0.97	1	1	1
	Power (*to detect OR*): 1.5	--	--	0.49	0.72	0.74	0.37	0.7	0.97	0.95	1	1	1	1	1	1
	Power (*to detect OR*): 1.7	--	--	0.86	0.97	0.98	0.66	0.94	1	1	1	1	1	1	1	1
	Power (*to detect OR*): 2	--	--	0.99	1	1	0.93	1	1	1	1	1	1	1	1	1
	Power (*to detect OR*): 2.5	--	--	1	1	1	1	1	1	1	1	1	1	1	1	1
	Power (*to detect OR*): 3	--	--	1	1	1	1	1	1	1	1	1	1	1	1	1

Fine Mapping (*n=17,126*)	Number of SNPs		22	139	187	12	28	28	18	54	70	20	206	80	215	219	153
	OR detectable	high power (0.8)	3.139	2.094	1.644	1.667	1.409	1.436	1.389	1.333	1.326	1.334	1.247	1.214	1.178	1.17	1.193
		moderate power (0.5)	2.586	1.847	1.511	1.528	1.33	1.35	1.314	1.27	1.264	1.27	1.201	1.175	1.146	1.139	1.158
	Power (*to detect OR*)	1.05	0.000057	0.00009	0.00017	0.00019	0.00037	0.00037	0.00046	0.00069	0.00087	0.00091	0.0018	0.0025	0.0054	0.0066	0.0067
		1.1	0.00013	0.00035	0.0011	0.0013	0.004	0.0042	0.0058	0.011	0.015	0.017	0.041	0.062	0.15	0.18	0.17
		1.2	0.00066	0.0035	0.018	0.025	0.094	0.1	0.14	0.25	0.33	0.34	0.63	0.72	0.88	0.9	0.76
		1.3	0.0026	0.02	0.11	0.15	0.44	0.43	0.53	0.73	0.76	0.75	0.92	0.97	0.99	0.99	0.95
		1.5	0.021	0.16	0.57	0.62	0.92	0.84	0.91	0.93	0.91	0.89	0.96	1	1	1	1
		1.7	0.078	0.43	0.84	0.82	0.97	0.95	0.98	0.98	0.98	0.96	0.99	1	1	1	1
		2	0.24	0.72	0.95	0.89	0.99	0.99	1	1	1	1	1	1	1	1	1
		2.5	0.53	0.9	0.99	0.95	1	1	1	1	1	1	1	1	1	1	1
		3	0.72	0.95	0.99	0.98	1	1	1	1	1	1	1	1	1	1	1

Notes:

1. Within each analysis, the number of SNPs whose mean SE was used to compute power is shown for each sub-range of MAF.
2. 'OR detectable' indicates the minimum risk effect detectable (expressed as the exponent of the beta from the meta-analysis) with high or moderate power. 'Power' indicates the study's power to detect the effect sizes (odds ratios) shown.
3. Effect sizes detectable or for which power is shown are expressed as the exponent of the absolute beta from the meta-analysis (i.e. the odds ratio computed with the lower risk group set as the reference group). Thus, in the CARDIoGRAM, Females, Males, and Fine Mapping analyses, these are the odds ratios associated for each additional copy of the risk allele; in the Gender*Genotype Interaction analysis these are the odds ratios for difference in risk between sexes.
4. Power does not increase linearly with increase in MAF because these data are based on empirical SE values, which may be affected by other factors (e.g. imputation quality, between-study heterogeneity in the meta-analysis, etc.) for SNPs in some sub-ranges of MAF compared to others.
5. In the computation of power for given effect size, scenarios with high power (≥80%) are shaded dark grey, those with moderate power (≥50% and <80%) are shaded light grey, and those with power lower than 50% are unshaded.

Supplementary Figures

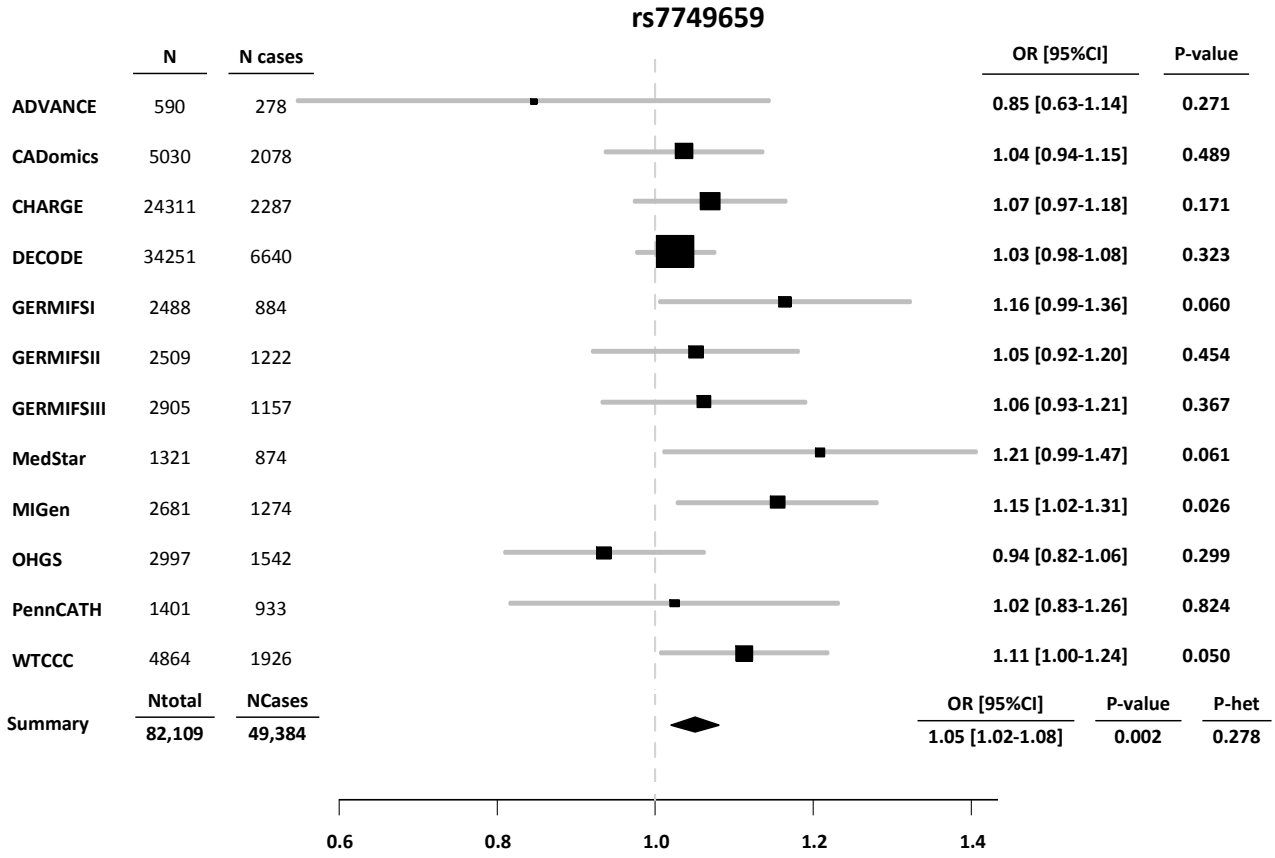
Supplementary Figure 1. Summary of quality control and analysis procedures in the fine mapping analysis.

<QC/analysis step>	MIGen	WTCCC	FHS	
Study design	case/control	case/control	cohort	Data availability
prior subject-level QC *(sample call rate)*	*≥0.95*	*≥0.95*	*≥0.95*	
Sample size	6042	7368	3717*	
N (cases/controls)	2967/3075	1988/5380	464/3253	
Number of genotyped SNPs in the region of interest	149	81	115	
SNP level QC *(SNPs removed: ≤95% call rate;HWE p≤10-6)*	*0;0*	*0;5*	*9;2*	SNP QC
Number of SNPs passing QC	149	76	105	
Imputation				Imputation
Number of genotyped and imputed SNPs	2473†	2472†	2477†	
post-imputation QC *(SNPs removed: IMPUTE2* INFO*<0.5)*	*891*	*970*	*814*	
Number of SNPs genotyped or imputed with high quality	1582†	1502†	1663†	
remove SNPs not present in all studies *(number of SNPs removed)*	*131*	*51*	*212*	Association and meta-analysis
Number of SNPs common to all studies	1451			
association testing				
meta-analysis	***17121 subjects in total***			
Gender-stratified analysis ***(n/cases/controls)***	Females: 6570/1270/5300; Males: 10540/4139/6401			
females (n/cases/controls)	1414/663/751	3053/406/2647	2103/201/1902	
males (n/cases/controls)	4611/2294/2317	4315/1582/2733	1614/263/1351	

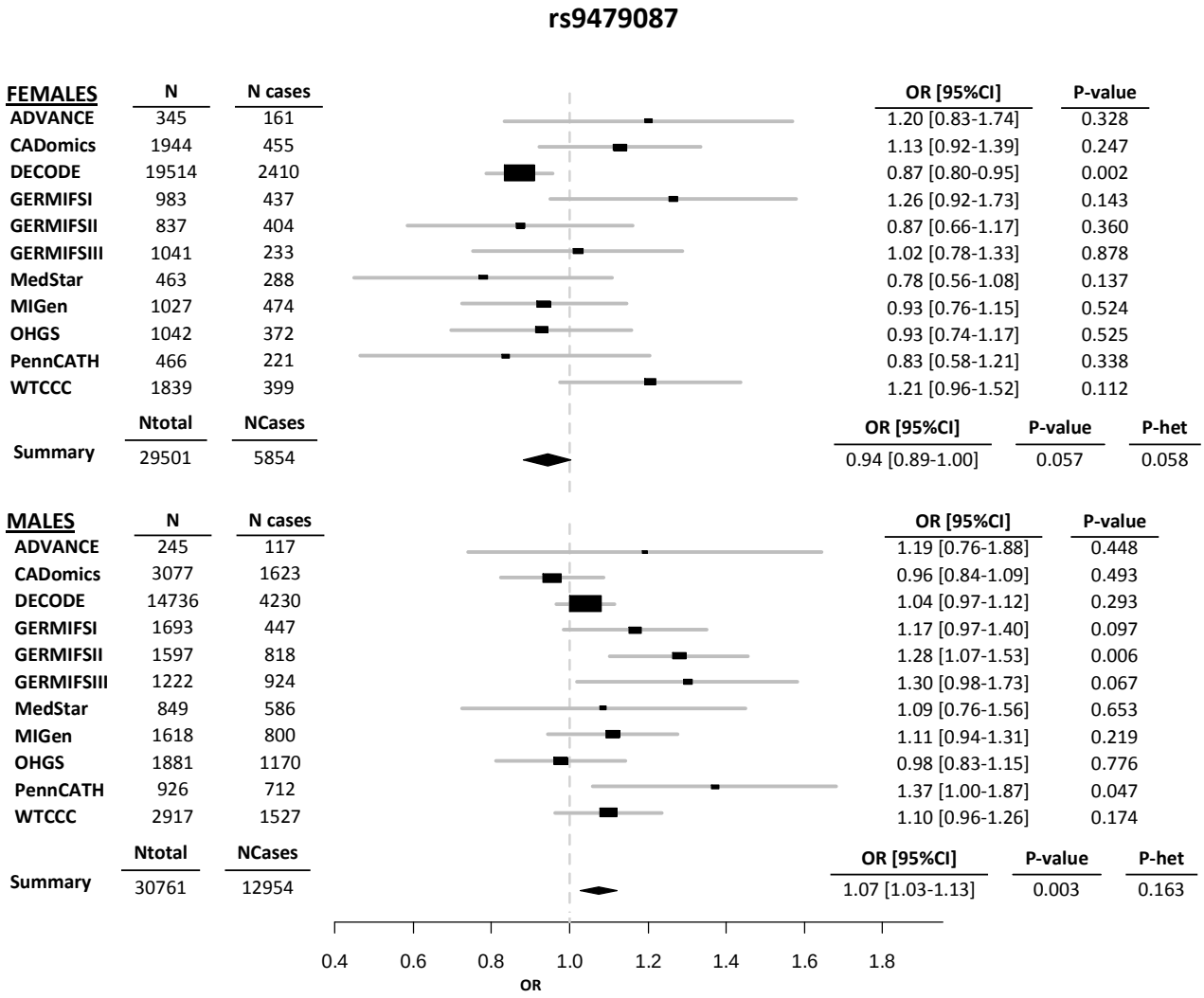
* The publicly available dataset for the Framingham study contained genotype and phenotype data for 9,270 individuals. In the current analysis, we included 3,717 individuals from the original and offspring cohorts for whom survival data were available for the follow-up periods beginning at visits 15 and 5 respectively. The sample selection and process used to filter these individuals is described in more detail in Lluís-Ganella *et al*. 2011 (submitted).

† For all three studies, all genotyped SNPs were also present in the 1kG reference panel. These values show the total number of SNPs, including genotyped SNPs, after imputation, post-imputation QC, and filtering to include SNPs common to all studies.

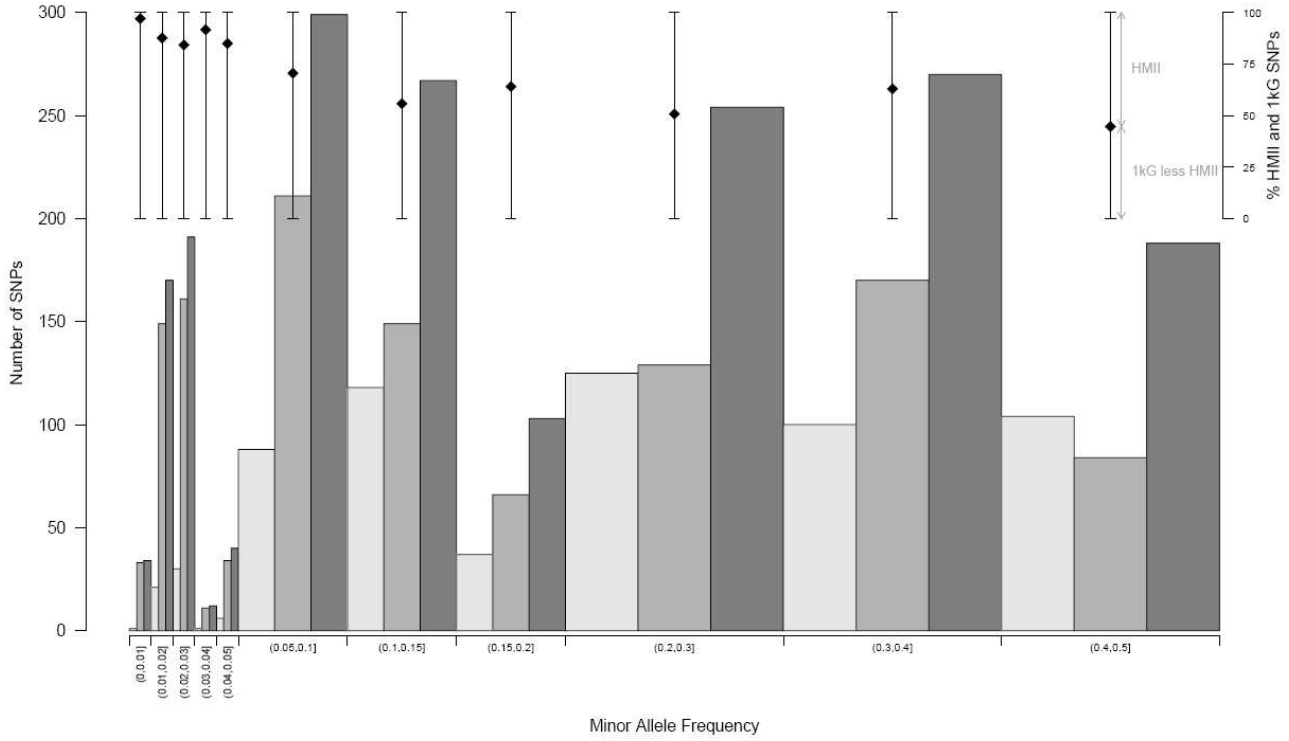
Supplementary Figure 2. CARDIoGRAM global meta-analysis results for the top SNP in the region of interest. Total sample size, number of cases, OR and 95% CI are shown for each contributing study, in addition to global sample sizes, OR, 95%CI, and p-values for association and heterogeneity. Note that only 12 of the 14 CARDIoGRAM studies are represented, as data for this variant was not available in the LURIC 1 and LURIC 2 samples.



Supplementary Figure 3. Meta-analysis results for the CARDIoGRAM SNP with the greatest difference in association between males and females (strongest interaction with gender). Total sample size, number of cases, OR and 95% CI are shown for each contributing study, in addition to global sample sizes, OR, 95%CI, and p-values for association and heterogeneity. Note that only 11 of the 14 CARDIoGRAM studies are represented, as data for this variant was not available in the LURIC 1, LURIC 2 and CHARGE samples.



Supplementary Figure 4. Distribution of minor allele frequencies (MAF) for SNPs analyzed in this study.
Data are shown as vertical bars whose widths are proportional to the ranges of MAF indicated on the x-axis, and whose heights correspond to the absolute number (left y-axis) of SNPs whose MAF falls within those ranges (MAF computed as the weighted mean in the MIGen, WTCCC and Framingham samples).
The number of SNPs within the region of interest that were genotyped or imputed in (a) the CARDIoGRAM meta-analysis (corresponding to the high-quality SNPs from the HapMapII reference panel) are indicated as white bars; (b) the number of additional SNPs imputed in the fine mapping analysis in this study are shown as light grey bars; the total the number of SNPs analyzed in the fine mapping analysis in this study (a plus b, corresponding to high-quality SNPs from the 1kG reference panel) are shown as dark grey bars.
Within each sub-range of MAF, the vertical black lines and diamonds at the top of the graph represent the proportions of SNPs analyzed in the fine mapping analysis (1kG panel, see (c) above). The portion of the line above the diamond represents the percentage (right y-axis) of these SNPs that were included in the HapMapII panel, and the portion below the diamond represents those additional SNPs that were imputed in the present study. This graph shows that many additional SNPs with a broad range of MAFs were imputed in this study, but that the greatest gain of information was obtained for rarer SNPs.



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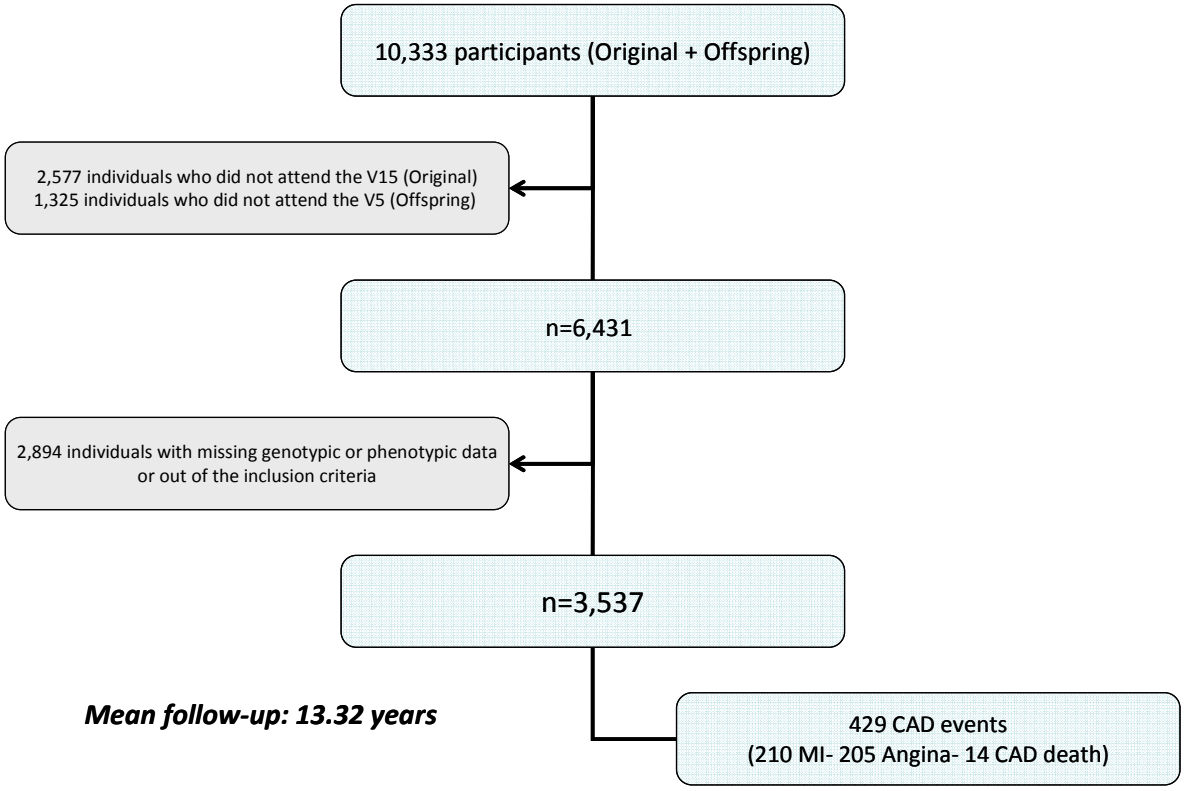
Supplementary Appendix 1. Process of selection of participants from the Framingham study (from Lluís-Ganella *et al.*, submitted).



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Post-Genomic Update on a Classical Candidate Gene for Coronary Artery Disease: *ESR1*

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