Prediction of Causal Candidate Genes in Coronary Artery Disease Loci


Objective—Genome-wide association studies have to date identified 159 significant and suggestive loci for coronary artery disease (CAD). We now report comprehensive bioinformatics analyses of sequence variation in these loci to predict candidate causal genes.

Approach and Results—All annotated genes in the loci were evaluated with respect to protein-coding single-nucleotide polymorphism and gene expression parameters. The latter included expression quantitative trait loci, tissue specificity, and miRNA binding. High priority candidate genes were further identified based on literature searches and our experimental data. We conclude that the great majority of causal variations affecting CAD risk occur in noncoding regions, with 41% affecting gene expression robustly versus 6% leading to amino acid changes. Many of these genes differed from the traditionally annotated genes, which was usually based on proximity to the lead single-nucleotide polymorphism. Indeed, we obtained evidence that genetic variants at CAD loci affect 98 genes, which had not been linked to CAD previously.

Conclusions—Our results substantially revise the list of likely candidates for CAD and suggest that genome-wide association studies efforts in other diseases may benefit from similar bioinformatics analyses. (Arterioscler Thromb Vasc Biol. 2015;35:0000-0000. DOI: 10.1161/ATVBAHA.115.306108.)

Key Words: coronary artery disease ▪ genome-wide association study ▪ MicroRNAs ▪ single-nucleotide polymorphism ▪ systems biology

The most recent meta-analysis of genome-wide association studies (GWAS) for coronary artery disease (CAD) identified 46 genome-wide significant and 104 genome-wide suggestive loci associated with increased risk. Together these loci explain ≈10% of the heritability. Although quantitatively the effects of common risk alleles identified by GWAS, for example, in the HMGCoR, LDLR or PCSK9 genes, are modest, the pathways tagged by these genes have utmost clinical importance as they constitute prime targets for preventive medication. Accordingly, the largest scientific relevance of GWAS discoveries is seen in elucidation of yet unknown causal mechanisms leading to CAD in the human population.

Twelve of the genome-wide significant loci are associated with blood lipid levels and 5 with blood pressure, suggesting that they function through these intermediate phenotypes to increase the risk for CAD.† However, the precise genetic mechanisms at the CAD loci affecting either intermediary traits or as of today unknown pathways leading...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AA</td>
<td>amino acid</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<tr>
<td>eQTL</td>
<td>expression quantitative trait loci</td>
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<td>GWAS</td>
<td>genome-wide association studies</td>
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<td>LD</td>
<td>linkage disequilibrium</td>
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<td>SNP</td>
<td>single-nucleotide polymorphism</td>
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to disease are largely unknown. Thus, translating GWAS loci into genes and pathways will help to provide novel insight into disease susceptibility and ultimately lead to novel treatments for patients with CAD that may also be tailored to the genetic and molecular makeup of individual patients.2

In theory, the process of moving from an associated genetic variant to a disease mechanism seems straightforward and linear: first, identify the causal (rather than the associated) variant, next identify how the causal variant alters gene function of the putative causal gene, and then work out how the altered function of an affected gene perturbs processes at the molecular, cellular, physiological, and whole organism levels that ultimately promote the development of atherosclerosis. However, barring a few notable examples where relevant intermediary phenotypes (eg, an effect on plasma cholesterol) already pointed to a pathway,3,4 experience has shown that dissecting the mechanism is complex. Most lead single-nucleotide polymorphisms (SNPs) identified by GWAS map outside protein-coding regions. Rather, accumulation of lead SNPs was found in regulatory elements that have been identified, for example, in the regions. Rather, accumulation of lead SNPs was found in regulatory elements that have been identified, for example, in the regions. However, barring a few notable examples where relevant intermediary phenotypes (eg, an effect on plasma cholesterol) already pointed to a pathway,3,4 experience has shown that dissecting the mechanism is complex. Most lead single-nucleotide polymorphisms (SNPs) identified by GWAS map outside protein-coding regions. Rather, accumulation of lead SNPs was found in regulatory elements that have been identified, for example, in the regions.

A systematic attempt predicting candidate causal genes and their functional mechanisms in all 159 CAD genome-wide significant and suggestive loci has not yet been carried out. In this study, we used a bioinformatics approach (Figure 1) to (1) catalog all the transcript-coding genes in these loci, (2) evaluate structural SNPs in the protein-coding genes, (3) identify expression quantitative trait loci (eQTL) that overlap CAD loci, and (4) prioritize candidate genes with respect to their likely functional relevance based on evidence from the literature and experimental results from our previous studies.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Locus Boundaries and Genes Within Loci

As a first step, we defined the boundaries of each of the 159 CAD loci by determining the locations of proxy SNPs ($r^2 > 0.8$) on either side of the lead SNP (Figure 1A in the online-only Data Supplement). There were 3432 proxy SNPs based on the 1000 genomes EUR genotypes determined by the SNAP bioinformatics tool.11 The SNPs were located in 135 nonoverlapping regions. Nine lead SNPs loci had no proxy SNPs suggesting that they probably represent the causal SNP (Table I in the online-only Data Supplement). The size of the remaining regions ranged from 488 bp to 566 kb with an average of 76.5 kb.

To catalog the genes within these CAD loci, we searched the latest release of the ENSEMBL database (release 75), which contains gene model annotations from RefSeq,12 GENCODE,13 and ENSEMBL/HAVANA14 databases. Collectively, within the boundaries of the CAD loci, there were 183 protein-coding genes along with genes for 29 antisense transcripts, 27 long intergenic noncoding RNAs, 15 miscellaneous RNAs, 12 miRNAs, 8 small nuclear RNAs, 8 small nucleolar RNAs, 3 intronic sense RNAs, 3 processed transcripts that do not contain an open reading frame, 2 long noncoding transcripts that contain a coding gene in their introns on the same strand, and 1 ribosomal RNA (Figure 1B in the online-only Data Supplement).

SNP Prioritization Pipeline Overview

To identify the most plausible causal SNP for each locus, we developed an SNP prioritization pipeline (Figure 1). The pipeline consists of 2 main parts: identification of candidate SNPs (Figure 1A) and the identification of genes functionally related to those SNPs (Figure 1B). We considered the 159 peak GWAS SNPs and the 3432 SNPs in high linkage disequilibrium (LD) with the peak SNPs. We assessed the functional implication of each of these SNPs based on 3 main criteria: (1) we checked whether the SNP cause a deleterious amino acid (AA) change; (2) we identified all SNPs that have an eQTL effect, and (3) we identified SNPs that lie within known regulatory regions of the genome. We further analyzed the SNPs that fulfilled at least of one these criteria as potential causal SNPs. The results of the annotation are shown in Table II in the online-only Data Supplement.

Each potential causal SNP assembled by these criteria and the gene it affects either because of AA change or eQTL were next analyzed in the second part of the pipeline. To establish a link between the disease and the candidate genes, we assessed all relevant information from published resources as well as experimental evidence from our laboratories.

CAD Loci With Predicted Nonsynonymous/Deleterious Mutations

To determine SNPs that have protein-altering effects, we annotated all SNPs using ANNOVAR software.15 The gene annotation was based on RefGene, KnownGene, and several functional prediction scores such as SIFT, PolyPhen, and Mutation Taster using the annovar LJB2 database16 as well as the CADD database.17
Of the 159 lead SNPs, 33 (or 20.7%) are exonic: 11 synonymous and 22 nonsynonymous (either the lead SNP itself or its proxy SNPs; Table III in the online-only Data Supplement); 66.7% of all exonic SNPs lead to nonsynonymous AA changes in CAD loci. This percentage was not statistically significantly enriched compared with all known nonsynonymous common SNPs (>1% MAF) in exonic regions as identified in the 1000 genomes data (48.9% of all exonic SNPs, Fisher \( P = 0.053 \)).

Next, we focused on the deleterious SNPs because they are likely to be causal (Table). Five lead SNPs (rs3184504, rs3825807, rs867186, rs2571445, and rs11556924) cause AA changes. Three of these are predicted to be deleterious. In addition, 2 of these had proxy SNPs that also cause deleterious AA changes. Furthermore, for 7 other lead SNPs, we identified proxy SNPs that are also predicted to be deleterious. In total, 12 SNPs predicted to be deleterious represent 10 independent loci.

Our results demonstrate the complexity for some of the loci. For example, 2 SNPs (rs1137524 and rs1060407) in the chromosome 3p21.31 locus affect the gene MAP4 complicating the identification of the causal SNP. Furthermore, the lead SNP, rs867186, in the 20q11.22 locus causes a deleterious AA change in the PROCR gene. This SNP is also in high LD with rs11906160 (\( r^2 = 0.92 \)), which causes a deleterious AA change in the MYH7B gene making it difficult to identify if one or both of these genes are causal for CAD in this locus.

**CAD Loci With Regulatory Effects on Gene Expression**

To determine SNPs that have effects on gene expression, we interrogated several eQTL results that are part of the Genome-Wide Repository of Associations between SNPs and Phenotypes (GRASP) database, Stockholm Atherosclerosis...
Gene Expression (STAGE) study, MGH liver/adipose study, and aortic endothelial cells study. These eQTL results are from >50 tissues and cell types, some are highly relevant to atherosclerosis, such as liver, adipose, and vessel wall as well as monocytes, macrophages, and endothelial cells. We looked for significant association between CAD loci and nearby gene expression (within 1 MB of the lead SNP). We eliminated the spurious associations by assessing if the most significantly associated expression SNP is among the 3591 CAD SNPs. The significant associations and the source of the eQTL association are presented in detail in Table II in the online-only Data Supplement. In total, we found significant associations between 66 CAD lead SNPs and nearby gene expression. This is in contrast to 10 CAD loci that are predicted to harbor complex mechanisms involving nearby gene expression.

AA Changes and eQTLs

Our analyses lead to the prediction of complex mechanisms in some of the loci. For example, CAD SNP rs2246833 at the 10q23.31 locus has a proxy SNP (rs1051338, r2 = 0.89) which leads to an AA change in the LIPA gene (Thr16Pro). The same SNP is also associated with the expression level of LIPA gene in the cardiogenics monocyte/eQTL data set from 758 individuals (P = 3.4 × 10−16). The risk allele (T) is associated with increased expression of the gene (β = 0.46), consistent with earlier reports. The risk allele also shows significant associations with increased LIPA expression in the MGH data set for liver, subcutaneous tissue, and omental adipose tissue, with the most significant association in the liver (P = 2.8 × 10−4; n = 741 individuals), followed by omental fat (P = 2.0 × 10−15; n = 567 individuals) and subcutaneous fat (P = 1.0 × 10−8; n = 612 individuals).

Multiple eQTL Genes in CAD Loci

Another example of complexity was the presence of multiple eQTL genes in CAD loci. In more than half of the loci (38 of 66), the risk SNP affected expression of multiple genes suggesting that several mechanisms, perhaps functioning in different tissues, could be influencing the disease susceptibility.

For example, CAD risk SNP rs17514846 in the 5q26.1 locus is located in the intron of the FURIN gene and is associated with its expression but also with 2 nearby genes, FES and MAN2A2.

Table. Predicted Deleterious CAD SNPs

<table>
<thead>
<tr>
<th>Proxy SNP</th>
<th>Lead SNP</th>
<th>Chr</th>
<th>SNP Position (hg19)</th>
<th>Gene</th>
<th>Transcript ID</th>
<th>Amino Acid Change</th>
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<td>rs35107735*</td>
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<td>47958037</td>
<td>MAP4</td>
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<td>32663496</td>
<td>ZC3HC1</td>
<td>NM_018256</td>
<td>p.R363H</td>
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<td>rs7074064</td>
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<td>rs867186</td>
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</table>

Of the 29 CAD-related nonsynonymous SNPs, 12 are predicted to have a functional effect. CAD indicates coronary artery disease; and SNP, single-nucleotide polymorphism.

*SNPs indicate that the lead SNP is the potential functional SNP.

Expression SNPs Located in Promoters

SNPs located in promoter or enhancer regions are likely causal variants for regulating the gene expression levels. Twenty-five CAD SNPs with eQTL effects are located in promoter histone marks based on HaploReg annotation (Table II in the online-only Data Supplement). For example, the CAD risk SNP rs590121 in the chromosome 11q13.4 locus is associated with the expression of multiple genes in different tissues, including liver, adipose, and vessel wall as well as monocytes, macrophages, and endothelial cells, as demonstrated by the eQTL results from the cardiogenics monocyte/eQTL data set from 758 individuals. The significant associations and the source of the eQTL association are presented in detail in Table II in the online-only Data Supplement.
with the expression of SERPINH1 and lies in the promoter of the same gene suggesting that the risk SNP alters the binding of transcription factors affecting SERPINH1 gene expression levels and is likely the causal SNP. However, rs2028900, in high LD \((r^2=0.93)\) with the CAD risk SNP rs1561198 (2p11.2 locus), lies in the promoter region of MAT2A and is associated with its expression but it is also associated with the expression of nearby genes VAMP8, VAMP5, and GGCX, making it difficult to predict the causal relationship between the risk SNP and nearby genes.

### Tissue-Specific eQTL Effects

In 2 loci, we observed tissue-specific effects of the risk SNPs. SNP rs602633, located between PSRC1 and CELSR2 genes, in the 1p13 locus has been associated with CAD and lipid levels.25 This locus regulates the expression level of PSRC1, CELSR2, and SORT1 genes in the human liver. In a recent study, hepatic expression of SORT1 has been shown to regulate lipid levels and, therefore, this gene has been predicted to be the causal gene in this locus.3 We observed that the lipid-lowering T allele (of rs602633) is associated with the higher expression of CELSR2 in liver tissue but is associated with lower expression levels in adipose tissue (Figure 2A) suggesting that tissue-specific eQTL effects need to be considered when dissecting the mechanisms of GWAS loci.

Similarly, in the chromosome 17p11 locus, rs4299203 has a suggestive association with CAD (Figure 2B). Expression levels of 5 genes, DRG2, C17orf39, MYO15A, TOM1L2, and SREBF1, are associated with this locus in various tissues (Table II in the online-only Data Supplement). The CAD risk allele (G) is associated with higher level SREBF1 expression in monocytes but is associated with lower expression in macrophages (Figure 2B). SREBF1 encodes the sterol regulatory element-binding protein 1 (SREBP-1), 1 of the 2 major transcription factors that regulate cellular cholesterol levels. This locus is not associated with plasma lipid levels25 suggesting that the SNP effect on SREBF1 is independent of the possible effects of the locus on lipid levels.

### CAD SNPs Affecting miRNA Binding

A possible mechanism by which risk SNPs affect gene expression is altering the affinity of miRNA binding to the 3′ untranslated regions (UTRs) of disease genes. For example, rs12190287 in the 6q23.2 locus resides in the 3′ UTR of the TCF21 gene and affects binding of miR-224.26 Therefore, we interrogated the CAD SNPs mapping to 3′ UTR region of genes using the microSNIPER database to assess whether they could reside in a predicted target miRNA-binding site.27 We restrained our analysis to miRNAs with predicted seed length of 7mers or more. Fifty-five 3′ UTR CAD SNPs from 33 distinct genes were predicted to lie within an miRNA-binding site for a total of 254 distinct miRNAs (Table IV in the online-only Data Supplement). The predicted number of miRNAs targeting the 3′ UTR region of a gene ranged from 1 (for BCAP29, MAP4, RND3, and WDR12) to 29 (for MRAS). Of note, 23 miRNAs were predicted to bind >1 candidate
CAD gene. For example, hsa-miR-130a-5p was predicted to bind UBE2Z (with the 3' UTR SNP rs15563) and SLC22A3 (with the 3' UTR SNP rs3088442), and hsa-miR-4722-5p was predicted to bind APOA5 (with the 3' UTR SNP rs2266788) and ICA1L (with the 3' UTR SNP rs7293707). In accordance with the expected effect on APOA5, rs2266788 was significantly associated with plasma triglyceride levels.25

Of the 55 SNPs affecting miRNA binding, 13 are also associated with the expression of the same gene. At the 11p15.4 locus, rs360137 affects the binding of hsa-miR-3198 to the 3' UTR of the SRA70 gene and is also associated with the expression of the same gene. Similarly, rs1058588 at the 2p11.2 locus affects the binding of hsa-miR-5197-3p to the 3' UTR of VAMP8 and is also associated with the VAMP8 expression. SNP rs12733378 at the 1q32.1 locus affects the binding of 5 miRNAs at the 3' UTR of CAMSAP2, and is also associated with the expression of the same gene. These examples suggest that eQTL effect may be because of altering miRNA binding to the target genes.

SNPs Affecting miRNA Binding and Promoter Regions
We also observed that SNPs that altered miRNA-binding sites in UBE2Z and MAP4 were in high LD with SNPs in their promoter regions. rs6442101 is predicted to be in the promoter region of MAP4 in various cell types and tissues examined in the ENCODE and NIH RoadMap Epigenome project.10 This SNP is in high LD with rs1061003 (r²=0.97), which is predicted to affect the binding of miR-378a-5p in the 3' UTR of MAP4. Similarly, rs999474 located in the promoter region of MAP4 is predicted to affect the binding of 8 different microRNAs (Table IV in the online-only Data Supplement). Therefore, it is plausible that the MAP4 and UBE2Z loci affect the expression of these genes by either altering the affinity of transcription factor binding in the promoter region or miRNA binding in the 3' UTR region. By studying the expression patterns of transcription factors or miRNAs whose binding would be altered, it might be possible to predict the tissue, which these genes would be functional in the context of CAD.

Candidate Gene Prioritization and Prediction of Novel CAD Genes
The CAD GWAS loci have been typically annotated based on their proximity to a gene, yielding a total of 161 genes. However, recent literature evidence26,28,29 suggests that the nearest gene is often not the target of a given GWAS association. Instead, the identification of eQTLs can be used for predicting the target genes. In this work, annotating the 159 CAD risk SNPs led to a list of 151 CAD candidate risk genes based on nonsynonymous AA changes and eQTL effects. Of note, we were not able to assign a gene to all loci. We compared our list of genes with the GWAS genes reported in the literature1 and identified 98 CAD genes hitherto not considered to be involved in the genetics of CAD for which our bioinformatics data provide suggestive evidence (Table V in the online-only Data Supplement; Figure 3A). Of the previously considered GWAS genes, 98 do not overlap with our annotation. These genes might be unrelated to CAD or missed by our annotation efforts. We attempted to prioritize the 98 novel genes using literature and database-based approaches (previous knowledge) or using analyses performed in this article and data from our laboratories (data driven).

For the previous knowledge approach, we first used a statistical text mining approach, Gene Relationships Among Implicated Loci (GRAIL),30 that assesses the degree of relatedness among putative candidate genes within disease regions using PubMed article abstracts. Second, we used another integrative tool, Data-driven Expression Prioritized Integration for Complex Traits (DEPICT),31 that predicts gene functions from manually curated pathways, protein–protein interaction screens and phenotypes from mouse gene knockout studies to prioritize the most likely causal genes at each associated loci, as well as performs pathways enrichment analysis and identifies tissues and cell types where genes from the associated loci are highly expressed. Third, we used the functional annotation information available from the public databases: (1) Mouse Phenotypes from the Mouse Genome Database,32 (2) Functional Disease Ontology,33 and (3) Biochemical Pathway information, as collected from the ConsensusPathDB database34 and the gene ontology annotation.35,36 If a gene was predicted to be a causal gene (eg, P≤0.05 assigned by GRAIL or DEPICT) or its functional annotation (eg, Mouse Phenotype, Biochemical Pathways, or Disease/gene ontology annotation) was CAD related (Methods and Data for the definition of CAD relatedness section in the online-only Data Supplement), we assigned a score of 1 for a total of 6 (Table VI in the online-only Data Supplement). Of note, the previous knowledge-driven approaches are biased because of the availability of literature-based information on well-studied genes. As a result, using previous knowledge-driven approach, 4 genes reached the maximum score of 6: LPL, CDKN2B, ALDH2, and PROC, all of them being among the 604 genes with CAD-related evidence manually extracted from scientific publications and deposited in the Coronary Artery Disease Gene Database (CADgene) V2.0.17

Because of the biased previous knowledge scoring, we also used an alternative, data-driven, approach to look for novel candidate genes. For the data-driven approach, we assigned scores to the genes if they harbored nonsynonymous SNPs, had eQTLs, had promoters with CAD SNPs, were members of a CAD-relevant Bayesian Network constructed from CAD-relevant tissue gene expression studies or had a significant correlation with aortic root lesion size in a systems genetics study of atherosclerosis in a mouse population.37 Hence, the total score a gene could achieve was 5 based on the data-driven approach. We present these prioritization results in Table VI in the online-only Data Supplement. For 69% of the genes, there was evidence from both the previous knowledge-based or data-driven approaches. However, for 31% of the genes, only the data-driven approach provided evidence for the involvement in CAD pathogenesis. The results of the prioritization approach can be found in the Table VI in the online-only Data Supplement. Here, we highlight some of the new potential CAD genes that were prioritized based on our data-driven approach. REST, GIP, and TMEM116 received the top 3 scores.
SNP rs17087335 at the 4q12 locus is located on the NOA1 gene but has proxy SNPs that lead to nonsynonymous AA changes in the REST gene. This CAD locus is also associated with the expression of REST in lung tissue and the CAD SNPs are located in its promoter. Furthermore, the aortic expression of REST is significantly correlated with lesion size in mice. REST encodes for a transcriptional repressor that has been shown to play a role in the phenotypic modulation of vascular smooth muscle cells. REST binds to the promoter of the potassium channel KCa3.1 and represses its expression during intimal hyperplasia. In humans, there seems to be an inverse correlation between REST expression and vascular smooth muscle cell proliferation. Consistently, in our mouse data set, we observed an inverse correlation between aortic expression of Rest and lesion size ($r=-0.24; P=0.03$).

SNP rs15563 at the 17q21.32 locus is located on the UBE2Z gene but has proxy SNPs that lead to nonsynonymous AA changes in the GIP gene. The locus is also associated with the expression of REST in lung tissue and the CAD SNPs are located in its promoter. Furthermore, the aortic expression of REST is significantly correlated with lesion size in mice. REST encodes for a transcriptional repressor that has been shown to play a role in the phenotypic modulation of vascular smooth muscle cells. REST binds to the promoter of the potassium channel KCa3.1 and represses its expression during intimal hyperplasia. In humans, there seems to be an inverse correlation between REST expression and vascular smooth muscle cell proliferation. Consistently, in our mouse data set, we observed an inverse correlation between aortic expression of Rest and lesion size ($r=-0.24; P=0.03$).

SNP rs3809274 at the 12q24.13 locus has previously been annotated with the ATXN2 gene. It is also in high LD with an SNP increasing the expression of the transmembrane protein 116 (TMEM116). Although little is known about this gene, in our data-driven approach, it is one of the highest ranked genes implying an influence on CAD. We do not observe an eQTL effect on ATXN2 but we observe an eQTL effect on TMEM116, C12orf30, SH2B3, BRAP, ALDH2, MAPKAPK5-AS1, HECTD4, and MAPKAPK5. We prioritized TMEM116 based on our data-driven approach because it harbored SNPs with nonsynonymous AA change and its expression level was associated with the CAD locus in multiple tissues (Table VI in the online-only Data Supplement).

In addition, we also highlight MYH7B here. The gene is linked to rs867186 at the 22q11.2 locus by a SNP predicted to cause a deleterious AA change. The lead SNP lies in the PROCR gene and also affects the expression of this gene. It is also associated with the expression of 8 other genes, including the MYH7B gene in his locus; however, rs867186 is located in the promoter of MYH1B. MYH7B encodes the heavy chain of myosin II and is expressed in heart and skeletal muscle. It is also reported to be expressed in smooth muscle cells in mice. Therefore, we prioritized MYH7B as the causal candidate gene out of the 8 genes in this locus.

Finally, to assess the information gain of our annotation effort, we compared the genes previously assigned to the loci and our annotations (Figure 3B; Table V in the online-only Data Supplement).
Recent genome-wide association efforts to understand the genetic architecture of coronary atherosclerosis and myocardial infarction have led to the identification of numerous novel DNA variants associated with disease risk. The main challenge for gaining biological insights from genetic associations is identifying which genes and pathways explain the associations. Only few studies partially identified the susceptibility mechanisms affected by CAD loci and thereby offer blueprints for subsequent efforts to explain disease pathogenesis. These include CAD risk alleles at the 1p13, 6q23, and 4q32 loci, which displayed functional links to gene expression and related disease mechanisms involving SORT1, TCF21, and GUCY1A3. The most robustly associated chromosomal region, the 9p21 locus, still remains a mystery after almost a decade of studies.

More comprehensive efforts are needed to translate the GWAS loci into actionable genes and pathways. Cell-type-specific eQTL or coding (nonsynonymous) variants in strong LD with associated variants can potentially link these variants to genes involved in atherosclerosis. Here, we queried publically available databases and our own experimental data sets to predict the functional genes in the CAD genome-wide significant and suggestive loci.

We observed that majority of the GWAS loci affect gene expression as opposed to leading to AA changes (41% versus 6%). This is in agreement with previous studies that predicted 70% to 80% of GWAS SNPs to be regulatory. Among the loci that lead to changes in gene expression, we revealed that several are associated with differential expression of multiple genes in multiple tissues. Moreover, we observed at a few loci both protein alterations and eQTL effects for significantly associated SNPs. The finding that 1 locus might harbor several proxy SNPs that have eQTL effects on different genes adds further to the complexity of elucidating genetic mechanisms underlying CAD. This is, for example, true for the 19q13.32 locus where the lead SNP is in LD with a missense SNP (in the APOC4 gene) and in LD with SNPs affecting the expression of 3 genes (APOC4, APOC2, and APOE). Second, we identified multiple SNPs that alter promoter and enhancer sequences. ENCODE data indicate that the average number of target genes of a distal regulatory element is 2.5, suggesting that the expression of >1 gene is affected. A potential example of this mechanism is the 10q22.3 locus. The CAD SNP lies in a potential enhancer and is associated with the expression of 2 genes, ANXA11 and MAT1A. Third, we observed loci that affect the expression of a transcription factor leading to changes in the expression of nearby genes. An example is the 17p11 locus that regulates the expression of the transcription factor SREBF1, as well as 4 other nearby genes. Hence, our annotation efforts show that the downstream effects of a locus may be highly complex, not fitting into 1 pathway or function (eg, the 9p21 locus), and that some loci may contain multiple causal genes. Further efforts to analyze pathways and gene networks affected by individual loci will be useful to understand if >1 gene is functional in a locus.

We relied heavily on eQTL data to identify likely causal genes at the CAD loci. Because a large fraction of the variation underlying common diseases seems to be regulatory, this is a sound strategy. But we note that the sample sizes in eQTL studies vary considerably; hence, there may have been insufficient power to detect the eQTLs at the CAD loci. In addition, there may have been confounding factors contributing to the detection of an eQTL, such as population structure or experimental heterogeneity. Importantly, only few studies used tissues relevant to atherosclerosis, such as endothelial cells or the vascular wall, to detect eQTLs as these tissue resources are difficult to obtain. Ongoing projects such as the Genotype-Tissue Expression will contribute to the identification of eQTLs in CAD-relevant tissues.

The complex expression patterns of multiple genes regulated by significantly associated SNPs at a single locus make it challenging to dissect the principle mechanism of the locus altering disease risk. A SNP affecting several genes (either the expression or the protein sequence) might increase the risk of the disease in an additive fashion. However, the disease might also only be caused by only 1 of the altered functions. Hence, it is not straightforward to identify the underlying disease mechanisms. In silico prediction can help to establish a link between an identified genetic effect and the disease. However, functional characterization using molecular biology and genetic approaches are required to understand the mechanisms in more detail.

Traditionally for locus annotations, the nearest gene to the identified risk SNP is reported. However, recent evidence suggests that because of the three-dimensional chromosomal confirmation, genomic locations that seem to physically distant can interact with each other. One such example is the FTO obesity locus that was shown to interact, at megabase distance, with the enhancer of transcription factor IRX3 and regulate its expression. The majority of GWAS-identified variants fall in noncoding regions of the genome, the most frequent elements affected being transcriptional enhancers and silencers, which are typically located >1 kb from their target genes and regulate transcription through long-range interactions. In fact, recent analysis by the ENCODE Consortium demonstrated that only ~27% of the distal regulatory elements have an interaction with the nearest promoter, suggesting that the nearest gene is often not the target of a given GWAS association. Therefore, we used local eQTL results from various resources and protein-altering information, and identified 98 genes that had not been linked to the CAD loci previously. However, this analysis typically provides indirect
evidence of an association, and the overlap of an eQTL with a disease locus may be coincidental. Our annotation is also limited because of lack of results from 5C or other chromatin capture methodology to assess long-range genomic interactions in CAD-relevant tissues. It is crucial to consider disease-relevant tissue types as some eQTLs are tissue dependent and trait-associated variants tend to exert more tissue-specific effects. Additional functional assays would be required for confirming the mechanistic relevance of these eQTLs to the disease or trait.

Gene assignment without functional evidence demonstrates the misleading potential of GWAS reports. It is biased by the biological relevance (and reported phenotypic effects) of the neighboring genes. For instance, the locus on chromosome 19p13.2 with the lead SNP rs1122608 spanning the SMARCA4 gene is also assigned to the neighboring LDLR, which has a well-established role in regulating plasma low-density lipoprotein (LDL) levels. In this work, we only identify an eQTL link between the locus and the SMARCA4 but not the LDLR gene. Our results imply that the disease-causing effect underlying the locus could be the altered expression of the SMARCA4 gene rather than influencing the LDLR. Alternatively, the tissue samples evaluated here for studying eQTL effects missed the interaction of the SNP and LDLR expression, which is less likely because LDLR is primarily expressed in the liver and our eQTL databases included ample liver eQTLs from multiple studies. After all, the LDLR gene is clearly a causal CAD gene, just perhaps not underlying this GWAS signal. Another example is the lead SNP at locus 12q24.12, rs3809274 (Figure 4). It is located between the genes ATXN2 (upstream) and BRAP (downstream) and was traditionally assigned to ATXN2. However, based on our annotation effort, we do not find a link between the locus and ATXN2, but instead on 6 other genes (Table II in the online-only Data Supplement). Another lead SNP, rs3184504, downstream of ATXN2 and located within SH2B3, is, however, associated with the expression of ATXN2 gene.

We used previous knowledge and data-driven approaches to prioritize the novel candidate genes. However, we note that using previous biological knowledge about the candidate genes undermines the agnostic nature of the GWAS approach. In addition, both the number of functional annotations per gene and the number of genes per functional annotation demonstrate scale-free properties, meaning that there is a small number of genes with a large number of functional annotations, whereas for a large number of genes there are only few or no functional annotations available. Hence, these approaches are limited by incomplete information about gene functions. From our pipeline, most functional annotations could be retrieved when searching for Biochemical Pathways in the ConsensusPathDB database and for the gene ontology annotations, where 75 of 154 (≈49%) genes could be mapped to at least 1 annotation term. However, this also indicates that about half (≈51%) of the candidate genes lacked any functional annotation and, therefore, could not be considered for prioritization here. Through our prioritization pipeline, we would again select only well-annotated genes for further studies (the rich get richer principle), whereas the biological function of underinvestigated and underannotated genes would further remain enigmatic; therefore, we highlighted some of the novel genes with top scores from only the data-driven prioritization approach.

Finally, we note that, although we were as comprehensive as possible in annotating the CAD loci, we are limited by the available data sets from previous studies. CAD loci may harbor coding variants that are not presently in databases or regulatory variants that may affect gene expression in a tissue or cell type that has not been examined. For example, the CAD loci, as defined by SNPs in high LD with the lead SNP contain a total of 291 genes, of which 140% of which are noncoding. Recent large RNA sequencing studies and integrative projects such as ENCODE suggest that noncoding RNAs constitute ≤60% of transcribed RNAs. Moreover, in recent years functional studies suggest that they play an important role in the regulation of transcription and translation. We do not have microarray probes measuring the expression levels of all protein-coding or noncoding genes. Furthermore, most eQTL studies were of moderate sample size; therefore, the power to detect significant associations is limited. In addition, we only considered SNPs in high LD (r²≥0.8) with the peak SNP. It is possible that the GWAS lead SNP imperfectly tags the causal SNP, which is moderate LD with the lead SNP, that is, r²<0.8. This could be the case if the causal variant has slightly different MAF compared with the lead SNP. Then by focusing on SNP with r²≥0.8 with the lead SNP may lead to improper conclusion.

Figure 4. Gene reassignment based on expression quantitative trait loci (eQTL) effects. The lead SNP rs3809274 was traditionally assigned to ATXN2. This link was not verified by our annotation effort. rs3809274 is associated with the expression of SH2B but not of ATXN2. The lead single-nucleotide polymorphism (SNP) rs3184504 was traditionally linked to SH2B, but the functional effect identified in this work, links the SNP to the ATXN2 gene.
about the functional variant and the causal gene. However, if we were to reduce the LD threshold, this could have led to spurious associations even though the eQTL and CAD locus are actually independent from each other but in low LD.

Ultimately, for a full understanding, each CAD locus will have to be individually investigated using tools, such as experimental organisms and iPS cells. In this study, we have used some relatively standard tools to refine the list of candidates. Additional approaches that could be useful at present include chromosome conformation analyses,57 application of novel algorithms for causal SNP analysis,58 network analyses, and identification of rare variants. Looking forward, new resources and tools, such as noncoding RNA annotation, RNA-binding maps, splicing variants and code annotation, and detailed enhancer and transcription maps in a variety of cell types relevant to atherosclerosis, will greatly assist such efforts.

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Disclosures

Dr Björkergen is the founder, main shareholder, and chairman of the board for Clinical Gene Networks (CGN). Dr Schadt is a board member of CGN.

References

Coronary artery disease remains the leading cause of death in the Western world despite significant advances in early detection and extensive use of lipid-lowering and antihypertensive drugs. The pathogenesis of atherosclerosis involves environmental factors, hundreds of genetic variants, and their interactions, each of which exerts a relatively small effect on disease susceptibility. A more complete understanding of the disease susceptibility is urgently needed to develop additional diagnostics and therapeutics. Genome-wide association studies identified numerous genetic loci associated with coronary artery disease. Translating these findings into therapies will require the identification of causal genes in the associated loci. In this study, we used publicly available and in-house functional information to systematically review evidence of the involvement of genes in and near the associated loci. Using this approach, we identified 98 possible novel candidate genes to be involved in the pathology of coronary artery disease.
Prediction of Causal Candidate Genes in Coronary Artery Disease Loci

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Supplementary Figure I: Definition of CAD locus boundaries (A) and the transcript coding genes in the CAD loci (B)
Supplemental Text

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Supplemental Material

Materials and Methods
Candidate gene prioritization using prior knowledge from published literature and public databases

GRAIL
Gene Relationships Among Implicated Loci (GRAIL)\(^1\) is a statistical text mining approach that quantifies the relatedness among genes within disease regions, where gene relatedness is defined as the degree of similarity in the text describing them within PubMed article abstracts. PubMed abstracts prior to December 2006 can be selected, in order to avoid publications that report on or are influenced by GWAS papers. In addition to the text-based similarity measure, GRAIL also uses two alternative measures of relatedness, including similarity in Gene Ontology (GO)\(^2\) annotations and gene expression data across multiple human tissues from the Novartis Gene Expression Atlas\(^3\). For each disease region, GRAIL selects the single most connected gene as the key gene and the disease region is assigned that key gene’s p-value. In addition, GRAIL also returns a set of keywords that may provide insight into the underlying biological pathways. Here, a candidate gene received a score of 1, if it had a significant p-value (p≤0.05) in one of the GRAIL’s similarity measure categories in the following order: (1) after the survey of literature prior to December 2006; (2) PubMed abstracts of August 2012; (3) tissue expression or (4) GO annotation. Otherwise, the gene was scored 0.

DEPICT
We used Data-driven Expression Prioritized Integration for Complex Traits (DEPICT)\(^4\), an integrative tool that employs predicted gene functions to prioritize the most likely causal genes at each associated loci, as well as performs pathways enrichment analysis and identifies tissues and cell types where genes from associated loci are highly expressed. DEPICT combines co-regulation of gene expression from 77,840 samples with 14,461 previously annotated gene sets, including manually curated pathways, data from protein-protein interaction screens and phenotypic gene sets from mouse gene knock-out studies, in order to predict gene function based on a ‘guilt-by-association’ procedure. Using these gene sets and a set of trait-associated loci, DEPICT assesses whether any of the gene sets are significantly enriched for genes in the associated loci and prioritizes genes that share predicted functions with genes from the other associated loci more often than expected by chance. In addition, 37,427 human microarrays are being utilized to identify tissue and cell types in which genes from associated loci are highly expressed. Moreover, DEPICT uses precomputed GWAS based on randomly distributed phenotypes (‘null GWAS’) to adjust the p-values from the above three analyses for null expectation. We assigned a candidate gene a score of 1, if DEPICT identified it as a prioritized gene (p ≤ 0.05) in the associated loci, otherwise, the gene was scored 0.

Mouse Phenotype
We downloaded Mouse Phenotype information from the Mouse Genome Database (MGD)\(^5\), which is a comprehensive, non-redundant catalog of mouse genome features obtained through loads from major data providers and experimental consortia, electronic submissions from laboratories and from the biomedical literature. For each of the candidate genes, we first collected all Mouse Phenotypes associated with the gene (Supplementary Table 6). This information was used in two ways. First, we performed Mouse Phenotype enrichment analysis in order to highlight the over-represented categories. Fisher’s exact test, as implemented in R was used to calculate the statistical significance of the overlaps, thereafter the false discovery rate (FDR) was controlled using the Benjamini-Hochberg (BH) procedure\(^6\). Second, Mouse Phenotype annotations were used in order to score the candidate genes within and across the CAD risk loci: each gene received a score of 1, if it was mapped to at least one CAD-relevant Mouse Phenotype, such as ‘MP:0005385-cardiovascular system phenotype’, ‘MP:0005375-adipose tissue phenotype’ or ‘MP:0005387-immune system phenotype’.

Disease Ontology
Functional Disease Ontology (FunDO)\(^7\) is a comprehensive disease to gene annotation, which uses the Disease Ontology (DO) to identify relevant diseases in GeneRIFs. Disease Ontology (DO) annotations of a human gene are complementary to Gene Ontology (GO) Consortium annotations\(^2\) and describe unique roles for genes in the context of disease. A GeneRIF (Gene Reference Into Function) is a brief (up to 255 character) Wiki-type annotation to a gene in the NCBI database and contains gene specific information including disease associations. Again, for each of the candidate genes, we first collected all FunDO disease categories associated with the gene (Supplementary Table 6). Thereafter, these FunDO annotations were used similarly to the Mouse Phenotype information: (1) disease enrichment analysis was performed and (2) each gene received a score of 1, if it was mapped to at least one CAD-relevant disease, e.g., ‘Atherosclerosis’, ‘Cardiovascular disease’, ‘Hyperglycemia’ or ‘Hypertension’.

Biochemical Pathways
Biochemical Pathway information was collected from ConsensusPathDB\(^8\), which is an integrative database currently containing 4,601 Biochemical Pathways in Homo sapiens from 32 public resources such as KEGG\(^9\), Reactome\(^10\), Wikipathways\(^11\) and NetPath\(^12\). In addition, we also included two CAD-related data sets: (1) 604 genes with CAD-related evidence manually extracted from over 5,000 scientific publications from the Coronary Artery Disease Gene Database (CADgene) V2.0\(^13\), which we designated as ‘CADgene’; (2) Cardiovascular Gene Ontology Annotation Initiative uses Gene Ontology (GO)\(^2\) terms to curate scientific literature and integrate results from high-throughput experiments in order to create an informative resource for the cardiovascular-research community. Over 4,000 cardiovascular-associated genes have been identified as targets for annotation with GO terms. We also included these genes in our Pathway set (‘Cardiovascular Gene’). Again, we first collected all Pathways associated with each of the candidate genes (Supplementary Table 6) and performed (1) enrichment analysis to highlight the over-represented categories, as described above for the Mouse Phenotype analysis and (2) to score the candidate genes based on their mapping to CAD-relevant pathways. In this case, in order to classify a Pathway as being CAD-relevant, it had to be identified as such by two out of the three strategies applied: (1) manual inspection; (2) significantly (FDR ≤ 0.05) over-represented in a list of CAD-related genes from the Coronary Artery Disease Gene Database (CADgene) V2.0; (3) selected as CAD-relevant based on a set of disease-related keywords from the scientific literature (e.g., ‘hypercholesterolemia’, ‘cholesterol pathway’, ‘vascular disorder’), whereas the keywords were selected by automatically searching the scientific literature using PolySearch\(^14\), a web-based text mining tool allowing the extraction of relationships between human genes, diseases, pathways, drugs and metabolites. This was followed by manual curation, in order to remove the false positives. In this case, we searched for all entities related to disease ‘Atherosclerosis/CAD/MI’, as well as to two well-known CAD genes apolipoprotein E (APOE)\(^15\)\(^,\)\(^16\) and the low density lipoprotein receptor (LDLR)\(^17\). Finally, this list was used in order to score the candidate genes: each gene received a score of 1, if it was mapped to at least one CAD-relevant Pathway, such as ‘Calcium Regulation in the Cardiac Cell’, ‘Cholesterol Biosynthesis’, ‘Complement and Coagulation Cascades’ or ‘Diabetes pathways’.

Gene Ontology
The Gene Ontology (GO)\(^2\)\(^,\)\(^18\) annotations were downloaded from the AmiGO 2 browser. The Gene Ontology (GO) project provides information about gene product function using ontologies to represent biological knowledge in the form of three classes: (1) molecular functions, (2) the biological processes these contribute to and (3) the cellular locations where these occur (cellular components). We first collected all GO terms associated with each of the candidate genes (Supplementary Table 12) and performed (1) enrichment analysis to highlight the over-represented categories, as described above for the Mouse Phenotype analysis and (2) to score the candidate genes based on their mapping to CAD-relevant GO categories, whereas CAD-relevance was determined similarly, as for the Biochemical Pathways described above and
summarized in Supplementary Table 13. Each candidate gene received a score of 1, if it was mapped to at least one CAD-relevant GO term, such as ‘GO:0002687-positive regulation of leukocyte migration’, ‘GO:0004465-lipoprotein lipase activity’, ‘GO:0005509-calcium ion binding’ or ‘GO:0006955-immune response’.

**Tissue-specific regulatory networks in human and key driver analysis**

In order to determine the gene regulations of CAD genes, we retrieved human tissue-specific Bayesian network models constructed from transcriptomic and genetic datasets from multiple human and mouse studies, including adipose tissue, liver, blood, brain, kidney, islet, and muscle\(^{19-27}\). The network topology of these tissue-specific Bayesian networks defines a partitioned joint probability distribution over all genes, capturing detailed gene-gene regulatory relationships of CAD genes.

We applied a previously developed key driver analysis algorithm of gene-gene interaction networks\(^{26-30}\) to CAD datasets in order to identify the key regulatory genes. We used Bayesian gene networks due to their ability to capture detailed gene-gene relationships with causal implications\(^{31}\). In particular, we analyzed networks from several tissues, including adipose tissue, liver, blood, brain, kidney, islet, and muscle. We explored the gene regulatory patterns of the detected CAD suspected genes above (Protein-coding + eQTL) in human tissue-specific Bayesian networks. The key driver genes for the CAD subnetworks are listed in Supplementary Table 6.

**Identifying mouse phenotypes from Systems Genetics Database (SGD)**

The Hybrid Mouse Diversity Panel Systems Genetics Database\(^{32}\) (http://systems.genetics.ucla.edu) contains multi-scale (clinical trait, transcriptomic, proteomic, metabolome, and epigenomic) data for several mouse crosses and a panel of 100 inbred strains of mice known as the Hybrid Mouse Diversity Panel. Among the clinical traits are atherosclerosis, heart failure, plasma lipids, and insulin resistance.
Materials and Methods References


