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Systems Genetics Analysis of GWAS reveals Novel Associations between Key Biological Processes and Coronary Artery Disease

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URLs

SNAP	https://www.broadinstitute.org/mpg/snap/
Gene Ontology	http://www.geneontology.org/
PLINK	http://pngu.mgh.harvard.edu/~purcell/plink/
MSigDB v3.1	http://www.broadinstitute.org/gsea/msigdb/index.jsp
i-GSEA4GWAS	http://gsea4gwas.psych.ac.cn/inputPage.jsp
ReactomeFI Cytoscape plugin	http://chianti.ucsd.edu/cyto_web/plugins/displaypluginfo.php?name=Reactome%20FIs
Cytoscape (v 2.8.2)	http://www.cytoscape.org/

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Abstract

Objective—Genome-wide association (GWA) studies have identified multiple genetic variants affecting the risk of coronary artery disease (CAD). However, individually these explain only a small fraction of the heritability of CAD and for most, the causal biological mechanisms remain unclear. We sought to obtain further insights into potential causal processes of CAD by integrating large-scale GWA data with expertly curated databases of core human pathways and functional networks.

Approaches and Results—Employing pathways (gene sets) from Reactome, we carried out a two-stage gene set enrichment analysis strategy. From a meta-analyzed discovery cohort of 7 CADGWAS data sets (9,889 cases/11,089 controls), nominally significant gene-sets were tested for replication in a meta-analysis of 9 additional studies (15,502 cases/55,730 controls) from the CARDIoGRAM Consortium. A total of 32 of 639 Reactome pathways tested showed convincing association with CAD (replication $p<0.05$). These pathways resided in 9 of 21 core biological processes represented in Reactome, and included pathways relevant to extracellular matrix integrity, innate immunity, axon guidance, and signaling by PDRF, NOTCH, and the TGF-β/SMAD receptor complex. Many of these pathways had strengths of association comparable to those observed in lipid transport pathways. Network analysis of unique genes within the replicated pathways further revealed several interconnected functional and topologically interacting modules representing novel associations (e.g. semaphorin regulated axonal guidance pathway) besides confirming known processes (lipid metabolism). The connectivity in the observed networks was statistically significant compared to random networks ($p<0.001$). Network centrality analysis (‘degree’ and ‘betweenness’) further identified genes (e.g. *NCAM1*, *FYN*, *FURIN* etc.) likely to play critical roles in the maintenance and functioning of several of the replicated pathways.

Conclusions—These findings provide novel insights into how genetic variation, interpreted in the context of biological processes and functional interactions among genes, may help define the genetic architecture of CAD.

Keywords

GWAS; coronary artery disease; pathway analysis; network analysis

Introduction

Meta-analysis of genome-wide association studies (GWAS) involving tens of thousands of subjects have provided a wealth of new information on the genetic basis of coronary artery disease (CAD), yet common susceptibility variants with achieved genome-wide significance explain only a small fraction of the heritability of CAD (~10.6%)[1, 2]. It has been proposed that much of the residual genetic risk may be attributable to rare variants with large effect[3, 4]. However, recent simulation, exome sequencing, and fine mapping studies of established GWAS loci support the hypothesis that joint contributions from common variants with modest effects are likely to account for a sizeable fraction of the missing heritability of complex diseases[5-7].

It is likely that many more common variants are linked to CAD but have not achieved genome-wide significance in GWAS because of small effect size and/or lower allele frequency and insufficient sample size. However, based on the premise that clinically informative polymorphisms related to complex disease occur in systems of closely interacting genes[8], even weakly associated variants may provide important information regarding the biological basis of disease when such variants cluster within a common functional module or pathway. One common approach for pathway-based analysis of genomic data is gene-set enrichment analysis (GSEA), originally developed and extensively utilized for the analysis of gene expression data[9]. In 2007, Wang et al.[10] described a modified version of the GSEA, designed to analyze genome-wide SNP associations rather than gene expression data. Since then, several other GSEA methods have been developed for this purpose[11-15]. The common goal of these analytic algorithms is to identify a subset of genes whose variants collectively demonstrate strong association with a trait of interest even if the component SNPs individually exhibit relatively modest or non-significant association. Importantly, pathway analysis can also place the set of validated SNPs for a trait of interest into a broader and clearer biologic context. A natural extension of this list-based pathway approach is the interrogation of molecular networks to unravel the architecture underlying complex diseases. A molecular network is based on ‘interactions’ among biomolecules (genes, protein, metabolites, etc.), where such interactions can take various forms (protein-protein interactions, coexpression, gene regulation, functional interactions, etc.). Efforts at the characterization of disease-associated genes reveal that genes associated with the same or similar disorders tend to occupy similar neighborhoods in molecular networks through physical or functional modules[16, 17]. Furthermore, the study of network topology suggests that key disease-related genes differ from other genes in terms of their network connectivity and network centrality properties[17]. Finally, molecular networks provide two distinct enhancements over traditional pathway based approach – (i) they provide additional information on interactions among gene subsets within a given pathway, and (ii) they allow for the identification of interactions between components of different biological pathways. Through these analyses, one is able to draw a clearer picture of the functional connectivities that influence pathway functions, and how multiple pathways may interact with one another to influence a phenotype.

Several studies have applied molecular networks for generating insights from GWAS data[8, 18-20] in disorders such as schizophrenia, multiple sclerosis and prostate cancer.

However, most of these approaches have relied mainly on protein-protein interaction networks, thereby missing the rich mechanistic information available from traditional biological pathway repositories and networks based on functional interactions. In this study, we have coupled the advantages of a well curated biological pathway repository with a similarly curated functional interaction network to identify mechanism-based processes that may underlie the genetic architecture of CAD. First, to identify novel associations between established biological mechanisms and CAD, we have carried out a two-stage pathway-based GSEA analysis of sixteen GWAS data sets for CAD using the i-GSEA4GWAS tool[15] and the Reactome pathway database[21]. Collectively, these GWAS include over 25,000 subjects with CAD and over 66,000 controls. We have then taken the replicated pathways as a starting point to explore functional interactions within and between pathways via interrogation of molecular interacting networks. Lastly, we have characterized the CAD-associated genes based on their topological properties within these networks as a way of prioritizing gene candidates for functional follow-up studies.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement. Briefly, employing pathways (gene sets) from Reactome, we first carried out a two-stage gene set enrichment analysis strategy. From a meta-analyzed discovery cohort of 7 CAD GWAS data sets (9,889 cases/11,089 controls), nominally significant gene-sets were tested for replication in a meta-analysis of 9 additional studies (15,502 cases/55,730 controls) from the CARDIoGRAM Consortium (Table 1). Genes from the replicated pathways were then mapped onto well-curated interaction networks.

Results

Significant Pathways

A total of 85 out of the 639 Reactome pathways tested in Stage 1 achieved a gene set enrichment p-value < 0.05 at a false discovery rate <0.25. Thirty two out of these 85 pathways were further replicated in Stage 2 at a nominal p<0.05 (Table 2). When the replicated pathways were compared to the full pathway content of Reactome, at least one replicated from 9 of the 21 core Reactome-defined biological processes. These included the core processes of *'metabolism', 'signal transduction', 'developmental biology', 'extracellular matrix organization', 'immune system', 'metabolism of proteins', 'cell-cell communication', 'transmembrane transport of small molecules'*, and *'gene expression'* (Figure 2). Due to the hierarchical organization of Reactome pathways, several replicated pathways were nested within larger gene sets, either completely or partially (Figure SII, online-only Data Supplement). This hierarchical structure enabled us to identify instances of pathway selectivity – for example, although the *'CRMPS in SEMA3A signaling', 'Sema4D in semaphorin signaling' and 'Sema3A PAK dependent axon repulsion'* pathways all nested completely within the *'Semaphorin Interactions'* pathway, only the former was significantly replicated (p<0.001) whereas the latter two pathways were not. In order to put the identified pathways in a broader context, we have also listed the non-replicated pathways that share

similar levels of hierarchy as the replicated pathways in Table SI (online-only Data Supplement).

About a third of the 32 replicated pathways were also significant in Stage 2 ($p<0.05$) after correcting for linkage disequilibrium between the *SNPs*, by analyzing SNPs pruned genome-wide at either $r^2>0.5$ or $r^2>0.2$ (Table SII, online-only Data Supplement). The pathways that were in common to all three pruned and unpruned SNP analyses were *'Toll receptor cascades'*, *'degradation of the extracellular matrix'*, *'lipid digestion, mobilization and transport'*, and *'lipoprotein metabolism'*, While the association of these pathways may be of higher confidence, pruning of SNPs may also lead to loss of power due to significant reduction in SNP number (5-15% of unpruned SNPs) and to the fact that the pruning was agnostic to the actual CAD SNP association p-values. Hence, for downstream gene and network analyses we chose to use the full set of 19 pathways that replicated with the unpruned list of SNPs.

Lastly, we examined the possible effect of LD among *genes* leading to inflated significance scores for the replicated pathways by considering the extent of LD among the gene-tagging (best scoring) SNPs for all genes in a pathway. The extent of LD among the most significant SNPs was found to be minimal. Specifically, of all the SNPs tested, we found only 2 SNP pairs with an $r^2>0.8$, observed across 3 pathways. Even at the more permissive r^2 threshold of 0.2, only 4 SNP pairs were observed across 5 pathways (Table SIII, online-only Data Supplement).

Gene and Pathway Prioritization

The 32 replicated pathways contained a total of 770 unique genes that were taggable by at least one SNP (no SNP tags were available for 83 genes). Figure SIII (online-only Data Supplement) summarizes the proportion of genes within the replicated pathways that were associated with CAD. All replicated pathways contained 50% or more genes above the significance threshold (range 50.0% to 92.3%), confirming that the pathway findings were driven by the combined contributions of multiple genes in each pathway and not due to large effects from a small minority of genes. For comparison purposes, we also analyzed a synthetic pathway derived from genes within the CARDIoGRAM loci reaching genome wide significance. This synthetic pathway contained the second highest proportion of genes reaching the significance threshold.

Network analysis

Statistical evaluation of network—A total of 770 genes from the replicated pathways were mapped to the InWeb PPI network and the observed network connectivity parameters ('degree', and number of edges) compared to random networks of similar size and degree distribution. A network of direct interactions could be created with 620 genes (assuming a minimum interaction size of 2 genes). The resulting network (Figure SIV) was significantly different with respect to random networks; thus there were 3726 direct edges in the network compared to only 1548 edges expected by chance ($p<0.001$), and the observed average connectivity per gene ('degree' of gene) was 12, compared to an expected 5.8 from random

networks ($p<0.001$). These results indicate that the networks constructed from the replicated pathway genes are likely not due to chance.

Mapping of replicated pathway genes to an interaction network—Although this PPI-based analysis provided confidence that the networks derived from the replicated pathway genes are unlikely to arise from chance, it allows only limited insights into the various biological mechanisms impacted by these pathways. Thus, in order to identify networks that contain more relevant information onbiological processes (including PPI), the genes from the replicated pathways were mapped to a functionally interacting network curated and maintained at Reactome. A total of 733 genes could be mapped to the larger network. This sub-network was further clustered to reveal within-network modules. Clustering resulted in the identification of 17 clusters with 10 clusters containing >10 gene members (Figure 3 and Table SIV). Within each cluster, a diverse array of interactions (reactions, complex formation, activation, etc.) was represented by the edges connecting the genes (nodes), as exemplified in Figure SV (online-only Data Supplement) for the genes in clusters 8 and 9. We also observed considerable inter-connectivity between the clusters; for example, the links between cluster 4 and other clusters are highlighted in Figure 3 (additional inter-cluster connectivities for each of the remaining clusters are shown in Figure SVI, online-only Data Supplement). Enrichment analysis within each cluster using Gene Ontology identified several cluster-specific overrepresentations of biological processes, as further highlighted in Figure 3. The following are some notable examples of functional enrichment within the clusters (FDR<0.001):‘innate immunity’ (cluster 1 and 4),‘Notch signaling’ (cluster 6), ‘extracellular matrix organization’ (cluster 7), ‘lipid metabolism’ (cluster 8) and ‘axon guidance’ (cluster 9). The full list of all significantly overrepresented GO-BP terms (FDR<0.001) is provided in Table SV (online-only Data Supplement).

Gene and pathway prioritization based on network topology

Network topology provides vital information towards the understanding of network architecture and performance and allows for the prioritization of genes based on their topological characteristics within the network. Thus, we interrogated the topological properties of the networks derived from the replicated pathways. Specifically, we investigated two key node centrality measures, namely ‘degree’ and “betweenness”, due to their reported significance in biological networks as drivers for gene/protein essentiality (see online-only Data Supplement for additional information on ‘degree’ and ‘betweenness’).[22] For this purpose, the replicated pathways were first converted into Reactome functional interaction networks (conversion was successful for 29 pathways, with the exclusion of *‘collagen formation’*, *‘metabolism of polyamines’* and *‘organic cation anion zwitterion transport’* pathways) and subsequently analyzed for the above two node centrality measures. Figure 4 depicts the ‘betweenness’ centrality measures for a merged network derived from two pathways related to cell-cell interactions (‘NCAM signaling for neurite outgrowth’ and ‘CRMPs in Sema3a signaling’). In this network, the NCAM1 and Fyn proteins display large ‘betweenness’ centrality and act as bridges connecting multiple other proteins in the network. Some additional genes with GWAS association $p<0.001$ that occupy potentially critical positions (betweenness > 100) in a subset of the replicated pathways include *FURIN (*component of *‘degradation of extracellular matrix’*, *‘extracellular matrix organization’*,

'signaling by NOTCH1' pathways), *MMP1* (*'degradation of extracellular matrix'* and *'extracellular matrix organization'* pathways), and *RPS6KA5* (*'Toll receptor cascades'* and *'NCAM signaling for neurite outgrowth'* pathways). Results for the remaining pathways are shown in Figure SVII and Table SVI (online-only Data Supplement).

Discussion

Despite the recent successes of large GWAS meta-analyses[1, 2], the genetic architecture of CAD remains poorly understood and the identified loci explain a small proportion of genetic risk. By integrating GWAS data with expertly curated databases of core human pathways as well as gene and reaction-based functional networks, we sought to obtain novel insights into the potential causal processes of coronary atherosclerosis. Additionally, the large size of the discovery population and replication sample (25,000 CAD cases and 66,000 controls) and the two-step discovery-replication strategy increases confidence in the results. The current analysis implicates 32 core human pathways representing 9 distinct biologic processes as being most etiologically relevant to CAD.

Notably, a number of replicated pathways from the two-stage GWAS analysis strategy converged on processes regulating cellular growth, migration and proliferation, such as the 'Signaling by TGF-β receptor' and 'Signaling by PDGF', pathways previously intensively investigated for their functional role in coronary atherosclerosis. By combining GWAS-based findings with such *a priori* information we obtained evidence that genetic variation in a critical number of genes representing these pathways contribute to the heritability of CAD. Moreover, these data support hypotheses that alterations in these pathways are potentially causally related to CAD. Specifically, TGF-β is known to control cell proliferation, cell migration, matrix synthesis, wound contraction, calcification and the immune response, all of which are major components of the atherosclerotic process[23]. *PDGF* is expressed in every cell type of the atherosclerotic arterial wall, as well as in infiltrating inflammatory cells[24] and plays a key role in the migration of vascular smooth muscle cells from the media into the intima and their subsequent proliferation. Although both pathways have been studied in animal models, animal data are often conflicting or inadequate and there are no data related to modulation of these pathways in humans. A number of pathways related to the integrity of the extracellular matrix were also highly significant including 'Extracellular matrix organization', 'degradation of the extracellular matrix, and 'Cell Extracellular matrix (ECM) interactions'. The ECM is responsible for maintaining not only the structural integrity of vessel wall plaques, but also participates in several key events such as cell migration, lipoprotein retention and thrombosis that are critically linked to plaque stability[25].

Two of the axon guidance pathway sub-classes, 'CRMPs in Sema3 signaling', and 'NCAM signaling for neurite out-growth' also replicated. The axon guidance pathways modulate diverse biological phenomena including cellular adhesion, migration, proliferation, differentiation, survival and synaptic plasticity through the participation of highly conserved families of guidance molecules including netrins, slits, semaphorins, and ephrins, and their cognate receptors[26]. Neural guidance cues such as netrin-1 and semaphorins have important roles outside the nervous system. Moore et al. provide compelling evidence that netrin-1 is secreted by macrophage foam cells in atherosclerotic plaques and acts to inhibit emigration

of these cells out of lesions by causing dysregulation of the actin cytoskeleton[27]. Oksala *et al*. reported that *NTN1* is downregulated in atherosclerotic plaques and its expression correlates negatively with inflammatory markers and M2 signals[28]. Like netrin-1, semaphorin 3A, encoded by *SEMA3A*, one of the top ranked genes in this analysis, is also expressed in coronary artery endothelial cells and potently inhibits chemokine-directed migration of human monocytes[29, 30]. The present study also provides further supportive evidence for a causal role of innate immunity in atherosclerosis and/or plaque rupture with significant pathways including both 'Toll receptor cascades' and 'initial triggering of complement'. Innate immune responses mounted by macrophages and other immune cells recruited to the arterial wall in response to an inflammatory challenge have a major role in the initiation of atherosclerosis[31].

An important advance encompassed in the current work is our further examination of the topological characteristics of genes comprising the replicated gene-sets and the potential implication of topology on biological function. Specifically, we applied the Reactome FI tool to identify gene-sets related to biological processes such as innate immunity, cell adhesion, and lipid metabolism that were further reorganized into functionally interacting networks and sub-network clusters demonstrating a high degree of inter-connectedness. Network clustering, followed by pathway enrichment analysis on the identified clusters via Gene Ontology, generated new insights on interrelationships among the enriched pathways, not available through our initial traditional gene-set analysis. For example, whereas the lipid-metabolizing genes were largely concentrated in a single cluster (cluster 8), genes related to innate immunity were, by contrast, distributed within three separate clusters (clusters 0, 1, 4), along with other biological processes, highlighting the possibility of extensive interactions among these processes. Lastly, through analysis of such networks, we were further able to evaluate the possible criticality of genes in network function, based on the'degree' and 'betweenness' centrality properties of the network genes.

Collectively, these additional analytic approaches provide important insights into the interrelationships among genes that are not usually available through conventional gene-set enrichment analysis, and could assist in the formation of testable hypotheses on areas of robustness and vulnerability in functional networks otherwise not intuitively evident. For example, topological analysis implicated a potential role for the axonal growth related pathways in CAD with NCAM1 (neural adhesion molecule 1) being a major hub in a network including plexins (PLXNA1, PLXNA2), neuropilin (NRP1) as well as adhesion molecules (CNTN2) and several members of the collagen family relevant to the extracellular matrix of the vessel wall (Figure 4). These data support the concept that neuronal guidance cues have important roles in both arteriogenesis[32, 33] and atherosclerosis by regulating macrophage retention in plaques[27, 29, 30]. Other studies demonstrate that semaphorin 3A and its receptors, neuropilin-1 and -2, plexins A1/A2/A3 are highly expressed in human monocyte derived macrophages and play a role in induction of macrophage apoptosis[34].

Despite these plausible observations, we are cognizant that 'betweenness' is but only one of several network centrality measures that could play critical roles in network function. As both fields of network biology and network pharmacology are currently evolving, our findings should be considered more as hypotheses-generating rather than conclusive

evidence of the importance of one gene or one pathway over another. Functional testing is necessary as the next step, and can take several forms, including (i) overexpression or knockdown of medium to high 'betweenness' genes in target pathways (e.g. *NCAM1, FYN*, for the network in Figure 4) in CAD-relevant cell models (e.g. human coronary artery endothelial or smooth muscle cells, macrophages, etc.) and to interrogate their effects on cell function (cell migration, lipid accumulation, etc.); (ii) testing the effects of candidate genes (e.g. *NCAM1, FURIN*) in knockout or overexpression mouse models (generated by somatic manipulation or transgene creation) on lesion formation (similar to studies on candidate GWAS genes for lipoprotein metabolism[35-38]); (iii) statistical epistasis analysis, limited to genes within a replicated pathway, to uncover functionally important interactions underlying the genetic basis of atherosclerosis, and (iv) prioritizing gene products from replicated pathways based on the availability of pharmacologic agents against them, and testing these for potential benefits in animal models of atherosclerosis (successfully demonstrated in identification of memory-modulating drugs[39]). We hope our approach stimulates extensive further discussion on how to experimentally interrogate CAD related networks and pathways.

We acknowledge potential caveats pertaining to this study. First, the number of pathways identified and replicated was modest but the pathways are biologically plausible. In the discovery analysis, 85 of the 639 (13%) pathways tested were significant at $p<0.05$ (and FDR <25%) with at least 50% of the genes in any given pathway being individually significant at a $p<0.05$. A total of 32 of these 85 (37%) pathways, achieved replication, a number somewhat lower than expected (75%) given the FDR threshold used in the discovery phase to select pathways for testing in the replication sample. This may reflect the less stringent criteria for age of onset of CAD cases applied in some of the replication studies as well as study-specific differences in inclusion/exclusion criteria and adjudication of outcomes, leading to increased sample heterogeneity[1]. Our study also highlights several generic issues that currently impose limitations on the conduct and interpretation of pathway analyses[40]. Some of these issues pertain to (i) the mapping of SNPs to genes, (ii) choosing the optimum 'pathway analysis' tool for GWAS, (iii) consequences of the permutation scheme used in i-GSEA4GWAS, and (iv) the effects of inter-SNP linkage disequilibrium on pathway analysis results. An additional caveat is the potential for bias in the network and topological analyses due to limitations in the extent and type of experimental data available in the source databases. We have provided a further detailed discussion of issues related to pathway and network analysis in the Results section of the online-only Data Supplement.

This is an area of emerging methodology and different approaches can yield complementary findings. Our findings extend gene-centric verification of CAD GWAS loci[41] and those recently reported by CARDIoGRAM+C4D, applying Ingenuity network analysis only on the top 239 candidate genes [2]. In another recently published study, based on this large-scale meta-analysis of GWAS studies for CAD, we used a very different approach[42]. Rather than a location based approach to map SNPs to genes, we used eQTL data from CAD-related tissues and primary cells to link CAD SNPs to their empirically defined target genes. We then created data-driven, tissue-specific gene expression networks from a multitude of human and mouse experiments [42]. These networks relied heavily on available gene expression data and did not involve other types of interactions such as protein-protein

interactions or biochemical reactions. In contrast, the present analysis is based on gene-to-SNP mapping methods for gene set enrichment rather than eQTL data and our analysis of the topological relationships among genes in the filtered, replicated pathways using Reactome FI and PID cover a more extensive array of molecular interactions, thus revealing important aspects that we failed to capture from the gene expression based networks. It is encouraging that these two approaches have yielded consistent results in terms of core processes related to lipid metabolism, immune system, Notch-HLH transcription and PPAR signaling. However, here we have identified additional biologically relevant pathways including extracellular matrix integrity, TGF-β signaling and axon guidance, the latter being of particular interest given recent laboratory findings[27-30, 32-34]. Many of these pathways had strengths of association comparable to those observed in known pathways related to lipoprotein metabolism.

The findings of this extensive but preliminary analysis do not imply causality. However, the utility of the integrative approach in elucidating the genetic bases of disease has been demonstrated by studies in several complex phenotypes. For example, in an investigation of the WTCCC Crohn's disease GWAS data set, only 3 genes at 2 loci showed GWAS significant signals but pathway analysis carried out by Wang *et al.* identified the 20 gene IL-12/IL-23 pathway to be associated with Crohn's disease that remained significant even when the 2 original loci were removed[11, 43]. In a similar vein, Holmans et al. provided supporting evidence for the immunogenetic origins of Parkinson's disease by identifying the regulation of leukocyte/lymphocyte activation and cytokine-mediated signaling as conferring increased susceptibility to Parkinson's disease, although none of the SNPs linked to genes within these pathways had achieved GWAS significance[44]. On the contrary, pathway analysis studies have had little success in generating new biological insights for other disorders including type 2 diabetes. Due to this variability, extensive mechanistic and functional validation of pathway and interactome-derived networks at multiple levels will be essential. An example of systematic experimental perturbation of interactome networks to understand cancer predisposition has been presented in Rozenblatt-Rozen et al.[45] and a framework for network inference and validation based on gene knock-down has been proposed in Olsen et al.[46]

In summary, the present analysis has provided potential new insights into mechanisms underlying atherosclerosis and its clinical sequelae. The results of this investigation suggest a possible link between several core human biological processes and CAD, including several with and several without a substantial body of prior experimental evidence. Further study of the genes within the highlighted pathways may facilitate the development of novel testable hypotheses that could ultimately improve our understanding of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations & Acronyms

GWAS	genome-wide association study
CAD	coronary artery disease
CARDIoGRAM	Coronary Artery DIsease Genome wide Replication and Meta-analysis
GSEA	gene-set enrichment analysis
CEU	Center d'Etude du Polymorphisme Humain
MSIGDB	Molecular Signatures Database v3.1
PID	Pathway Interaction Database

Significance

Genome-wide association studies (GWAS) have identified over 45 loci associated with CAD risk but provide limited insight into causal mechanisms. Furthermore, the identified signals explain little more than 10% of the predicted heritability of CAD. Part of this "missing heritability" It is likely because many more common variants are linked to CAD but have not achieved genome-wide significance in GWAS because of small effect size and/or lower allele frequency and insufficient sample size. However, even weakly associated variants may provide important information regarding the biological basis of disease when such variants cluster within a common functional module or pathway. By integrating GWAS data with extensive databases on core biological processes, we have identified novel biological pathways relevant to the pathogenesis of CAD. These findings provide new insight into how genetic variation, interpreted in the context of biological processes and functional interactions among genes, may help define the genetic architecture of CAD.

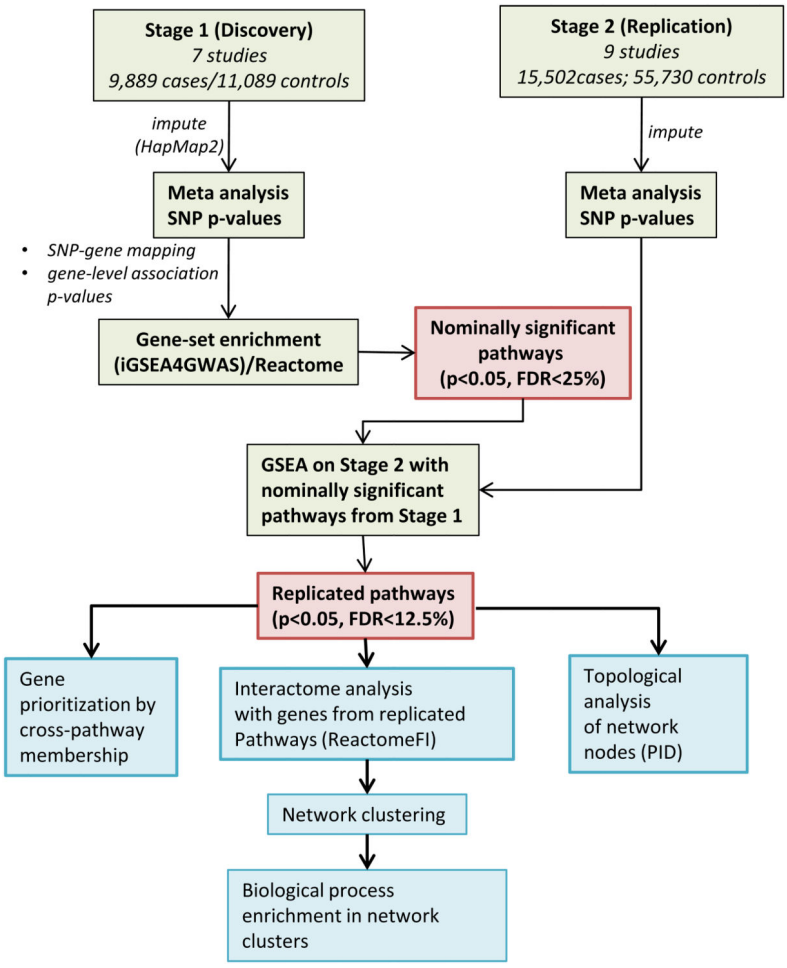


Figure 1. Analytical approach

Schematic of analytical approach as described in detail in Methods section.

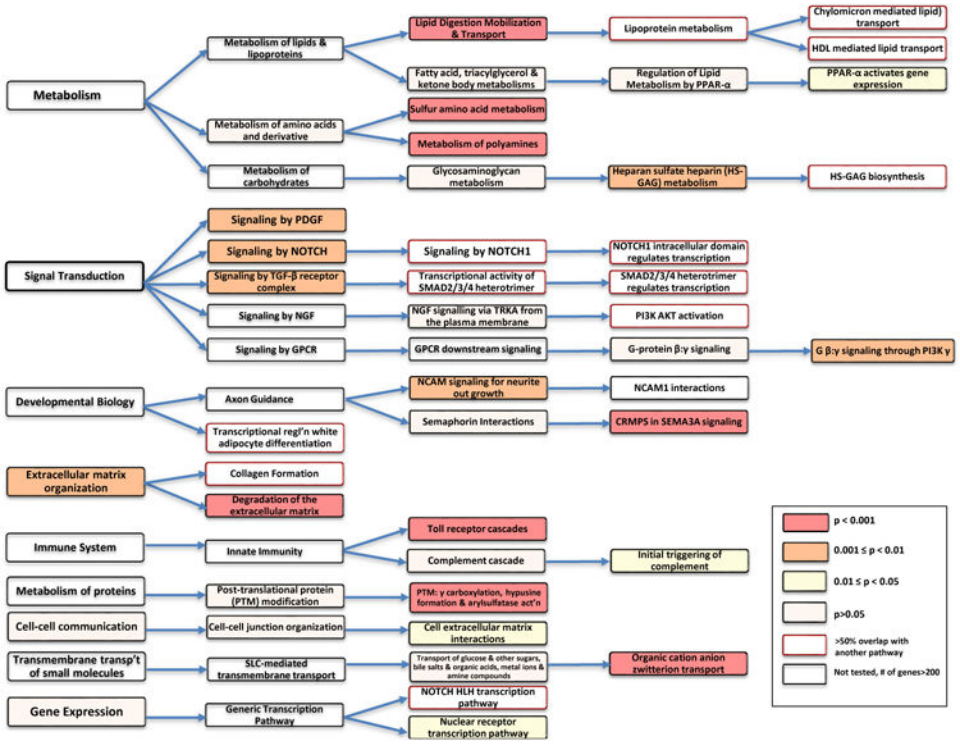


Figure 2. Replicated Reactome pathways for CAD using i-GSEA4GWAS with a 100kb mapping interval

Replicated pathways are represented in a hierarchical Reactome pathway diagram. Top-level pathways, representing core biological processes, are listed to the left, and sub-levels corresponding to each top level are illustrated progressively to the right. The 9 top-level pathways that contain at least one replicated pathway (top-level and/or sub-levels) are shown. No sub-level pathways are shown to the right of the last replicated pathway. Pathways are color coded according to their gene-set enrichment p-value from the replication stage as indicated in the legend. A $p < 0.05$ corresponds to an FDR <12.5%. Pathways containing less than 10 or greater than 200 genes were not tested. Replicated pathways with >50% overlap of genes with other replicated pathways are also identified as indicated in the legend.

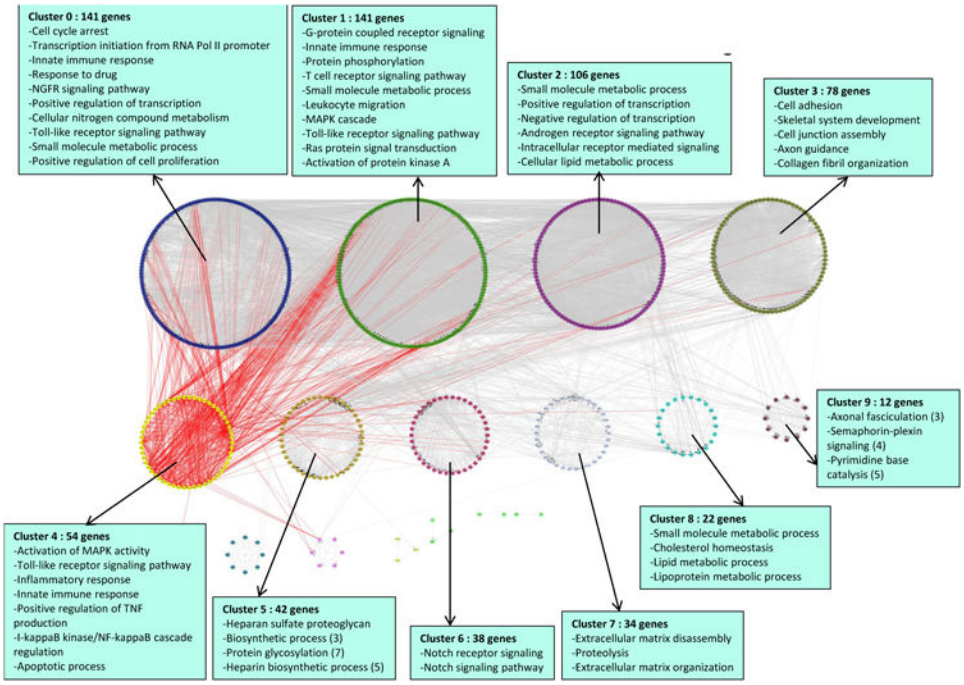
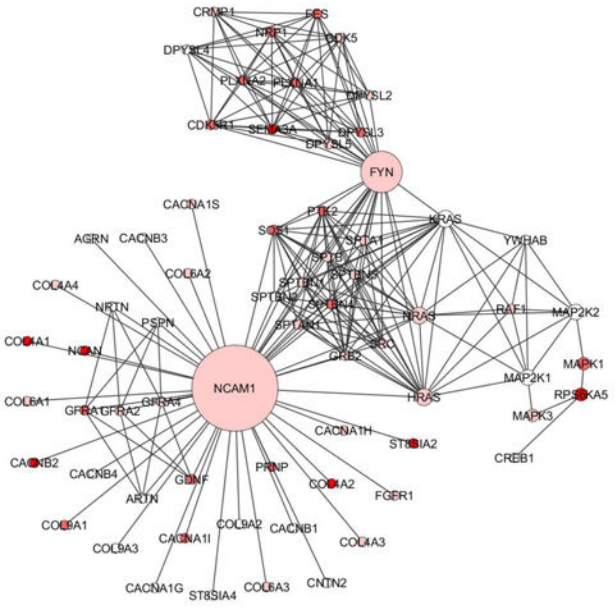


Figure 3. Functionally interacting network modules constructed from genes belonging to the replicated, CAD-associated pathways

Functional interactions among the genes from all replicated pathways were analyzed and clustered by the ReactomeFI tool and visualized in Cytoscape. Genes are represented as nodes and interactions among genes are represented as edges. The parent network was further analyzed to yield sub-network clusters; each cluster is shown separately and color coded for clarity. Inter-cluster connectivity is exemplified in red for cluster 4. The top GO-BP terms that are enriched in each cluster are listed in the blue boxes. For each cluster, all terms are at FDR<0.0001 and contain a minimum of 10 genes (unless otherwise indicated in parentheses). A maximum of 10 GO-BP terms are shown for each cluster. Genes that were not linked to at least one other gene were excluded from the network diagram.



Gene	Betweeness
SEMA3A	3.1
FES	2.5
PLXNA2	3.1
DPYSL3	0.6
CDK5R1	2.5
NRP1	3.1
PLXNA1	3.1
DPYSL2	0.6
DPYSL5	0.6
FYN	1330.4
CDK5	2.5
CRMP1	0.6
DPYSL4	0.6
COL4A2	0
CACNB2	0
COL4A1	0
RPS6KA5	134
NCAN	0
COL3A1	#N/A
ST8SIA2	0
SOS1	4.7
COL9A1	0
PTK2	7.3
PRNP	0
CACNA1I	0
SPTAN1	2.6
NRAS	308.7
CACNA1S	0
COL6A1	0
FGFR1	0
COL1A1	#N/A
KRAS	308.7
CNTN2	0
CREB1	0
CACNB4	0
CACNB3	0
COL2A1	#N/A

Gene	Betweeness
GDNF	1.2
COL5A1	#N/A
MAPK1	128
COL1A1	#N/A
GFRA1	3
SPTBN4	2.6
COL6A1	0
COL5A1	#N/A
SRC	4.7
GFRA4	3
GFRA2	3
RAF1	0
SPTA1	2.6
NCAM1	3201.5
MAPK3	128
COL4A1	0
FYN	1330.4
SPTB	2.6
SPTBN5	2.6
HRAS	308.7
GRB2	4.7
CACNA1H	0
COL6A3	0
SPTBN1	2.6
COL4A3	0
CACNA1G	0
CACNB1	0
MAP2K2	250
SPTBN2	2.6
MAP2K2N	250
NRTN	1.2
ST8SIA4	0
YWHAB	0
PSPN	1.2
COL9A2	0
COL9A3	0
AGRN	0
ARNT	1.2

Figure 4. Topology based network analysis in replicated pathways

Topological relationships among genes are shown for a merged Reactome functional interaction network created in Cytoscape from two replicated pathways associated with cell-cell interactions (*NCAM signaling for neurite outgrowth and CRMPs in Sema3a signaling*). Genes (nodes) in the network are color coded by their replication p-values (deep red, $p<0.001$; lighter red, $0.001<p<0.01$; lightest red, $0.01<p<0.05$; white, $p>0.05$) and sized by their "betweenness" network centrality score (calculated via Centiscape 2.0). The individual gene names and their "betweenness" scores are listed beside the network diagram. Betweenness scores are not calculated for genes that do not connect to at least one other gene in the network (these genes are indicated with #N/A for betweenness).

Table 1
Demographics of Discovery & Replication Cohorts

GWAS Data Set	Number Cases/controls	Age (mean±SD) Cases/controls	% male Cases/controls	% MI cases
Stage 1 Studies				
OHGS_A	921/994	48.2±7.0/74.9±4.9	78.1/54.6	54.6
OHGS_CCGB-B	2,688/1,819	49.8±7.7/74.8±5.4	75.1/49.0	59.8
DUKE_2	1,200/648	56.7±9.7/63.3±8.7	69.4/42.0	48.0
GerMIFs I	875/1,644	50.2±7.8/62.6±10.0	50.6/49.2	100
GerMIFs II	1,222/1,298	51.4±7.5/51.2±11.9	66.9/51.7	100
GerMIFs III (KORA)	1,157/1,748	58.6±8.7/55.9±10.7	79.9/51.1	100
WTCCC	1,926/2,938	49.8±7.7/N/A	79.3/50.0	71.5
Total Stage 1	**9,889/11,089**			
Stage 2 Studies				
ADVANCE	278/312	45.8±6.2/45.3±5.7	42.1/41.0	50.4
CADomics	2,078/2,952	60.8±10.1/55.3±10.8	78.1/49.5	58.3
CHARGE	2,287/22,024	60.0±7.9/63.1±8.0	66.6/40.4	48.0
deCODE CAD	6,640/27,611	74.8±11.8/53.7±21.5	63.7/38.1	54.7
LURIC/AtheroRemo 1	652/213	61.0±11.8/58.3±12.1	79.7/54.0	71.9
LURIC/ AtheroRemo 2	486/296	63.7±9.4/56.4±12.7	76.6/51.4	79.0
MedStar	874/447	48.9±6.4/59.7±8.9	67.0/45.4	48.1
MIGen	1,274/1,407	42.4±6.6/43.0±7.8	62.8/60.1	100
PennCATH	933/468	52.7±7.6/61.7±9.6	76.3/48.1	50.3
Total Stage 2	**12,501/55,730**			
Total Stage 1 & 2	25,491/66,819			

Table 2

List of replicated Reactome pathways enriched for genetic association to CAD. Pathway names are listed in *column 1*; *column 2* lists the nominal p-value for pathway enrichment; *column 3* lists the corresponding false discovery rates (FDR); *column 4* records the number of genes in each pathway.

Reactome Pathway	Enrichment p-value	FDR	# genes/ pathway
CHYLOMICRON_MEDIATED_LIPID_TRANSPORT	<0.001	0.000	16
CRMPS_IN_SEMA3A_SIGNALING	<0.001	0.000	14
DEGRADATION_OF_THE_EXTRACELLULAR_MATRIX	<0.001	0.000	29
LIPID_DIGESTION_MOBILIZATION_AND_TRANSPORT	<0.001	0.000	46
LIPOPROTEIN_METABOLISM	<0.001	0.000	28
METABOLISM_OF_POLYAMINES	<0.001	0.000	15
NOTCH1_INTRACELLULAR_DOMAIN_REGULATES_TRANSCRIPTION	<0.001	0.000	46
SULFUR_AMINO_ACID_METABOLISM	<0.001	0.000	24
TOLL_RECEPTOR_CASCADES	<0.001	0.000	118
ORGANIC_CATION_ANION_ZWITTERION_TRANSPORT	<0.001	0.001	13
SMAD2_SMAD3_SMAD4_HETEROTRIMER_REGULATES_TRANSCRIPTION	<0.001	0.001	27
NOTCH_HLH_TRANSCRIPTION_PATHWAY	<0.001	0.002	13
EXTRACELLULAR_MATRIX_ORGANIZATION	<0.001	0.003	87
HS_GAG_BIOSYNTHESIS	<0.001	0.003	31
PTM_GAMMA_CARBOXYLATION_HYPUSINE_FORMATION_AND_ARYLSULFASE_ACTIVATION_ACTIVATION	<0.001	0.004	27
SIGNALING_BY_NOTCH	0.003	0.005	70
TRANSCRIPTIONAL_ACTIVITY_OF_SMAD2_SMAD3_SMAD4_HETEROTRIMER	0.001	0.005	38
SIGNALING_BY_PDGF	0.003	0.017	122
SIGNALING_BY_NOTCH	0.007	0.019	103
G_BETA_GAMMA_SIGNALING_THROUGH_PI3KGAMMA	0.002	0.020	25
HEPARAN_SULFATE_HEPARIN_HS_GAG_METABOLISM	0.004	0.020	52
NCAM_SIGNALING_FOR_NEURITE_OUT_GROWTH	0.003	0.022	64
SIGNALING_BY_TGF_BETA_RECEPTOR_COMPLEX	0.007	0.033	63
PI3K_AKT_ACTIVATION	0.014	0.048	38
PPARA_ACTIVATES_GENE_EXPRESSION	0.014	0.048	104
HDL_MEDIATED_LIPID_TRANSPORT	0.016	0.052	15
NUCLEAR_RECEPTOR_TRANSCRIPTION_PATHWAY	0.022	0.061	49

Reactome Pathway	Enrichment p-value	FDR	# genes/ pathway
TRANSCRIPTIONAL_ACTIVATION_OF_WHITE_ADIPOCYTE_DIFFERENTIATION	0.019	0.063	72
INITIAL_TRIGGERING_OF_COMPLEMENT	0.032	0.082	16
NCAM1_INTERACTIONS	0.023	0.092	39
CELL_EXTRACELLULAR-MATRIX_INTERACTIONS	0.032	0.102	14
COLLAGEN_FORMATION	0.041	0.124	58