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From Loci to Biology: Functional Genomics of Genome-Wide Association for Coronary Disease

Sylvia T Nurnberg[1,*], **Hanrui Zhang**[2,*], **Nicholas J Hand**[3], **Robert C Bauer**[1], **Danish Saleheen**[4], **Muredach P Reilly**[2,**], and **Daniel J Rader**[1,2,3,**]

[1]Division of Translational Medicine and Human Genetics, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, PA, USA

[2]Penn Cardiovascular Institute, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, PA, USA

[3]Department of Genetics, Perelman School of Medicine, University of Pennsylvania, PA, USA

[4]Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, PA, USA

Abstract

Genome-wide association studies (GWAS) have provided a rich collection of ~58 CAD loci that suggest the existence of previously unsuspected new biology relevant to atherosclerosis. However, these studies only identify genomic *loci* associated with CAD and many questions remain even after a genomic locus is definitively implicated, including the nature of the causal variant(s) and the causal gene(s), as well as the directionality of effect. There are a number of tools that can be employed for investigation of the functional genomics of these loci, and progress has been made on a limited number of novel CAD loci. New biology regarding atherosclerosis and CAD will be learned through the functional genomics of these loci and the hope is that at least some of these new pathways relevant to CAD pathogenesis will yield new therapeutic targets for the prevention and treatment of CAD.

Keywords

atherosclerosis; genome-wide; functional; coronary artery disease; genomics

Introduction

Efforts to perform unbiased discovery using the tools of human genetics to uncover novel pathways underlying complex diseases and traits have been pursued extensively over the last decade. In particular, genome-wide association studies (GWAS), reviewed elsewhere in this compendium, have capitalized on the millions of common single nucleotide polymorphisms (SNPs) to identify those SNPs that are genome-wide significantly associated with a disease

**communicating authors: muredach@mail.med.upenn.edu, rader@upenn.edu.
*equal contribution

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or trait. More than 20 thousand genetic loci have been identified to be associated with diseases or traits (http://www.ebi.ac.uk/gwas December 2015). In particular, myocardial infarction (MI), and more generally coronary artery disease (CAD), have been the focus of intense discovery using GWAS. In the most recent and largest meta-analysis of GWAS for CAD[1], 10 new CAD loci were identified, bringing the total number of CAD loci to 58 (Table 1).

It is interesting to examine this list of loci in light of known risk factors for CAD. For example, 10 of the loci are also genome-wide significantly associated with LDL-cholesterol (LDL-C)[2–5], a known causal risk factor for CAD. The causal genes at these loci exert their effects through their expression in hepatocytes or enterocytes, consistent with their role in regulating LDL metabolism. Another locus, *LPL,* harbors the gene encoding the enzyme lipoprotein lipase (LPL), the most important regulator of triglyceride-rich lipoprotein (TRL) metabolism. In addition, the *APOA1/C3/A4/A5* locus associated with LDL-C is also associated with triglycerides (TG) and harbors two genes, *APOC3* and *APOA5*, in which coding variants have been shown to be associated with both TG levels and CAD.[6–9] These and other observations have helped to confirm the causal role of TRLs in CAD. Another five loci are genome-wide significant for association with blood pressure, consistent with the causal role of elevated blood pressure in CAD. Also there is some overlap with GWAS studies for other vascular diseases such as stroke[10]. Interestingly, none of the 58 loci are associated with type 2 diabetes mellitus (T2DM), raising interesting questions regarding the genetic overlap between T2DM and CAD and whether T2DM per se is causally related to CAD. Importantly, the majority of CAD GWAS loci are not associated with known risk factors for CAD (Table 1) and thus have the potential to provide novel insights into the biology and pathophysiology of CAD.

Issues and challenges for functional genomics of CAD GWAS loci

Common variant GWAS studies only identify genomic *loci* associated with disease or trait. However, many questions remain even after a genomic locus is definitively implicated, including the nature of the causal variant(s) and the causal gene(s), as well as the directionality of effect. For the majority of the CAD GWAS loci, the answers to these questions are unknown, and after excluding the loci associated with lipids or blood pressure virtually none of the remaining loci have answers to these fundamental questions. Identification of the causal variant is challenging, because of linkage disequilibrium (LD) and the possibility that the variant(s) at a given locus with the lowest p-values for association with CAD may simply be proxies for the causal variant (see Figure 1). Furthermore, although the majority of variants with the lowest p-values fall in non-coding intergenic regions, they usually do not fall within a well-established *cis*-regulatory element such as a known promoter, and thus challenge predictions of their impact on regulatory elements, like disruption of transcription factor binding or function of a long non-coding RNA (lncRNA). Below we discuss approaches to elucidating the causal variant at a GWAS locus.

Arguably the most important biological question to be addressed at each CAD GWAS locus is what the causal gene(s) at the locus are. By convention, GWAS loci are tabulated by the coding gene closest to the ‘lead SNP’ with the lowest p-value. However, it is becoming clear

that this approach does not always identify the causal gene[11]. Because of chromatin looping that places regulatory enhancer elements in proximity to the promoters of genes that may be quite distant on the physical map, a causal variant may influence expression of distant genes (Figure 1). Furthermore, the 'causal gene' at a GWAS locus need not necessarily be a protein-coding gene, but could be a lncRNA (for example *ANRIL*, a lncRNA at the 9p21 CAD locus), a microRNA, or some other transcribed or regulatory element. Finally, some loci may not have a *single* 'causal gene' but in fact may be characterized by the coordinate regulation of several genes, potentially in different contributing cell types, that have additive effects on disease phenotype. Below we discuss in some detail the methodological approaches to solving these critical biological conundrums.

The directionality of effect at the locus is a critically important issue, particularly with regard to the question of whether the biology represented by that locus can be approached from a therapeutic targeting standpoint. For example, if the minor allele at a locus is associated with protection from CAD, it is essential to know if the minor allele is associated with increased or decreased expression of the causal gene in the relevant cell type. Approaches such as expression quantitative trait loci (eQTL) and allele-specific expression (ASE) can be used to establish directionality of effect. However, the effects of many variants on differential gene expression are cell type specific and in most cases we don't know with any confidence the relevant cell type for the genetic effect. Again, below we discuss the experimental approach to establishing directionality of effect given these challenges.

The tools of functional genomics

There is large variety of experimental tools available to investigate the mechanism by which GWAS loci exert their effect on biological phenotype. They are tailored to the scale of interrogation, sampling on the genome-wide level, targeting a gene, a genomic region or even a specific SNP of interest. Figure 2 gives an overview of the experimental techniques which will be discussed in detail below.

Next-generation sequencing

Most polymorphisms associated with risk for disease lie in non protein-coding regions of the human genome. A major mechanism of how these SNPs affect disease phenotype is by disruption of a transcription factor binding site within a *cis*-regulatory element. The subsequent dysregulation of gene expression leads to a changed cellular response. The first step in establishing a causal chain of evidence from association SNP to disease phenotype lies in the definition of genomic regions with *cis*-regulatory potential in a disease-relevant cell type. Genome-wide methods such as 'Formaldehyde-Assisted Isolation of Regulatory Elements' (FAIRE-Seq)[12], DNase Hypersensitivity sequencing[13] and the novel 'Assay for Transposase-Accessible Chromatin using Sequencing' (ATAC-Seq)[14] assess the general accessibility of chromatin. However, these patterns only portray a general 'openness' of the genome and include *cis*-regulatory regions as well as promoters and protein-coding regions. Also, these techniques cannot distinguish between poised, primed and actively functional regulatory regions – they only map the general regulatory potential.

In contrast, several different histone modifications have been shown to sample distinct fractions of the human genome, such as promoters (Histone 3 Lysine 4 trimethylation - H3K4me3), transcribed genes (Histone 3 Lysine 36 trimethylation - H3K36me3), primed (Histone 3 Lysine 4 monomethylation - H3K4me1) and active (Histone 3 Lysine 27 acetylation - H3K27ac) enhancers.[15] Chromatin immunoprecipitation with massively parallel sequencing (ChIP-Seq) is the method of choice to identify these chromatin marks. This technique can also be used to identify the binding events of specific transcription factors of interest.

Active enhancers are identified with the highest confidence when datasets are intersected, for example by combining H3K27ac abundance with Polymerase II binding patterns from ChIP-seq as well as Gro-Seq. The latter technique maps actively transcribed RNAs from enhancers (eRNAs)[16] through the isolation of newly synthesized RNA from nuclei incubated with bromouridine. Large-scale consortium data for histone marks and transcription factor binding is now publicly available from the ENCODE project (https://genome.ucsc.edu/ENCODE/dataMatrix/encodeChipMatrixHuman.html). The experimental data stems from *in vitro* cultured cells, many of them immortalized human cell lines. Of particular interest for the annotation of CAD GWAS loci is data generated from HepG2 cells (a hepatocellular carcinoma line that serves as a proxy for hepatocytes), endothelial HUVECs, CD14+ monocytes, and aortic smooth muscle cells. Similarly, the Roadmap Epigenomics Project (www.roadmapepigenomics.org/data/tables/adult) compiled a large portfolio of histone marks from human tissues, including data from liver, aorta and primary CD14+ monocytes.

In addition to revealing transcription factor occupancy and histone modification at sites of interest, ChIP-Seq data can also be interrogated for allelic imbalance on an individual basis, if paired with corresponding genotype information.[17,18] In this case sequencing reads from ChIP-Seq experiments are assigned to either the maternal or paternal allele. A significant difference in coverage on either allele is indicative for sequence dependent differential transcription factor binding or histone abundance in this region and provides direct evidence for the functional role of a polymorphic site.

A second step in elucidating the mechanism by which non-coding polymorphisms affect disease risk lies in the analysis of transcriptome data from disease-relevant cells and tissues. Whole-transcriptome RNA-Seq data provides useful information on the transcriptional repertoire of a cell of interest. No meaningful association of regulatory variants with disease risk genes is possible without knowledge of the cell's transcriptome. Publicly available expression data from relevant cells and tissues is provided by the ENCODE consortium and to a larger extent the GTEx consortium (www.gtexportal.org), which generated transcriptome data from primary human post mortem tissue.[19]

Technical advances such as single-cell based RNA-Seq[20] and single-cell ChIP-Seq[21] allow for the interrogation of the transcriptional machinery in single cells. Future applications may allow simultaneous derivation of both datasets from the same cell, which would greatly reduce the information lost due to sampling heterogeneous pools of cells at different cell cycle and developmental stages. This could be particularly useful for the characterization of the diverse cell populations within the atherosclerotic neointima.

An important consideration in functional transcriptomic analysis is that since both transcriptional regulation and gene expression are tissue-specific processes, particular attention should be paid to the suitability of cell type from which the data is generated. Early datasets have depended heavily on samples generated in cell culture for practical reasons, namely the accessibility of long-established cell lines, and the ability to generate homogeneous material, reproducibly, and in large scale. However, all cultured cells, whether they be immortalized or cancer cell lines, induced pluripotent stem cell-derived cells, or cultured primary cells, display to some extent rather an immature, precursor-like phenotype in comparison to the corresponding fully differentiated primary cell *in vivo*. Additionally, cancer cell lines in particular, often carry genomic rearrangements leading to artifacts that are not representative of their cell type of origin. On the other hand, primary *ex vivo* tissues, whilst displaying the most 'authentic' transcriptional profiles, are often comprised of multiple cell types which complicates analysis and can mask subtle effects within data noise, or due to numerically underrepresented cell types. The best approach when dealing with tissues of mixed cell types such as coronary arteries may be to focus on effects also observed in *in vitro* cultured pure cell populations.

Furthermore primary tissues need to be extensively phenotyped as to their disease status to enable identification of changes within the transcriptome between the healthy and diseased state. Additionally, the exact tissue origin can play an important role. For example aortic, coronary or femoral artery transcriptomes, although often very similar, can show distinct differences in their expression profiles which may be based on their differing tissue environments or developmental origins. Differences in gene expression are well established along the different section of the aorta itself and follow embryological and hemodynamic patterns.

For meaningful correlation of ChIP-Seq and RNA-Seq data to identify SNP-to-gene interactions, data should be generated from the same cell type, if possible even from the same individual culture. In case of primary tissue, combined data sets from the same individual are particularly informative. The association of *cis*-regulatory regions with one or multiple nearby regulated targeted gene or genes is however problematic. Frequently employed distance-based methods do not adequately reflect the true biology. More meaningful approaches to directly link SNP genotype with gene expression levels include expression quantitative trait loci (eQTL) and allele-specific expression (ASE) studies.

eQTLs and ASE

Quantitative trait loci are polymorphic sites within a genome which show significant association with a quantitative trait such as plasma lipid levels[22], carotid intima-media thickness[23] or gene expression levels (eQTL) [24, 25]. eQTL studies combine genotype with gene expression level information - the latter usually assessed using expression microarrays or RNA-seq - and can detect both local *cis*-effects as well as distal *trans*-effects of regulatory elements on gene expression. eQTL data has been generated from multiple human cells and tissues[26–28]. Of particular relevance is data derived from liver, which has proven especially successful for loci involved in dyslipidemia, as well as data from peripheral blood monocytes[29] and *in vitro* cultured human aortic endothelial cells[30].

eQTL studies require sampling from several hundreds of individuals to identify the majority of loci with statistical significance as inter-individual noise arises from differences in genetic background, host factors such as age or gender as well as environmental factors like diet and lifestyle. The large sample sizes needed for eQTL analysis are particularly problematic when interrogating tissues that are rare or difficult to sample. Additional challenges such as insufficient platform coverage and batch variation stem from the use of microarrays to determine gene expression levels. These issues can be circumvented in large part by the use of RNA-Seq as a data source, which greatly increases statistical power, and is more compatible with meta-analysis.

eQTL studies have been successfully used to identify functional SNPs and directionality from GWAS studies for a variety of diseases, due to changes in the expression of their downstream putative risk genes[31]. Since CAD is a complex disease involving multiple tissues, recent studies have aimed at integrating expression data from several tissues for a more comprehensive annotation of CAD GWAS loci[32,33]. However, the portfolio of tissues used included several human tissues not relevant to CAD, which may have introduced bias in the SNP-to-gene association process. Fortunately, the GTEx consortium is generating publicly available RNA-Seq data from currently under represented CAD-relevant tissues, including coronary artery and aorta.

Allele-specific expression has emerged as an alternative to eQTL analysis for linking genetic variation in *cis*-regulatory regions to gene expression[34–37]. It is based on the identification of allelic imbalance, showing differences in gene expression levels between the two alleles in a single heterozygous individual. The within person allelic analyses greatly reduces impact of inter-individual variation from environmental and genetic *trans* effects thus enhancing statistical power. ASE requires allele-specific transcriptomic data such as stranded RNA-Seq data, but in contrast to eQTL analysis, a relatively small number of samples that carry the same heterozygous site of interest are needed. The information value of each sample is limited by the number of heterozygous sites of its genome. Additionally, the data sets that are generated need to be of high read coverage to ensure presence of multiple reads at interrogated sites of interest. With regards to CAD, to date ASE has for been applied to assess general principles of the regulation of gene expression in mouse liver[38, 39].

Additionally, Chromosome Conformation Capture (3C) can be used as a complementary technique to associate *cis*-regulatory regions with their target genes. This method captures the physical interaction between two genomic regions such as enhancers with promoters, and has recently been applied to study human liver and aorta[40]. Several different variations of the technique are in use, with Hi-C and 5C as genome-wide methods. A major obstacle is however their low resolution of tens to hundreds of kilobases. Targeted approaches such as Capture-C[41] and Capture Hi-C[42] that can interrogate hundreds of select loci simultaneously are displaying higher resolutions of down to 1 kilobase (kb) and single cell approaches are starting to emerge[43]. The improved availability of tissues relevant to CAD and increasingly sensitive methods at hand to link association SNPs to downstream risk genes, suggest that a comprehensive re-annotation of all 58 known CAD association loci in disease-relevant cells and tissues using the latest methods may be worthwhile.

Non-coding RNAs

A substantial proportion of trait-associated SNPs identified by GWAS lie outside of protein coding regions and map to the non-coding intervals[44]. However, the mechanistic relationship of trait-associated SNPs with the non-coding functional genome is poorly understood. Since protein-coding genes account for only a very small proportion of the transcribed human genome, non-coding (nc) RNA are now emerging as alternative functional genomic elements underlying GWAS hits. Along with microRNAs, long non-coding RNAs (lncRNAs), ncRNAs defined as transcripts larger than 200 nucleotides (nt) in length, are emerging as important regulators involved in cancer as well as in neurological, cardiovascular, developmental and other human diseases[45], highlighting the need to investigate the possible contributions of variations in ncRNAs to human diseases.

Systematic analyses are emerging that evaluate the potential association of regulatory ncRNAs, with complex traits. miRNAs comprise a class of short (20–24 nt) regulatory RNAs that modulate mRNA translation and turnover. A recent study leveraged GWAS meta-analysis in more than 188,000 individuals to identify 69 miRNAs located in genomic regions associated with abnormal blood lipid levels[46]. The work identified four miRNAs (miR-128-1, miR-148a, miR-130b and miR-301b) that are associated with LDL-C uptake and cholesterol efflux by possibly controlling the expression of the LDL receptor (LDLR) and the ATP-binding cassette transporter A1, respectively. miR-QTL studies using liver tissue from 424 morbidly obese individuals revealed an association of miR-128-1 and miR-148a expression with SNPs linked to abnormal human blood lipid levels, suggesting the relevance of these miRNAs identified by GWAS studies to human cardiometabolic disorders[46].

A subset of lncRNAs, intergenic lncRNAs (lincRNAs), represents a rapidly evolving catalog of lncRNA species that does not overlap with exons of protein-coding genes.[47] A number of studies examined the implication of lincRNAs in complex diseases based on GWAS studies (LincPoly[48], LincSNP[49]). These studies represent initial efforts to integrate disease-associated SNPs and human lincRNAs, but both datasets do not include complete GWAS SNP data, and focus on only a few thousand lincRNAs. A recent study identified 495,729 and 777,095 SNPs in more than 30,000 lncRNA transcripts in human and mouse, respectively. A large number of SNPs were predicted to impact the lncRNA secondary structure and modulate lncRNA–miRNA interactions. By mapping these SNPs to GWAS results, 142 human lncRNA SNPs are GWAS tagSNPs and 197,827 lncRNA SNPs are within these GWAS LD regions.[50] Kumar et al. examined the association of SNPs with expression of lincRNAs in human blood, and identified 112 *cis*-regulated lincRNAs. A considerable number of the observed lincRNA *cis*-eQTLs had disease- or trait-associations[51] suggesting that intergenic GWAS-associated SNPs may act by modulating expression of specific lincRNAs.

Although functional roles of most lincRNAs remain elusive, mechanistic insights into distinct nuclear and cytoplasmic actions[47, 52, 53] for a small number of well-studied lincRNAs strongly suggest that some lincRNAs play major regulatory roles in a variety of cellular processes, such as X chromosome inactivation[54], embryogenesis[55], cell pluripotency[56], cell development and differentiation[57]. Depending on their subcellular

localization, lincRNAs can mediate gene expression through distinct mechanisms. In the nucleus, they are involved in co-transcriptional regulation, recruitment of proteins complexes to specific loci for *cis* or *trans* regulation of gene expression or scaffolding of nuclear complexes[47, 52]. In the cytoplasm, lincRNAs can function as competitive endogenous RNAs that bind miRNAs and inhibit their activity, pair with mRNAs to trigger post-transcriptional regulation or interact with target proteins to modulate their function[47, 52]. LincRNAs are increasingly implicated in human diseases, including cancer,[58] neurological diseases[59] and cardiovascular disorders[60–66], and also modulate physiology and pathophysiology in cells relevant to cardiometabolic disease. For example, cardiac lncRNAs like *Braveheart, CHRF and Mhrt* regulate cardiomyocyte differentiation and cardiac hypertrophy[60, 63, 64]. LincRNAs in smooth muscle and endothelial cells (*MALAT*, linc-p21) regulate proliferation[65, 66]. A few lincRNAs have been implicated in macrophage functions: *lincRNA-Cox2* in mouse represses the basal expression of interferon-stimulated genes (ISGs) by partnering with the heterogeneous nuclear ribonucleoproteins (hnRNPs) hnRNPA/B and hnRNPA2/B1[67] and a human monocytic THP-1 lincRNA called TNF and hnRNPL-related immunoregulatory lincRNA (*THRIL*) regulates expression of tumor necrosis factor (*TNF*) through its interactions with hmRNPL[68]. Conserved adipose lincRNAs, such as *Firre* and *Blnc1*[69–71], and species-specific lincRNAs, such as lnc-BATE1 and *ADINR*[72, 73], regulate adipogenesis of white and brown adipocytes in mouse and human.

Several studies have tried to identify and validate the causal GWAS variants that regulate lncRNA expression and function. A well-known example on chromosome **9p21** that encompasses an antisense lncRNA, ANRIL (antisense ncRNA of the INK4 locus) has been significantly associated with susceptibility to coronary disease as well as abdominal aortic and intracranial aneurysms[74]. Some associated SNPs in this region have been shown to alter the transcription and processing of ANRIL transcripts[75].

To further uncover the effects of GWAS-associated genetic variants on ncRNAs function, future studies are needed to: 1) Define a comprehensive genome-wide set of human lncRNAs across all disease-relevant tissues. Because lncRNAs have lower and more tissue-specific expression patterns than mRNAs, this requires deeper RNA sequencing of multiple human cells and tissue than is currently available in GENCODE[76] or the human bodymap[77]; 2) Investigate ncRNA enrichment and association in targeted yet genome-wide approaches within catalogued GWAS and particularly whole genome sequencing projects as they emerge, for example NHLBI's Trans-Omics for Precision Medicine (TOPMed) Program (https://www.nhlbiwgs.org); 3) establish bioinformatics pipelines to prioritize trait-associated ncRNAs (systematically using synteny and conservation, tissue expression, eQTL and ASE, and ChIP-Seq promoter, enhancer and transcription factors marks at trait-associated lncRNAs); and 4) Establish high-throughput pipelines for human-relevant functional follow-up of trait-associated ncRNAs in disease-relevant cell types and in *in vivo* animal models.

Taken together, ncRNAs, such as miRNAs and lncRNAs may be important for interpreting GWAS data and may in many cases act as the causal genomic element in contributing to human cardiometabolic diseases. The therapeutic tractability of potent and specific antisense

technologies targeting single or multiple ncRNAs implicated in human cardiometabolic diseases may thus have important clinical ramifications for the treatment of these diseases.

Genome editing

The putative functional variants identified by fine mapping overlapping with regulatory marks, eQTL and ASE analysis, and other bioinformatic approaches require further experimental validation to establish causality. Genome engineering and human induced pluripotent stem cells (hiPSCs), when combined, represent powerful tools to accelerate GWAS-driven functional validation of causal variants at trait-associated loci.

Genome engineering represents strategies and techniques developed in recent years for the targeted modification of the genetic information. The type II clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein 9 (Cas9), an RNA-guided nuclease, are based on a bacterial system that has been modified for genome engineering in mammalian cells[78, 79]. Due to its ease of adaptability and improved efficiency, CRISPR/Cas9 has rapidly become one of the most popular approaches for genome engineering[80]. Consisting of Cas9 and a short guide RNA (gRNA), it generates site-specific DNA breaks, which are repaired by either non-homologous end-joining (NHEJ), creating insertions or deletions at the site of the break, or homology-directed repair (HDR) by precise change of a genomic sequence using an exogenously introduced donor template[81]. In addition to the disruption of genomic sequence through nucleases, the CRISPR-associated catalytically inactive Cas9 protein, termed dCas9, can be fused to repressor[82] or activator domains[82–84], named CRISPRi[82] and CRISPRa[82–84], respectively. Such modified CRISPR/dCas9-fusion proteins, together with guide RNA, can then be introduced to control the expression or activity of candidate gene or regulatory elements that harbor GWAS signals[85].

Although the CRISPR/Cas9 system is highly efficient in human cell lines, gene editing in primary human cells is very challenging. Human iPSCs have the potential to be differentiated to all adult cell types, including rare or inaccessible human cell populations, for reliable disease modeling. By generating clonal lines carrying desired genetic modification introduced by CRISPR/Cas9 and then differentiating to somatic cells relevant to atherosclerosis (for example hepatocytes, macrophages, smooth muscle cells, endothelial cells, cardiomyocytes), genome editing in hiPSCs provides a unique platform for functional validation of GWAS CAD loci across multiple disease-relevant cell types. Applications include: 1) Double strand break repaired by NHEJ pathway may introduce frame-shift mutation or early stop codon, which, if in a critical coding exon, likely causes nonsense-mediated decay of the mRNA and effectively eliminates gene function for loss-of function (LOF) studies; 2) By HDR-mediated precise nucleotide alteration using a donor template, it is feasible to generate hiPSC lines in which the disease-associated SNP is the sole experimental variable, thereby investigating the causal role of genetic variants - for instance the targeted alteration of specific transcription factor binding site motifs in otherwise intact loci could reveal the functional contribution of transcription factor binding to the function of a regulatory element; 3) The unique multiplexing capabilities of the CRISPR/Cas9 system facilitate the deletion of a large stretch of genomic DNA, enabling the functional

interrogation of non-coding regulatory elements and non-coding transcripts. In addition, because most disease-associated SNPs confer only modest risk, the relevance of multiple monoallelic and biallelic combination can be addressed by multiplexing of CRISPR/Cas9 gene editing.

One example of such an interrogation investigates the role of an intronic variant (rs9349379) in the CAD/MI risk locus PHACTR1 prioritized by genetic fine-mapping and eQTL in human coronary arteries. The study using endothelial cell extracts first showed that alleles at rs9349379 are differentially bound by the transcription factors myocyte enhancer factor-2 (MEF2). The deletion of this MEF2-binding site using CRISPR/Cas9 in hiPSC and subsequent differentiation to endothelial cells then revealed that heterozygous endothelial cells carrying the deletion express 35 percent less PHACTR1 transcript[86].

Despite being powerful, precise editing of human genomes in pluripotent stem cells by HDR of targeted nuclease-induced cleavage has been hindered by the low efficiency of HDR over NHEJ, making the screening of clones containing desired genotypes time-consuming and labor-intensive. However, great strides have been made to improve the efficiency of HDR. A Cas9D10A mutant functioning as a nickase yields similar HDR but lower NHEJ mutation rates[81]. The establishment of the iCRISPR platform through targeting of inducible Cas9 expression cassettes into the AAVS1 locus in human embryonic stem (ES) and iPS cell lines has increased markedly the efficiency of genetic modifications for both knockout or knockin of genetic variants[87]. The overall gene editing efficiency can be further enhanced by transfection of ribonuclear protein (RNP) complex that is comprised of the recombinant Cas9 protein and synthetic gRNAs[88], either *in vitro* transcribed or chemically synthesized and modified[89], the latter further improving efficiency. Several studies have also found that inhibition of DNA ligase IV[90] and DNA-PKcs (DNA-dependent protein kinase, catalytic subunit)[91], key players in the NHEJ pathway, promote HDR while reducing the frequency of NHEJ[90–92].

Future studies need to: 1) Further improve the efficiency and reduce the cost of hiPSC differentiation; 2) Optimize differentiation protocols to produce mature cells phenotypically, functionally and transcriptomically highly similar to primary somatic cells; 3) Improve the efficiency of HDR-mediated precise nucleotide alteration over NHEJ; 4) Apply CRISPRi and CRISPRa in hiPSC for dynamic and precise control of expression of individual transcripts in hiPSC and differentiated cells; 5) Adapt conditional knockout for the assessment of gene function in different lineages of differentiation; 6) Establish more advanced techniques to facilitate rapid screening of rare iPS clones carrying the desired genotypes[93], and thoroughly evaluate potential off-target effects.

In summary, facile high efficiency genome-editing coupled with hiPSC differentiation can pave the way for functional interrogation of GWAS variants and loci of complex non-Mendelian diseases such as CAD, and can help delineate human genotype-phenotype relationship in human cellular disease models, and potentially, in genetically-modified mice carrying mutations, reporter or conditional alleles for *in vivo* modeling using CRISPR/Cas9-mediated genome engineering[94, 95].

Somatic gene targeting (siRNA, ASO, and AAV)

As mentioned above, transgenic mouse models that are genetically predisposed to develop atherosclerosis due to partial or complete loss of ApoE or Ldlr function, or that have been modified to have plasma lipid profiles which more closely reflect human biology are invaluable in the functional study of candidate GWAS genes. Mouse lines with 'humanized' lipid profiles include the Apobec knockout and the human apolipoprotein B(100) transgenic mouse models, which have been combined with haploinsufficient Ldlr deficiency in the LAhB-H mouse strain[96]. Genetic ablation remains the gold standard for the characterization of gene function: combining conditional aproaches and tissue-specific Cre drivers allow precise interrogation of the potential role of a gene to the phenotype of interest. On the other hand, even with the advent of facile CRISPR/Cas9 genome editing, the development of genetic models is laborious, expensive, and time-consuming.

Alternatives to genetic approaches include the use of small interfering RNA (siRNA) or antisense oligonucleotide (ASO) inhibitors. In addition, adeno-associated virus (AAV) platforms can be used for either overexpression, or for permanent loss of function by expression of short hairpin RNAs (shRNA). Combining these approaches with the existing genetic atherosclerosis models is a potent way to accelerate GWAS functional analysis, but compared to more rigorous genetic approaches involves some compromises and limitations in interpretability. These approaches are therefore not a substitute for subsequent genetic validation of promising putative causal genes, but rather a way to quickly prioritize candidate genes for further study. ASO and siRNA have in common that they are systemically delivered, modified nucleic acids that target the gene of interest through complementary base-pairing between their primary sequences and those of their target transcripts. Despite these similarities, the two forms differ in their mechanism of action. In the case of **siRNA**, the technology takes advantage of the RNA-direct RNA endonuclease activity of Argonaute2 (Ago2), the miRNA binding component of the RNA-interference Silencing Complex (RISC). The cytoplasmic RNase III endonuclease, Dicer1, cleaves double-stranded or short-hairpin RNA, and concomitantly loads one strand of the circa 22 basepair cleavage product into a binding cleft in Ago2. The solvent-exposed bases of the Ago-loaded RNA serve to target RISC to complementary sequences[97]. Endogenous microRNAs in mammals have imperfect complementarity with their targets: down regulation occurs by a combination of mRNA destabilization due to the recruitment of decapping and de-adenylation factors, and to the inhibition of translational initiation [98]. In contrast, artificial shRNA and siRNA systems take advantage of an evolutionary remnant activity of Ago2, which cleaves the paired target strand where perfect complementarity exists between itself and the loaded RNA [99]. The interfering RNA serves only as a targeting component and is not cleaved itself. The two types of RNA interference most significantly differ in their entry points to the endogenous system: while exogenous siRNAs can be transfected directly in cell culture, the shRNA are supplied as transgenes (usually along with a reporter gene, such as eGFP). As such, the shRNA genes must be transcribed in the nucleus, and the hairpin RNAs exported to the cytoplasm, cleaved by Dicer and loaded into RISC. The advantage of the shRNA approach is that candidate inhibitory RNAs can be validated in cell culture, and then readily adapted to viral or transgenic applications *in vivo*. However, the nuclear export protein, Exportin-5, which shuttles shRNA to the cytoplasm has

been shown to be limiting in the biogenesis of shRNA, leading to initial limitations of this approach[100]. A number of subsequent innovations have alleviated these concerns, which were partly due to saturation of the miRNA biogenesis pathway, but also to off-target effects of the passenger strands of the shRNAs[101–103].

In contrast to shRNA, siRNA bypasses the requirement for nuclear transcription and export: upon entry into the cytoplasm; the siRNA is rapidly loaded into RISC and interference begins. Since naked RNA has an extremely low half-life in plasma, and since endocytosed RNA is targeted to the lysosome and degraded, a variety of strategies have been developed to evade these obstacles to *in vivo* use[104]. In addition to its usefulness in basic research, RNA interference has already shown therapeutic potential in CVD, for example by targeting PCSK9[105].

ASOs are also short nucleic acids, but do not depend on the RISC complex for their action. In the context of functional analysis of GWAS hits, ASOs may be deployed in three ways: 1) targeted to a transcript of a protein-coding gene to interfere with the initiation of translation; 2) targeted to intron-containing genes to block splicing, and 3) targeted against microRNAs to block their inhibitory effects on target genes. In each of these cases, pairing of the ASO with its target physically precludes the interaction of the target RNA with another molecule, elongation initiation factors, splicing factors, or target mRNAs respectively. In addition, the specialized class of ASOs termed ‘gapmers’ are designed with a central stretch of unmodified DNA nucleotides, which when base-paired to a complementary RNA target yields a heteroduplex that is recognized as a substrate by the ubiquitous intracellular ribonuclease, RNase H1. The resulting cleavage of the RNA strand of the heteroduplex by RNase H1 is analogous to that of siRNA, albeit by a completely different mechanism, and induces rapid turnover of the cleaved RNA. It should be noted that mipomersen, a therapeutic ASO targeting apoB, which is approved for the treatment of homozygous familial hypercholesterolemia, is based on a gapmer strategy[106].

The advantage of RNA-targeting strategies as an approach, irrespective of the precise mechanism, is that they are relatively straightforward in design and validation. There are however key limitations of this approach. Systemic delivery of nucleic acids has been shown to induce an inflammatory response - for any given inhibitor there is a possibility of off-target effects of the artificial RNA, and the biodistribution of the oligonucleotides can be influenced slightly, but not tightly controlled. The inflammatory side effects have been largely mitigated by successive innovations in the chemistry of the synthetic nucleic acids used, and concerns over off-target effects can be addressed by the separate use of independent ASOs targeting a given gene. The limitations concerning the delivery and distribution are harder to address: the fact that most oligonucleotides end up by default in the liver (predominantly in hepatocytes) is less of a concern in the functional analysis of CVD GWAS candidate risk genes that are liver-expressed, than it might be in other biological contexts. In addition, both siRNA and ASO approaches only permit gene knockdown but not upregulation.

Adeno-associated virus (**AAV**) vectors provide a tractable system to perform the reciprocal experiment: overexpression of candidate causal genes for functional analysis *in vivo*[107]. As

noted above, they can also be adapted for corresponding loss of function experiments using shRNA expression cassettes. As the name suggests, AAV was identified as a coinfecting parvovirus with adenovirus. To date AAV has no identified role in any human disease, and does not replicate in the absence of adenovirus. These characteristics have made it an attractive platform for candidate gene therapy development, which has been a boon for parallel uses in basic science. AAV induces minimal immune response compared to other viral vectors commonly used for somatic gene expression (specifically adenovirus and lentivirus). An additional advantage is the availability of multiple serotypes with varying tropisms, which addresses a significant shortfall in the application of system siRNA/ASOs: combining serotyped-limited tropism with tissue-restricted promoters allows a significantly nuanced expression of genes or shRNAs that target them. Furthermore, significant efforts have been made to improve and refine the naturally isolated serotypes by repeated rounds of *in vivo* selection and expansion, suggesting that even more tissue-selective versions will be available in the near future[108]. A significant validation of this platform is that AAV-driven expression of PCSK9 when coupled with a dietary stress (high fat diet) induces atherosclerosis in mice[109].

Hepatocyte-expressed genes are particularly amenable to study using AAV: AAV8 transduction rates of hepatocytes are high and the relatively quiescent nature of the adult liver allows expression to be maintained for many months. One significant limitation is however that the packaging capacity of AAV is not very large: approximately 4.8 kb for AAV and about 2.4 kb for its self-complementarity derivative. While this precludes the use of AAV for some genes, the platform is nonetheless very powerful and widely used. To date, clinical AAV gene therapy has been approved only in Europe, and only for the treatment of familial lipoprotein lipase deficiency[110], but homozygous familial hypercholesterolemia is a CVD-relevant condition that is an excellent candidate for an AAV-based therapeutic[111].

Examples of functional genomics at selected CAD GWAS loci

Recent progress has been made in understanding the biology underlying some of the genes which have been implicated in risk for CAD. Being expressed in distinct cell types involved in atherosclerosis (Figure 3), these genes exert their effects in a cell type specific manner, which determines their specific contributions to disease. We provide here five selected examples of CAD GWAS loci for which the tools described above have been variably used to identify the causal gene at the locus and probe the underlying biology linking the gene to CAD.

SORT1 (sortilin)

A compelling and now widely replicated novel locus associated with plasma lipid traits is the chromosome 1p13 locus, which had the lowest p-value of association in the Global Lipids Genetics Consortium (GLGC) study[4]. Notably, this locus had been independently and genome-wide significantly associated with MI/CAD, suggesting that it is of high importance to human cardiovascular health[112–115]. The locus harbors a high density of genes that might plausibly contribute to the phenotype, which necessitated thorough functional analysis. Fine-scale mapping of the locus refined the signal to a 6.1 kb genomic region containing six SNPs

in high LD. Cloning of this region into a luciferase reporter construct, and the separate replacement of each SNP with the corresponding minor allele variant identified rs12740374 as the causal SNP. The mechanism by which it exerts its effect is due to the creation of a novel C/EBPa binding site, which was functionally validated by gel shift assays[115]. Nonetheless, the causal gene remained ambiguous: *SORT1* and *PSRC1* both had strong eQTLs in liver and a priori, neither gene could be eliminated as causal. To address this, the genes were separately overexpressed using the hepatocyte-tropic AAV8 system. SORT1 overexpression, but not that of PSRC1, substantially decreased plasma LDL-C in a mouse model with a humanized lipid profile (LahB, as described above), identifying SORT1 as the causal gene[115].

Sortilin is a type I transmembrane multi-ligand receptor that is synthesized in the ER as a propeptide and is further processed to an active, mature form in the Golgi. It localizes to both the Golgi and plasma membranes, and facilitates trafficking of a variety of proteins bidirectionally between the Golgi lumen and the extracellular environment. Sortilin can also facilitate protein degradation by shuttling proteins from the Golgi to the lysosome through the endolysosome. Preliminary characterization of the role of sortilin in regulating very low-density lipoprotein (VLDL) secretion was performed through a series of *Sort1* overexpression studies in hepatocytes and hepatocyte-like cell lines, and in a variety of mouse models. *Sort1* expression was shown both to decrease VLDL secretion rates and increase plasma LDL turnover, thereby reducing plasma cholesterol additively[116]. Surface plasmon resonance demonstrated a high affinity pH-dependent interaction between sortilin and apoB-containing lipoproteins; and mutants defective in their ability to traffic to the endolysosomal system were used to show that sortilin serves as a bona fide cell surface LDL receptor. Wild-type sortilin binds LDL at the cell surface in an LDL receptor-independent manner and delivers the LDL to the endolysosomal system for degradation[115, 116].

Based on the concordance of the human GWAS and mouse overexpression data it was hypothesized that genetic knockout or knockdown of Sort1 would have the opposite effect, increasing plasma cholesterol and VLDL secretion. However, the reported effects of the genetic loss of sortilin on VLDL secretion have been contradictory and perplexing: loss of sortilin has been shown in different studies to result in either increased and decreased VLDL secretion[116–118]. These discrepancies likely reflect the differences in models, methods of ablating sortilin function, lengths of time under diet-induced lipid overload, and technical approaches to measuring outcomes. Further complicating the story, *Sort1*−/− mice have recently been shown to be more insulin sensitive than wild-type mice on a high fat diet[119], and loss of sortilin in 3T3-L1 adipocytes and C2C12 myotubes decreases insulin-stimulated glucose uptake due to decreased transport of Glut4 to the plasma membrane[120, 121]. While these data suggest that sortilin influences insulin signaling, the sortilin protein is itself regulated by insulin signaling[122]. Increased insulin sensitivity in extrahepatic tissues in *Sort1*−/− mice could be responsible for a decrease in FFA flux to the liver, a major contributor to hepatic lipid accumulation and a driver of VLDL secretion during insulin resistance. The role of sortilin in the tissues other than the liver under these conditions is unknown and confounds the interpretation of the effect of sortilin knockout and knockdown in the liver on VLDL secretion. In addition, there may be other aspects of extrahepatic sortilin biology that could influence disease risk: in a mouse model with a humanized

plasma lipid profile, whole body knockout Sort1−/− had no effect on plasma lipids, but nonetheless was associated with decreased atherosclerosis. This effect was found to be attributable to the ability of sortilin to serve as a receptor of LDL: macrophages lacking sortilin have reduced LDL uptake, which led to decreased foam cell formation[123].

The detailed molecular mechanisms by which sortilin influences the complex processes of hepatic and plasma lipid metabolism, VLDL secretion, and MI/CVD risk have not yet been elucidated, but under conditions consistent with western lifestyle it clearly impacts ApoB100 secretion, LDL clearance and foam cell formation. While the roles of sortilin in diverse CVD-relevant cell and tissue types have confounded the analysis of its biological function, this underscores the importance of the gene, and likely explains its robustness as a GWAS signal.

TRIB1 (Tribbles-1)

GWAS have consistently associated variants at the 8q24 locus containing the gene *TRIB1* with multiple human metabolic phenotypes. The *TRIB1* locus was first implicated in plasma lipid metabolism by two papers published simultaneously that showed non-coding variation in the *TRIB1* gene locus was associated with circulating TG levels in humans[2, 124]. The landmark GLGC meta-analysis of more than 100,000 individuals further illustrated the importance of *TRIB1* in lipid metabolism by associating the locus with not only TGs but also total cholesterol (TC), HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and CAD[4], making *TRIB1* the only novel locus from these studies to be associated with all four lipid traits and CAD. The plasma lipid associations were replicated in a GLGC follow-up meta-analysis of about 200,000 individuals[125], while the CAD association was confirmed in two recent GWAS from the CARDIoGRAMplusC4D consortium, in which researchers investigated the association of genome-wide sequence variation with atherosclerosis regardless of plasma lipid phenotype[1, 22]. The significantly associated SNPs in all instances fall around 20 kb upstream of the *TRIB1* gene, suggesting a role in the regulation of *TRIB1* gene expression. One recent study showed that significantly associated SNPs in the *TRIB1* locus alter the expression of a long non-coding RNA named TRIBAL, although the role of TRIBAL in any disease pathology is currently unclear[126].

The *TRIB1* gene encodes a protein known as Tribbles-1, which was originally identified in a drosophila mutagenesis screen which revealed that the protein Trbl (the drosophila homolog of *TRIB1*) participates in oogenesis via promoting the proteasomal degradation of String, Twine and Slbo, the latter of which is the Drosophila homolog of the human transcription factor CCAAT/enhancer binding protein alpha (*C/EBPa*)[127–130]. Subsequent work in the myeloblast 32D cell line showed that human Tribbles-1 can induce the proteasomal degradation of C/EBPa and C/EBPb by promoting their ubiquitination by the E3 ligase COP1 through direct binding to both targets and the ligase[131, 132]. This function is critical for Tribbles-mediated leukemogenesis[133, 134], and also coordinates Tribbles regulation of macrophage polarization and differentiation[135]. Prior to the GWAS described above, *TRIB1* had not been implicated in cardiometabolic disease pathology.

The human genetic findings have spurred a great deal of research into *TRIB1* aimed at elucidating the mechanism through which it may participate in CAD pathogenesis. Studies

using AAV-mediated overexpression of mouse *Trib1* (AAV_mTrib1) to investigate this association found that increasing levels of hepatic *Trib1* decreased plasma TC, HDL-C, LDL-C, and TG levels in a dose-dependent manner[96]. AAV-treated mice showed a decrease in hepatic lipogenic gene expression, and *ex vivo* studies of primary hepatocytes from those mice showed reduced cellular TG production and secretion. Furthermore, LAhB mice treated with AAV_mTrib1 had decreased plasma ApoB levels, and HepG2 cells overexpressing *TRIB1* had decreased ApoB secretion. These data suggest that *TRIB1* can modulate VLDL secretion from the liver, presumably by affecting the level of Triglycerides available for efficient VLDL assembly. More recent work from our group in a liver-specific KO of *Trib1* (Trib1_LSKO) established that C/EBPa is the mechanistic link between *TRIB1* and hepatic lipogenesis[136]. Trib1_LSKO mice have increased hepatic TG content, lipogenic gene expression, and *de novo* lipogenesis. They also display increased hepatic C/EBPa protein, and this increase is both necessary and sufficient to drive the lipogenic phenotype. The Trib1_LSKO mice also have increased plasma lipids, however this appears to be a C/EBPa-independent affect and suggests that *TRIB1* regulates plasma lipid metabolism via other mechanisms independent of lipogenesis. TRIB1 has also recently been shown via *in vitro* overexpression assays to interact with the transcription factor *ChREBP*[137] as well as *SAP18*, a component of the Sin3A-HDAC co-repressor complex[138]. The role of these interactions *in vivo* and the extent to which they all participate in plasma lipid regulation remains to be determined.

The association of the *TRIB1* gene locus with CAD is likely driven in large part by its putative regulation of VLDL secretion. However, it remains possible that other mechanistic links between the gene and CAD contribute to this genetic association. The aforementioned role of TRIB1 in macrophage polarization[135] is one potential link, as the M1/M2 status of macrophages in the lesion can contribute to plaque progression[139].

Human genetics have implicated the *TRIB1* locus in a host of other human phenotypes, including levels of circulating adiponectin[140] and liver enzymes[141] - an association functionally confirmed by the Trib1_LSKO mouse - as well as the onset of metabolic syndrome in humans[142]. Each of these human traits could by themselves contribute to CAD, either directly or indirectly. Thus it is possible that pleiotropic effects of *TRIB1* contribute to CAD, and careful work in animal models of metabolic disease with *TRIB1* tissue-specific deletion will be required to determine the specific contribution to disease burden by each specific function of TRIB1.

LIPA

A number of GWAS studies have identified *LIPA* as a novel locus for CAD[18, 171, 172]. Meta-analyses revealed that *LIPA* CAD risk alleles rs1412444T and rs2246833T (clustered in introns 2 and 3 in high linkage disequilibrium, r^2=0.985) were associated with higher *LIPA* expression in monocytes[143] but not in liver[26], nor did they alter plasma lipids[125]. Fine mapping of the *LIPA* region by the CARDIoGRAM+C4D consortium[22, 144] failed to reveal additional variants with stronger signals than the original GWAS SNPs, and rs2246833 had the strongest CAD association (P=4.9x10^{-12}). Both aforementioned SNPs show strong

H3K27Ac enrichment, and are in and near DNase I hypersensitivity site and TF binding sites (ENCODE),[145, 146] suggesting possible regulatory roles.

LIPA encodes an enzyme called lysosomal acid lipase (LAL) which catalyzes the hydrolysis of cholesteryl ester (CE) and triglycerides in intracellular lysosomes after their internalization via receptor-mediated endocytosis of lipoprotein particles. Human LAL is encoded by the *LIPA* gene on chromosome 10q23.2–23.3[147], and is a 46 kDa glycoprotein. After undergoing co-translational glycosylation in the endoplasmic reticulum and attachment of mannose-6-phosphate residues in the Golgi apparatus, LAL is targeted to pre-lysosomal compartments[148, 149].

Prior to the GWAS discovery for CAD, loss-of-function (LOF) mutations in *LIPA* were identified as causes of rare lysosomal disorders. Wolman disease is an infantile-onset disorder with massive infiltration of CE/TG filled macrophages in multiple organs due to complete *LIPA* LOF. Cholesteryl ester storage disease (CESD) is a later-onset disorder with incomplete *LIPA* LOF mutations resulting in hepatomegaly, hyperlipidemia and premature atherosclerosis[150, 151]. The most common mutation seen in CESD patients is a splice junction mutation at exon 8 of *LIPA*, which leads to about 3 to 5 percent of normally spliced LAL protein and similar low levels of LAL activity[152]. Data of CESD fibroblasts suggest that *LIPA* deficiency leads to lysosomal CE accumulation that limits lysosomal FC release[153, 154] and cytosolic cholesterol esterification[21, 22], and impaired ATP binding cassette transporter A1 (*ABCA1*)-mediated cholesterol efflux[155, 156]. In a phase 3 trial of enzyme-replacement therapy in children and adults with lysosomal acid lipase deficiency, recombinant human LAL Sebelipase Alfa resulted in a reduction in multiple disease-related hepatic and lipid abnormalities[157]; with the long term effects of Sebelipase Alfa on cardiovascular events undetermined.

Although recombinant human LAL (rhLAL) enzyme replacement therapy is likely to exert protective effects against premature atherosclerosis in CESD patients, it is unclear what the effects of rhLAL treatment would be in CAD patients without LAL deficiency. Indeed, our understanding of the role of *LIPA* in the progression of atherosclerosis is far from complete. eQTL studies have suggested that the GWAS risk alleles for CAD are associated with increased *LIPA* mRNA in monocytes.[143] How the higher *LIPA* mRNA in monocytes relates to increased risk of CAD is unclear: whether the GWAS CAD variants associated with higher *LIPA* mRNA is correlated with higher LAL protein levels or enzymatic activity, and if it is a true gain of function (GOF) mutation has yet to be determined. The GWAS CAD alleles are in LD with a missense coding variant (rs1051338) in the signal peptide that may alter post-translational trafficking and secretion, so it is possible that the CAD signal marks a loss of normal LAL processing and function despite higher mRNA. Thus, whether increased or decreased monocyte-macrophage activity of LAL beyond the normal cellular response, in the general population and in CAD patients is atherogenic or protective remains a completely open and controversial question.

A comprehensive understanding of the impact of *LIPA* on CAD pathogenesis relies on *in vivo* modeling. *Lipa* knockout mice ($Lipa^{-/-}$) display shortened life span, tissue CE accumulation and hepatosplenomegaly.[158] Furthermore, $Lipa^{-/-}$ accelerates atherosclerosis

in the *ApoE*$^{-/-}$ hyperlipidemic mouse model.[159] These findings recapitulate the pathological phenotypes of human *LIPA* LOF in CESD.[160] Although systemic rhLAL administration reduces hyperlipidemia and atherosclerosis in *Ldlr*$^{-/-}$ mice,[159] the atheroprotection was most likely attributable to the reduction in plasma lipids.[159] Surprisingly, transgenic (Tg) mice with whole body *Lipa* overexpression apparently have elevated plasma VLDL-C and hepatocellular lipids on western diet.[161] In context of the lack of association between GWAS CAD risk alleles and plasma lipid levels or liver *LIPA* expression,[125] the whole body knockout or Tg mice do not serve as an appropriate model of the CAD-associated locus identified in GWAS studies. Indeed, because the CAD risk alleles are specifically associated with higher monocyte *LIPA* mRNA, it is now imperative to define the monocyte/macrophage-specific role of *LIPA* CAD risk alleles *in vitro* in macrophage function using isogenic hiPSC lines carrying risk or non-risk alleles with subsequently differentiation to macrophages - or *in vivo* in the progression of atherosclerosis using monocyte/macrophage-specific GOF of *LIPA* in mice models of atherosclerosis.

ADAMTS7

The association of the *ADAMTS7* locus with CAD risk has been identified and replicated through GWAS.[1, 162–164] This locus for coronary atherosclerosis was discovered in the PennCath cohort using angiographic CAD as the primary outcome,[162] and subsequent studies have shown that *ADAMTS7* also relates to MI.[1, 163, 164] Its association is most robust for angiographic CAD, a marker of coronary atherosclerotic burden, suggesting that *ADAMTS7* is likely to relate to clinical events through the development and progression of atherosclerosis. Recent findings in mouse vascular injury and atherosclerosis models[165, 166] are consistent with such an action in the humans. Genetic variation at the *ADAMTS7* locus has no relationship with traditional risk factors or mechanistic biomarkers;[163, 164] hence the directional impact of *ADAMTS7* expression on CAD risk and the underlying biological mechanisms have been unclear. Functional studies suggest that *ADAMTS7*, a metalloproteinase expressed in vascular smooth muscle cells (VSMC) and endothelial cells (ECs), is the probable causal proatherogenic gene at this locus.[165–168] Briefly, the top CAD-risk SNPs at this locus are eQTLs for higher *ADAMTS7* expression while allelic variation at a non-synonymous variant (rs3825807, Ser214Pro) in *ADAMTS7* associates with reduced CAD risk and may impair ADAMTS7 function in VSMC (7). Our recent work demonstrated that deletion of *Adamts7* is atheroprotective in both *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ mouse models.[166] Thus, blockade of ADAMTS7 expression or inhibition of its function presents novel therapeutic opportunities for prevention and treatment of CAD.

Understanding the relationship between CAD risk alleles at the *ADAMTS7* chr15q21.1 region and expression levels of *ADAMTS7* in human disease-relevant cells has not been straightforward. Interestingly, in available eQTL datasets with large sample sizes[169] the lead SNPs from the PennCath (rs1994016), CARDIoGRAM (rs3825807), and C4D (rs4380028) GWAS studies demonstrate a significant association with *ADAMTS7* expression and match the directionality and causality of *in vivo* data using mouse model, with the CAD risk alleles being associated with higher *ADAMTS7* expression. However, currently there are no large eQTL or RNA-Seq based ASE data that provide adequate power to determine eQTL directionality in the most pertinent human vascular cells and tissues. Individual laboratories

and the GTEx consortia[170] are now generating datasets from a large enough sample pool of human vasculature to address this question. In the ENCODE Project,[171] the NIH Roadmap Epigenomics Mapping data and in our own ChIP-Seq experiments, the top CAD SNPs fall in *ADAMTS7* 5′ and 3′ regions that overlap regulatory elements in VSMC and aortic tissues and are, for instance, close to binding sites for TCF21, a VSMC transcription factor that regulates coronary development and is itself a GWAS locus for CHD.[163, 172, 173] In unpublished data, several of these regions have been found to have enhancer activity in rat A7r5 VSMC suggesting that this chr15q21.1 CHD SNPs may act on CHD by regulating human coronary arterial smooth muscle cells (HCASMC) *ADAMTS7* expression.

ADAMTS7 (or the A disintegrin and metalloproteinase with thrombospondin motifs-7) is a member of the ADAMTS family of secreted zinc metalloproteases with characteristic protein domain composition including at least one thrombospondin type I repeat (TSPI).[174–177] The family of ADAMTS proteases degrades extra-cellular matrix and several ADAMTS family members have been implicated in human diseases including thrombotic thrombocytopenic purpura (TTP),[178] Weill-Marchesani syndrome[179] and atherosclerosis.[180] Unlike other metalloproteinases, ADAMTS family members demonstrate narrow substrate specificity due to their C-terminal exosites (13–16).[174–177] Previous research on *ADAMTS7* has mainly centered on its role in bone and cartilage growth because cartilage oligomeric matrix protein (COMP) has been identified as a substrate.[181] *ADAMTS7* can regulate endochondral bone formation through interactions with COMP. COMP is also expressed in VSMC and vasculature and additional studies with viral-mediated overexpression and knockdown *in vivo* and *in vitro* suggests that ADAMTS7 might modulate VSMC phenotype switching and migration via interactions with COMP.[180]

Most domains in human and mouse *Adamts7* are highly conserved rendering the mouse as a useful model for actions in human disease. The first evidence that *Adamts7* deficiency (*Adamts7*$^{-/-}$) attenuates atherosclerosis *in vivo*, in both the *ApoE*$^{-/-}$ and *Ldlr*$^{-/-}$ mouse models, and *Adamts7*$^{-/-}$ confers a specific loss of VSMC migration in response to inflammatory signals was recently published.[166] It has been shown that *Adamts7*$^{-/-}$ also reduces vascular response to mechanical injury.[165, 166] *Adamts7* gene expression was induced transiently in the mouse vasculature in response to stress, both in the wire injury model and in the atherosclerosis experiments, that TNFa induces *Adamts7* expression in primary VSMCs, and that VSMC of *Adamts7*$^{-/-}$ mice show reduced TNFa-induced migration.[166] Immunostaining in human diseased coronary arteries reveals colocalization of ADAMTS7 with cells positive for VSMC markers, and immunofluorescence in human aortic smooth muscle cells shows subcellular localization with leading edges of migrating VSMCs. These data suggests that Adamts7 modulates VSMC phenotype and migration during inflammatory stress and mechanical injury and that *Adamts7* deficiency markedly reduces atherosclerotic lesions in hyperlipidemic mice.

Human eQTL interrogations reveal that common alleles that relate to lower CAD risk are also associated with reduced *ADAMTS7* expression. This is consistent with rodent studies and supports a pro-atherogenic role of *ADAMTS7* in humans. Because ADAMTS7 has narrow substrate specificity, it has promise as a potentially safe drug target. Thus, inhibition of ADAMTS7 is a potential novel therapeutic strategy for CAD in humans. Howevver,

several important questions still need to be addressed in order to accelerate clinical and therapeutic translation related to this locus.

TCF21 (Pod1, capsulin, epicardin)

The *TCF21* gene codes for a basic helix-loop-helix transcription factor known to bind *cis*-regulatory elements as heterodimers with TCF3 or TCF12. It is expressed in the mesenchyme of developing organs including the lung, kidney, gut and heart, and constitutive *Tcf21* knockout mice die at birth due to missing alveoli in their lungs[182]. In addition, Tcf21 has been shown to play a role in vascular development: Tcf21 is highly expressed in the proepicardial organ which contains progenitor cells of coronary artery smooth muscle and endothelial cells and cardiac fibroblasts. It is necessary for epithelial-to-mesenchymal transition of epicardial fibroblasts and their subsequent migration into the cardiac interstitium[183, 184].

The *TCF21* gene locus has been linked to CAD risk by GWAS which first reported rs12190287 as the association lead SNP[163]. This polymorphism lies in the 3′UTR of one of the two *TCF21* transcript variants. It has been shown to disrupt an AP-1 binding site inside an enhancer in vascular smooth muscle cells[172]. Interestingly, this variant also alters a mir-224 binding site inside a *TCF21* transcript variant, suggesting additional miRNA-dependent regulation of *TCF21* on the post-transcriptional level[185]. More recently, the 1000 Genomes based CARDIoGRAMplusC4D GWAS meta-analysis reported rs12202017, which lies 3.7kB upstream of the *TCF21* gene within the TARID lncRNA locus, as the lowest p-value association SNP[1]. This locus also harbors a separately reported CAD association signal in Han Chinese, with rs12524865 as the strongest association SNP in this study[186]. The potential role of TARID in the vasculature has so far not been interrogated and presents an attractive target for future study. The contribution of TCF21 to disease appears to be coronary artery specific: a meta-analysis for shared susceptibility reported that the TCF21 association signal is confined to CAD and is not implicated in risk for ischemic stroke[10].

The role of the Tcf21 protein in CAD has recently been investigated *in vivo* using a lacZ reporter and a lineage tracing model in mouse. These studies show that Tcf21 expressing cells migrate into the forming atherosclerotic lesion and contribute to the fibrous cap[187]. Further studies are needed to investigate how lack of TCF21 contributes to lesion size and composition. To identify TCF21 transcriptional target genes, ChIP-Seq studies have been carried out in HCASMCs[173]. Enrichment analysis showed that TCF21 binding sites are enriched for CAD GWAS association SNPs. This suggests a role of TCF21 as regulator of genomic loci such as ADAMTS7 conferring risk for atherosclerosis in coronary artery SMCs.

Summary

GWAS have provided a rich collection of CAD loci that suggest the existence of exciting new biology relevant to atherosclerosis that we never suspected but that require extensive functional follow-up studies. However, causality of a specific gene cannot be inferred solely based on proximity to a region of statistical association with disease. A thorough and meticulous annotation of the region with data generated in relevant cell types is paramount.

Specific targeting of the SNP or region of association needs to be combined with the interrogation of changes in transcription of *all* genes in the haplotype block of interest. Far too often are candidate genes chosen for functional studies without solid evidence for their definitive association with the GWAS signal, and poorly characterized or long-distance genes are rarely followed up upon. Combinatorial effects of more than one gene within a locus, possibly across multiple tissues, could be one mechanism by which common, non protein-coding variation contributes to CAD. We may eventually be surprised by the complexity with which the implicated genomic regions modulate the CAD phenotype. In any case, it remains early days for the functional genomics of CAD GWAS loci. The likelihood is very high that fundamentally new biology regarding atherosclerosis and CAD will be learned through the interrogation of CAD GWAS loci. Furthermore, the hope remains that at least some of these new pathways relevant to CAD pathogenesis will yield new therapeutic targets for the prevention and treatment of CAD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

3C	Chromosome Conformation Capture
AAV	adeno-associated virus
ASE	allele-specific expression
ASO	antisense oligonucleotide
ATAC-Seq	Assay for Transposase-Accessible Chromatin using Sequencing
Cas9	CRISPR-associated protein 9
ChIP-Seq	Chromatin immunoprecipitation with massively parallel sequencing
CRISPR	type II clustered regularly interspaced short palindromic repeats
CRISPRa	activation of gene transcription using the CRISPR/Cas9 technology
CRISPRi	inhibition of gene transcription using the CRISPR/Cas9 technology
eGFP	enhanced Green Fluorescent Protein
eRNA	enhancer RNA
eQTL	expression quantitative trait loci
FAIRE	Formaldehyde-Assisted Isolation of Regulatory Elements
GOF	gain of function
gRNA	guide RNA

GWAS	genome-wide association studies
H3K4me1	Histone 3 Lysine 4 monomethylation
H3K4me3	Histone 3 Lysine 4 trimethylation
H3K27ac	Histone 3 Lysine 27 acetylation
H3K36me3	Histone 3 Lysine 36 trimethylation
HDR	homology-directed repair
hiPSC	human induced pluripotent stem cell
hnRNP	heterogeneous nuclear ribonucleoprotein
iCRISPR	inducible genome editing using the CRISPR/Cas9 technology
LD	linkage disequilibrium
lincRNA	intergenic lncRNA
lncRNA	long non-coding RNA
LOF	loss-of function
LSKO	liver-specific gene knockout
NHEJ	non-homologous end-joining
shRNA	short hairpin RNA
SNP	single nucleotide polymorphism

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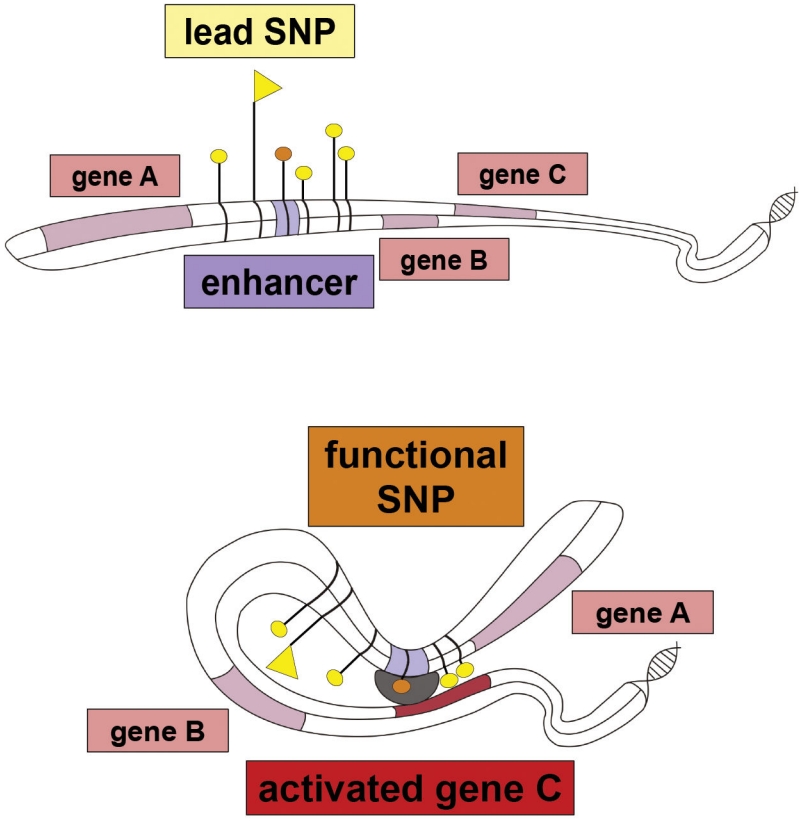

Figure 1. Mechanism by which non-coding risk SNP can affect phenotype

Top: multiple SNPs associated with disease are located in the intergenic region proximal to genes A, B and C. One of the SNPs with genome-wide significance is situated within a *cis*-regulatory element (orange tag). The lowest P-value SNP ('lead SNP', flag tag) lies outside the regulatory element.

Bottom: Through bending of the DNA molecule the regulatory element gets into physical contact with the promoter of its target gene, in this case gene C which is not the gene in closest proximity, leading to regulation of its expression ('activation' or upregulation in case of an enhancer element). The SNP located within the regulatory element ('functional SNP', orange tag) can now affect transcription by for instance altering transcription factor (TF) binding affinity based on genotype via disruption of a TF binding motif

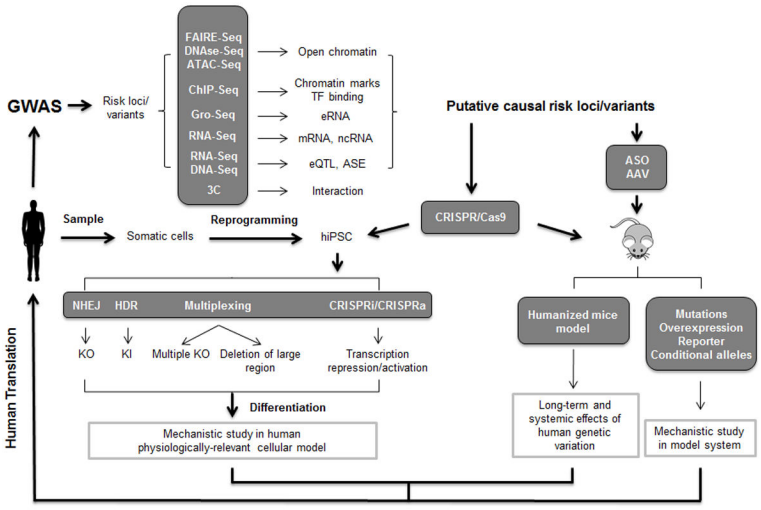

Figure 2. Experimental tools for GWAS functional follow-up studies

GWAS findings can be functionally annotated using genome-wide methods, which help prioritize loci with likely biological function. These putative risk loci can be further interrogate by genome editing using the CRISPR/Cas9 system *in vitro* and *in vivo*. Additionally, adeno-associated virus (AAV) and antisense oligos (ASO) can be employed to study candidate gene knockdown and overexpression in the mouse model. TF: transcription factor; eRNA: enhancer RNA; ncRNA: non-coding RNA; eQTL: expression quantitative trait loci; ASE: allele-specific expression; iPSC: induced pluripotent stem cells; NHEJ: non-homologous end joining; HDR: homology-directed repair; KO: knockout; KI: knockin.

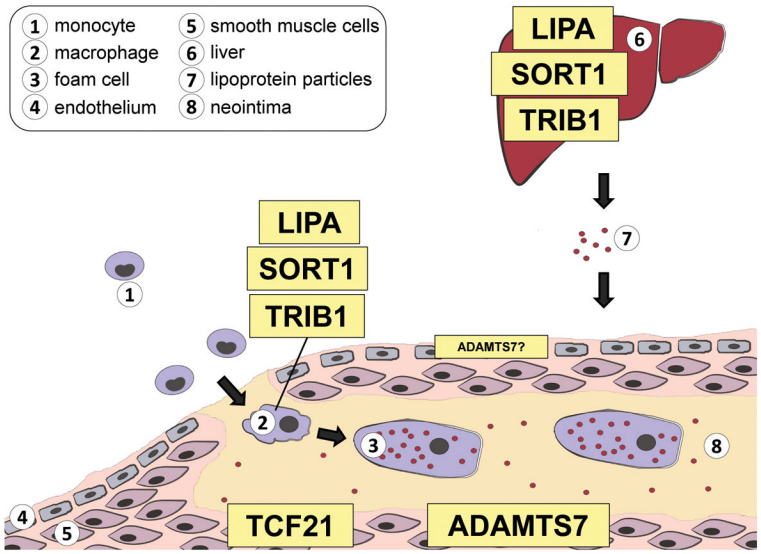

Figure 3. CHD GWAS risk genes are active in selective cell types involved in atherosclerosis

Coronary Heart Disease follow-up studies have demonstrated roles for LIPA, SORT1 and TRIB1 as plasma lipid regulators in the liver, as well as in macrophages biology. Within the vessel wall, TCF21 is upregulated in de-differentiated smooth muscle cells which migrate to the forming fibrous cap. Adamts7 is also a regulator of smooth muscle migration but also a role in endothelial cells has been suggested

Table 1

Overview of 58 previously reported loci with genome-wide significance for Coronary Artery Disease[1]

reported gene	first reported lead SNP	chr	location*	1000G p-value	new lead SNP	updated location*	updated 1000G p-value	reported phenotypes**
PCSK9	rs11206510	1	intergenic	2.34E-08				MI,[188] LDL-C[2]
PPAP2B	rs17114036	1	PPAP2B	2.22E-13	rs9970807	PPAP2B	5.00E-14	MI[163]
SORT1	rs646776	1	CELSR2	9.01E-19	rs7528419	CELSR2, PSRC	1.97E-23	MI,[188] LDL-C,[124] Phospholipase A2[189]
IL6R	rs4845625	1	IL6R	3.93E-08	rs6689306	IL6R	2.60E-09	MI[22]
MIA3	rs17464857	1	TAF1A	4.18E-05	rs67180937	MIA3	1.01E-12	MI[22,190]
	rs17465637	1	MIA3	3.52E-12				
AK097927	rs16986953	2	intergenic	1.45E-08				MI[22]
APOB	rs515135	2	intergenic	3.09E-08	chr2:21378433:D	intergenic	2.89E-08	MI,[22] LDL-C,[124]
ABCG5-ABCG8	rs6544713	2	ABCG8	8.88E-07	chr2:44074126:D	ABCG8	2.60E-08	MI,[22] LDL-C[124]
VAMP5-VAMP8-GGCX	rs1561198	2	VAMP5, VAMP8	6.37E-10	rs7568458	GGCX, VAMP8	3.62E-10	MI[22]
ZEB2-ACO74093.1	rs2252641	2	TEX41	5.16E-04	rs17678683	LINC01412, ZEB2	3.00E-09	MI[22]
WDR12	rs6725887	2	WDR12	9.51E-18	chr2:203828796:I	ALS2CR8	2.15E-18	MI[188]
MRAS	rs9818870	3	MRAS	2.21E-06	chr3:138099161:I	MRAS	2.89E-09	MI[191]
REST-NOA1	rs17087335	4	NOA1	4.6E-08				MI[1]
EDNRA	rs1878406	4	intergenic	1.24E-06	rs4593108	intergenic	8.82E-10	plaque,[192] stroke[10]
GUCY1A3	rs7692387	4	GUCY1A3	7.35E-09	rs72689147	GUCY1A3	6.07E-09	MI,[22]
SLC22A4-SLC22A5	rs273909	5	SLC22A4, LOC553103	1.24E-04				MI[22]
*ADTRP-C6orf105****	rs6903956	6	ADTRP	0.96				MI[193]
PHACTR1	rs12526453	6	PHACTR1	2.14E-20	rs9349379	PHACTR1	1.81E-42	MI,[188] calcification,[112] artery dissection,[194] migraine[195]
ANKS1A	rs17609940	6	ANKS1A	0.03				MI[163]
KCNK5	rs10947789	6	KCNK5	1.63E-06	rs56336142	intergenic	1.85E-08	MI[22]
TCF21	rs12190287	6	TCF21,TARID	1.07E-03	rs12202017	TARID, LINC01312	1.98E-11	MI[163]
SLC22A3-LPAL2-LPA	rs2048327	6	SLC22A3	2.46E-09	rs55730499	LPA	5.39E-39	MI[196,163]
	rs3798220	6	LPA	4.66E-09				

reported gene	first reported lead SNP	chr	location*	1000G p-value	new lead SNP	updated location*	updated 1000G p-value	reported phenotypes**
PLG	rs4252120	6	PLG	3.32E-03	rs4252185	PLG	1.64E-32	MI[22]
HDAC9	rs2023938	7	HDAC9	1.36E-04	rs2107595	intergenic	8.05E-11	stroke,[197] MI[22]
7q22	rs10953541	7	BCAP29	1.02E-05				MI[164]
ZC3HC1	rs11556924	7	ZC3HC1	5.34E-11				MI[163]
NOS3	rs3918226	7	NOS3	1.7E-09				MI[1]
LPL	rs264	7	LPL	1.06E-05				MI[22]
TRIB1	rs2954029	8	RP11-136O12.2	2.61E-06				Triglycerides,[124] LDL-C,[4] HDL-C,[4] total cholesterol,[4] MI[22]
9p21	rs3217992	8	CDKN2B, CDKN2B-AS1	1.03E-42	rs2891168	CDKN2B-AS1	2.29E-98	MI[22,195,188]
	rs4977574	9	CDKN2B-AS1	6.35E-98				
ABO	rs579459	9	ABO	1.14E-10	rs2519093	ABO	1.19E-11	MI,[163] venous thromboembolism[198]
KIAA1462	rs2505083	9	KIAA1462	1.57E-10	rs2487928	KIAA1462	4.41E-11	MI[164]
CXCL12	rs2047009	9	intergenic	2.75E-11	rs1870634	intergenic	5.55E-15	MI[22,190]
	rs501120	10	RP11-20J15.2	1.39E-11				
LIPA	rs11203042	10	LIPA	1.22E-04	rs1412444	LIPA	5.15E-12	MI,[22,164]
	rs1412444	10	LIPA	5.15E-12				
CYP17A1-CNNM2-NT5C2	rs12413409	10	CNNM2	1.07E-07	rs11191416	PFN1P11	4.65E-09	MI,[163] intracranial aneurysm[199]
SWAP70	rs10840293	10	SWAP70	1.3E-08				MI[1]
PDGFD	rs974819	10	RP11-563P16.1	2.44E-10	rs2128739	RP11-563P16.1	7.05E-11	MI[164]
ZNF259-APOA5-APOA1 (ZPR1)	rs964184	11	ZNF259	5.60E-05				MI,[163] Triglycerides,[124] HDL-C,[124] LDL-C[4]
ATP2B1	rs7136259	11	ATP2B1	2.45E-05	rs2681472	ATP2B1	6.17E-11	MI, blood pressure[200]
SH2B3	rs3184504	11	SH2B3	1.03E-09				MI,[163] blood pressure[200]
KSR2	rs11830157	12	KSR2	2.12E-09				MI[1]
FLT1	rs9319428	12	FLT1	7.13E-05				MI[22]
COL4A1/A2	rs4773144	12	COL4A1, COL4A2	3.87E-07	rs11838776	COL4A2	1.83E-10	MI[163,22]
	rs9515203	13	COL4A2	9.33E-10				
HHIPL1	rs2895811	13	HHIPL1	1.86E-05	rs10139550	HHIPL1	1.38E-08	MI[163]
SMAD3	rs56062135	13	SMAD3	4.5E-09				MI[1]

reported gene	first reported lead SNP	chr	location*	1000G p-value	new lead SNP	updated location*	updated 1000G p-value	reported phenotypes**
ADAMTS7-MORF4L1	rs7173743	14	MORF4L1	5.55E-16	rs4468572	MORF4L1	4.44E-16	MI[22]
MFGE8-ABHD2	rs8042271	15	intergenic	3.7E-08				MI[1]
FURIN-FES	rs17514846	15	FURIN	3.10E-07				MI[22]
SMG6-SRR	rs216172	15	SMG6	5.07E-07				MI[163]
RASD1, SMCR3, PEMT	rs12936587	15	intergenic	8.24E-04				MI[163]
UBE2Z, GIP, ATP5G1, SNF8	rs46522	15	UBE2Z	1.84E-05				MI[163]
BCAS3	rs7212798	15	BCAS3	1.9E-08				MI[1]
PMAIP1-MC4R	rs663129	17	intergenic	3.2E-08				MI[1]
LDLR	rs1122608	17	SMARCA4	2.73E-11	rs56289821	intergenic	4.44E-15	MI[188]
ZNF507-LOC400684	rs12976411	17	LOC400684, ZNF507	1.18E-14				MI[1]
APOE-APOC1	rs2075650	17	TOMM40, PVRL2	1.61E-06	rs4420638	APOC1	7.07E-11	Total Cholesterol,[201] LDL-C,[202,203] Phospholipase A2,[204] plaque,[192] Triglycerides[205]
	rs445925	18	APOC1, APOE	4.23E-06				
SLC5A3-MRPS6-KCNE2	rs9982601	19	intergenic	1.33E-13	rs28451064	AP000320.7	1.33E-15	MI[188]
POM121L9P-ADORA2A	rs180803	19	POM121L9P	1.6E-10				MI[1]

*gene body +-5kb (hg19, UCSC genes, Ensembl genes, Refseq genes)

**CAD lead SNPs with genome-wide significance in published GWAS studies for CAD relevant phenotypes

***only significant in East Asians

novel loci