



From genotype to phenotype in human atherosclerosis - recent findings

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Purpose of review

Since 2007, genome-wide association studies (GWAS) have led to the identification of numerous loci of atherosclerotic cardiovascular disease. The majority of these loci harbor genes previously not known to be involved in atherogenesis. In this review, we summarize the recent progress in understanding the pathophysiology of genetic variants in atherosclerosis.

Recent findings

Fifty-eight loci with $P < 10^{-7}$ have been identified in GWAS for coronary heart disease and myocardial infarction. Of these, 23 loci (40%) overlap with GWAS loci of classical risk factors such as lipids, blood pressure, and diabetes mellitus, suggesting a potential causal relation. The vast majority of the remaining 35 loci (60%) are at genomic regions where the mechanism in atherogenesis is unclear. Loci most frequently found in independent GWAS were at Chr9p21.3 (*ANRIL/CDKN2B-AS1*), Chr6p24.1 (*PHACTR1*), and Chr1p13.3 (*CELSR2, PSRC1, MYBPHL, SORT1*). Recent work suggests that Chr9p21.3 exerts its effects through epigenetic regulation of target genes, whereas mechanisms at Chr6p24.1 remain obscure, and Chr1p13.3 affects plasma LDL cholesterol.

Summary

Novel GWAS loci indicate that our understanding of atherosclerosis is limited and implicate a role of hitherto unknown mechanisms, such as epigenetic gene regulation in atherogenesis.

Keywords

1p13.3, 6p24.1, 9p21.3, atherosclerosis, GWAS

INTRODUCTION

Findings from genome-wide association studies (GWAS) are a treasure trove for our understanding of the pathophysiology of atherosclerosis. The first GWAS in 2007 identified a locus on chromosome 9p21.3 (Chr9p21.3), which is the strongest genetic factor of atherosclerosis known today [1–4]. Since then, additional loci have been constantly added, resulting in over 50 loci. The majority is completely novel and the current challenge in the ‘post GWAS era’ is to identify the responsible genes and integrate them into our understanding of the pathophysiology of this frequent disease.

Here, we focus on the most robust loci identified by GWAS and review some of the approaches recently used to tease out their complex pathophysiology. These approaches include expression quantitative trait loci (eQTL) and functional studies in tissues from patients with defined genotypes, which are essential to single-out the culprit gene at loci usually containing multiple transcripts. Moreover, overlap with GWAS hits of cardiovascular risk

factors and seemingly unrelated phenotypes gives hints to potentially causal relations. Finally, cell culture studies and mouse models using knockout and overexpression strategies are essential, in particular at loci involving completely novel pathophysiology. Understanding the mechanisms of these loci in atherogenesis is a prerequisite for later therapeutic targeting.

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KEY POINTS

- GWAS have identified 58 loci of CHD and MI.
- Forty percent overlap with genomic loci for classical risk factors, whereas mechanisms at the remaining 60% are unclear.
- The three most frequently found loci are at Chr9p21.3, Chr6p24.1, and Chr1p13.3.
- Recent work suggests a role of epigenetic gene regulation by a noncoding RNA as a novel mechanism of atherogenesis at Chr9p21.3.
- Mechanisms at Chr6p24.1 are unclear, and Chr1p13.3 likely works through affecting plasma LDL-cholesterol.

Atherosclerosis is a disease affecting arterial blood vessels, leading to different disease phenotypes depending on the anatomical location and stage of the disease process. Most GWAS have been performed for the phenotype of coronary heart disease (CHD), which includes a broad spectrum of patients with stable and unstable coronary disease, myocardial infarction (MI) survivors and patients undergoing coronary angiography (Table 1) [3–8,9[■],10[■],11–17,18[■],19–21]. A smaller number of GWAS has specifically dealt with the phenotype MI, which overlaps with CHD because CHD almost always precedes MI. However, MI clearly involves additional mechanisms, such as thrombosis. In this review, we are not covering stroke, which requires differentiation into several subtypes of ischemic and hemorrhagic stroke with different underlying pathophysiology [22]. We are also not explicitly covering peripheral atherosclerosis and its surrogate marker ankle brachial index, where until now GWAS have only revealed the Chr9p21.3 locus with genome-wide significance in a study of more than 40 000 individuals [23].

GWAS LOCI OF CORONARY HEART DISEASE AND MYOCARDIAL INFARCTION

Searching the GWAS catalogue (www.genome.gov/gwastudies; accessed May 2013; [21]) with a stringent cutoff ($P < 10^{-7}$), we have assembled 58 loci from 18 publications for the phenotypes of CHD and MI (Table 1) reporting the best P values (including combined analyses with replication) [1,3–8,9[■],10[■],11–17,18[■],19,20]. A predominant number of these variants has been identified by the CARDIoGRAM consortium [10[■]]. A total of 6220 single-nucleotide polymorphisms (SNPs) with $P < 0.01$ from this analysis were followed-up in the

CARDIoGRAMplusC4D consortium in 63 746 coronary artery disease cases and 130 681 controls, adding 15 additional loci to the list [18[■]]. Whereas earlier GWAS were mainly performed in cohorts of European decent, a number of novel loci were recently identified in Asian and Middle Eastern populations [13,15–17,20] (Table 1).

The Chr9p21.3 (*CDKN2B-AS1*) locus, which is the strongest genetic marker of human atherosclerosis and which is generally considered the ‘gold standard’ for any association study of atherosclerosis-related traits, is listed in 12 independent GWAS publications (Table 1) [1,3,4,9[■],10[■],11–13,16,17,18[■],19]. Chr9p21.3 stands out because of its relatively large effect size [odds ratio (OR) 1.3 per allele], and its allele frequency of ~50%. The second most frequently identified GWAS locus is on Chr6p24.1 (*PHACTR1*), which has been described in six publications (Table 1, OR 1.10) [9[■],10[■],15,17,18[■],19]. The third most often found locus is on Chr1p13.3 (Table 1, OR 1.11) [3,9[■],10[■],18[■],19]. Despite harboring at least four transcripts, *SORT1* is currently considered the prime candidate gene at Chr1p13.3 and was investigated in several functional studies [24–26,27[■]]. The current advances in understanding the pathophysiology at each of these three major loci identified so far will be discussed later in this review.

UNDERSTANDING FUNCTION BY CO-SEGREGATION ANALYSIS WITH OTHER TRAITS

A promising strategy for inferring function from a locus is to search for overlap with GWAS loci for other traits. We systematically screened the 58 loci in Table 1 for overlap with GWAS hits for classical risk factors of CHD or MI (lipids, blood pressure/hypertension, diabetes-related phenotypes) and added information from the CARDIoGRAMplusC4D consortium [18[■]], which also tested for overlap with genetic variants for established risk factors (Table 1). As summarized in Fig. 1, we found that GWAS loci for CHD and MI overlap with 14 loci for lipids (24% of all risk loci), six loci for blood pressure/hypertension (10%), one locus for diabetes mellitus (2%), and two loci with at least two risk factors (4%). Thirty-five (60%) loci did not co-segregate with loci of classical risk factors but out of these, six overlapped with loci from seemingly unrelated GWAS (Table 1; Supplementary material).

Another approach to get insights into function is to investigate the effects of the genotype on mRNA expression of genes at GWAS loci and to map eQTLs. This might be particularly helpful to identify the culprit gene(s) at loci harboring many

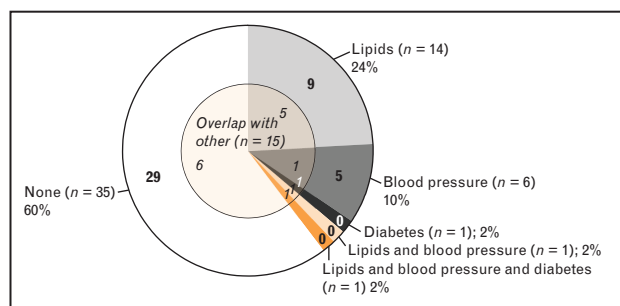


FIGURE 1. Overlap between atherosclerosis loci and loci for common risk factors. Out of 58 loci for coronary heart disease (CHD) and myocardial infarction (MI), 24% overlapped with lipid loci (LDL cholesterol, HDL cholesterol, total cholesterol, triglycerides), 10% with blood pressure, 2% with diabetes-related traits, 2% with lipids and diabetes-related traits, and 2% with all three risk factors. Sixty percent ($n=35$) of CHD and MI loci did not overlap with loci for common risk factors suggesting novel pathophysiology. The inner circle shows additional overlap with genome-wide association studies hits for other nonrisk factor-associated traits (26% of all loci).

genes. Testing *cis*-regulation of a genetic variant at a genome-wide level requires large cohorts where transcriptome-wide mRNA expression has been assayed in each individual and where genome-wide SNP data are also available. Folkersen *et al.* [28] have systematically tested lead SNPs from GWAS of CHD and MI and found evidence for *cis*-regulation at five loci in different vascular tissues and liver samples (Chr1p13.3: *SORT1*, *PSRC1*, *CELSR2*; Chr2q33.2: *NBEAL1*; Chr3q22.3: *MRAS*; Chr6q25.1: *MTHFD1L*; Chr21q22.11: *SLC5A3*). Wild *et al.* [11] performed a comparable analysis using mRNA expression data from monocytes of 1494 individuals from a population-based study [29] and found three eQTLs (Chr1p13.3: *PSRC1*; Chr2q33.2: *WDR12*; Chr10q23.31: *LIPA*). Results at Chr2q33.2 are particularly interesting since expression analysis in different tissues apparently led to different findings. A similar approach was taken by the C4D consortium, which systematically tested for eQTLs at newly identified loci [9^a]. A current limitation of this very promising approach is the limited availability of large cohorts with tissue collections for transcriptome-wide expression analysis.

Chr9P21.3 (ANRIL): ROLE OF A LONG NONCODING RNA (ncRNA) IN ATHEROGENESIS

Chr9p21.3 is the most replicated locus of human atherosclerosis (reviewed in [30,31]). The locus lacks associations with common cardiovascular risk

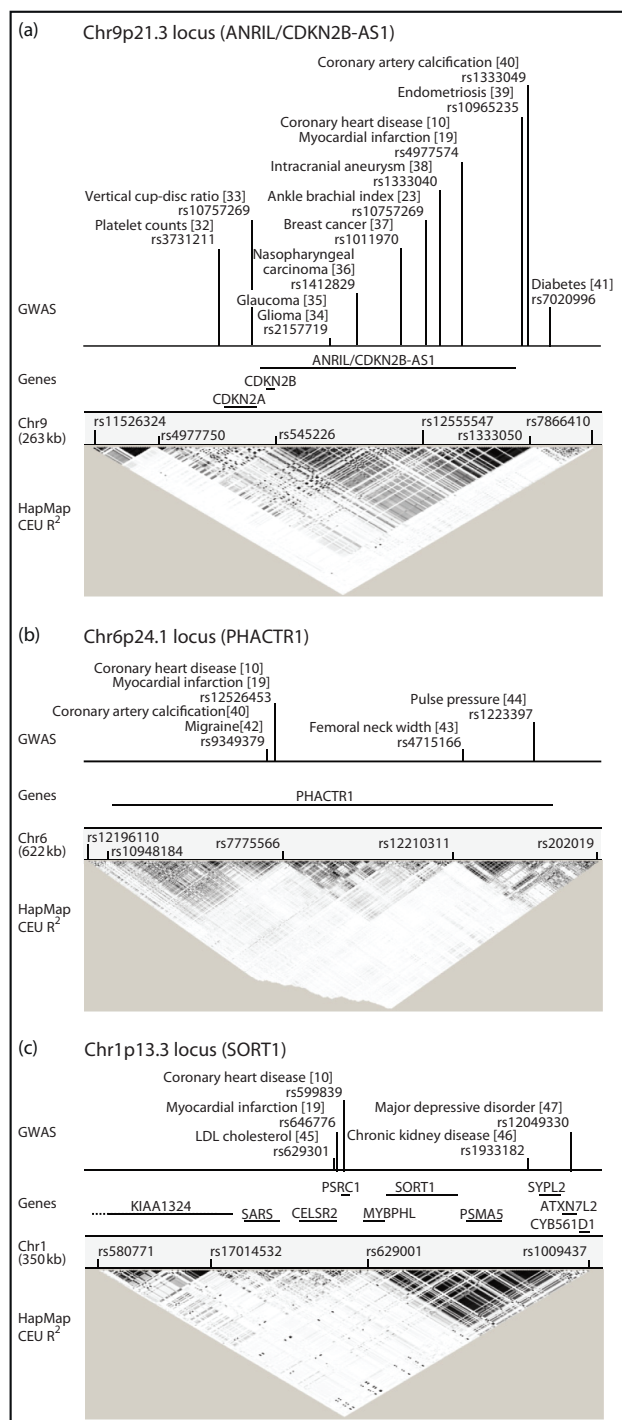


FIGURE 2. Haplotype analysis (HapMap CEU) and annotated genes at the three most frequently identified loci for coronary heart disease (CHD) and myocardial infarction (MI). Single-nucleotide polymorphisms with strongest signals of the respective phenotype and corresponding references are given. (a) Chr9p21.3 CHD and MI locus and adjacent hits for cancer, diabetes, and other traits. (b) Chr6p24.1 CHD and MI locus overlapping with migraine. Significance of pulse pressure and femoral neck width loci is unclear. (c) Chr1p13.3 CHD and MI locus co-segregating with genome-wide association studies (GWAS) hits for lipids.

factors suggesting that it exerts its effect through an alternative mechanism. The core risk haplotype spans approximately 50 kb [10¹¹,19,22,32–47] (Fig. 2a) and does not contain protein-coding genes but the 3' end of the long ncRNA *antisense noncoding RNA in the INK4 locus* (*ANRIL*). The synonyms *CDKN2B antisense RNA 1* (*CDKN2B-AS1*) and *CDKN2BAS* are used for *ANRIL* and refer to its antisense orientation to *cyclin-dependent kinase inhibitor 2B* (*CDKN2B*), which is located proximal to the core CHD region. Together with *CDKN2A*, which is located further proximal of *ANRIL*, this region depicts a GWAS hotspot for different tumor entities [30,34–37] and other phenotypes [32,33], which is in line with loss of function of these genes in many human cancers (Fig. 2a) [48]. In an adjacent haplotype block, an independent locus for diabetes was identified [41]. Despite their expression in human plaques [49], several lines of evidence argue against a role of *CDKN2A* and *CDKN2B* as major Chr9p21.3 effector genes. First, SNPs within these genes are not in linkage disequilibrium with the lead CHD SNPs (Fig. 2a). Second, *cis*-regulation of these genes is lacking in the majority of human studies (reviewed in [30]). Third, mouse models speak against a causal role of *CDKN2B* in atherosclerosis [50¹,51] and yielded conflicting results for *CDKN2A* [50¹,52–54].

In contrast, there is growing evidence for a role of *ANRIL* in modulating atherosclerosis susceptibility at Chr9p21.3. *ANRIL* expression is tightly regulated by the Chr9p21.3 genotype [55–58,59¹, 60–62] (for review see [30]). In addition, a positive correlation of *ANRIL* expression with atherosclerosis severity has been described [58]. Transcription of *ANRIL* is complex and more than 20 linear and several circular isoforms are known today [55,57,59¹]. As a mechanism for differential expression, Harismendy *et al.* [63] proposed that *ANRIL* expression in Chr9p21.3 risk allele carriers was induced by disruption of an inhibitory STAT1-binding site. Functional studies in mammalian cells revealed that *ANRIL* knock-down led to decreased proliferation [64–67]. Recent work has extended these findings, showing that *ANRIL* overexpression not only led to accelerated proliferation but also increased adhesion and decreased apoptosis [59¹]. These are key mechanisms of atherosclerosis and the direction of effects would be in line with the pro-atherogenic role of *ANRIL* suggested from expression studies (Fig. 3) [59¹].

But how does *ANRIL* exert these effects at the molecular level? *ANRIL* belongs to the group of large noncoding RNAs which have been shown to regulate gene expression through RNA–RNA, RNA–DNA, or RNA–protein interactions [68–70]. For

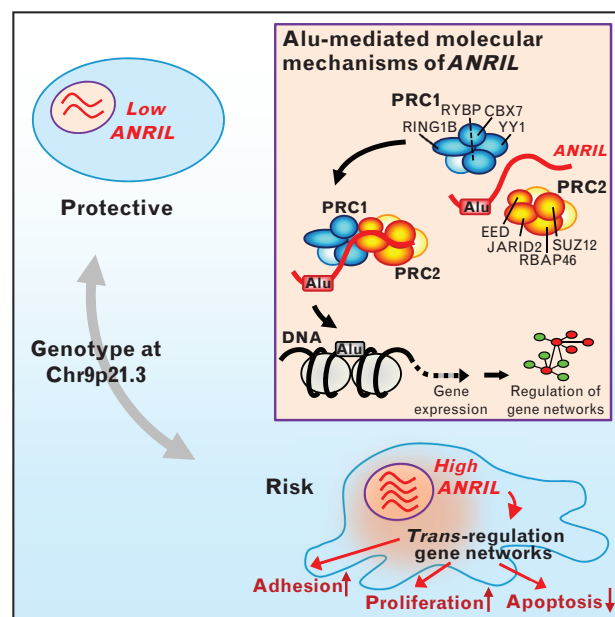


FIGURE 3. Model of *ANRIL/CDKN2B-AS1* function at Chr9p21 according to [59¹]. The atherosclerosis risk allele leads to up-regulation of the long ncRNA *ANRIL*. Increased *ANRIL* expression modulates networks of genes *in-trans*, leading to pro-atherogenic cell properties (increased cell adhesion, increased proliferation, decreased apoptosis). On the molecular level, *ANRIL* may act as a scaffold, guiding epigenetic modifier proteins of Polycomb repressive complexes 1 and 2 (PRC1, PRC2) and potentially others to chromatin. These functions depend on Alu motifs, which mark the promoters of *ANRIL* target genes and are mirrored in *ANRIL* RNA, suggesting an Alu-mediated RNA-DNA interaction as effector mechanism.

ANRIL, binding to epigenetic silencer Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) [59¹,66,67] and to PRC-associated activating proteins RYBP and YY1 [71,72] has been demonstrated (Fig. 3) [59¹]. In accordance, modulation of *ANRIL* expression led to the epigenetic regulation of target genes expression *in cis* [66,67] and *in trans* [59¹,64,73]. We have recently shown that *trans*-regulation was dependent on an Alu-DEIN motif [74,75], which marked the promoters of *ANRIL* target genes and was mirrored in *ANRIL* RNA transcripts (Fig. 3). The functional relevance of Alu motifs in *ANRIL* was confirmed by deletion and mutagenesis, reversing *trans*-regulation and restoring normal cellular functions [59¹]. Recent work by Jeck *et al.* has also demonstrated that Alu motifs are preferably incorporated in noncoding RNA lariats, which might represent inactive isoforms and were also shown to exist for *ANRIL* [55,76]. Whether integration of Alu motifs in ncRNA lariats leads to silencing of the effector sequences remains to be determined.

In summary, the robust association of *ANRIL* with the risk genotype, its correlation with atherosclerosis severity, and functional data strongly support *ANRIL* as Chr9p21.3 effector gene. Recent work has not only broadened our understanding of *ANRIL*'s function but also suggested a novel molecular mechanism for long ncRNA-mediated *trans*-regulation.

Chr6p24.1 (*PHACTR1*): FREQUENTLY REPLICATED BUT POORLY UNDERSTOOD

Chr6p24.1 is the second most often identified GWAS hit for CHD and MI. The locus was found in European, Asian, and Middle Eastern populations and therefore appears to be relevant across ethnicities [9[•],10^{••},15,16,18^{••},19]. Chr6p24.1 is also associated with coronary calcification [40]. Until now, virtually nothing is known about the mechanism of Chr6p24.1 in atherogenesis. The region contains a single gene, *protein phosphatase and actin regulator 1* (*PHACTR1*), spanning a very large genomic distance of ~500 kb, and extending over three haplotype blocks (Fig. 2b). Lead SNPs for CHD and MI are in the proximal haplotype block and the same SNPs were independently identified in a GWAS for migraine [42]. Intriguingly, alleles conferring migraine susceptibility were also associated with risk for CHD suggesting a common pathophysiology. The distal haplotype block of *PHACTR1* also contains hits in the GWAS catalogue (www.genome.gov/gwastudies; accessed May 2013; [21]), originating from a 100k GWAS for femoral neck width in females of the Framingham Heart Study [43] and a linkage study for pulse pressure in 63 Chinese sib-pairs [44] (Fig. 2b). However, these findings have not been firmly replicated and their significance is still unclear. In addition, these SNPs are ~300-kb apart and seemingly unrelated to the lead atherosclerosis SNPs, speaking against a causal relation.

PHACTR1 is highest expressed in human heart and brain [77] and is a member of a family of proteins that bind actin and interact with protein phosphatase 1 (PP1) [78]. PP1 is an ubiquitous enzyme, regulating essential cellular processes such as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, and neuronal signaling (reviewed in [79]). For *PHACTR1*, a role in cell migration, motility and invasiveness of breast cancer, and melanoma tumor cells was described [80,81]. Moreover, *PHACTR1* is expressed in endothelial cells and involved in regulation of endothelial tubulogenesis and apoptosis [82,83]. In summary, even though *PHACTR1* is an obvious candidate gene at Chr6p24.1, current data on its function is scarce and its mechanism in atherogenesis is still unclear.

Chr1p13.3 (*PSRC1/CELSR2/MYBPHL/SORT1*): LIPIDS AND CORONARY HEART DISEASE

The Chr1p13.3 locus has been discovered in the first surge of GWAS for CHD even before it was also identified as one of the top GWAS hits for plasma LDL cholesterol concentrations [84–86]. Genetic variation at the locus is associated with reduced plasma LDL-cholesterol and reduced risk of coronary artery disease [10^{••},25,45] suggesting that Chr1p13.3 exerts its effect on atherosclerosis by modulating LDL-cholesterol levels. The lead SNPs of CHD and LDL-cholesterol are located in a haplotype block encoding three genes, *cadherin EGF LAG seven-pass G-type receptor 2* (*CELSR2*), *proline/serine-rich coiled-coil 1* (*PSRC1*), and *myosin binding protein H-like* (*MYBPHL*) (Fig. 2c). Wild *et al.* found differential expression of *PSRC1* in monocytes at the locus [11]. The majority of functional work, however, has focused on *sortilin 1* (*SORT1*), which is located in a haplotype block distal of *PSRC1*, *CELSR2*, and *MYBPHL* (Fig. 2c) containing GWAS hits for major depressive disorder [46] and chronic kidney disease [47]. Schadt *et al.* [87] and Folkersen *et al.* [28] found that mRNA expression of *CELSR2*, *PSRC1*, and *SORT1* were all strongly associated with Chr1p13.3 in liver. Although *SORT1* was highly expressed in many tissues, genotype-dependent differential regulation was only seen in liver [28]. Musunuru *et al.* [26] identified a SNP in linkage disequilibrium with the lead SNP, creating a C/EBP transcription factor binding site in the 3' UTR of *CELSR2* and altering expression of *SORT1*. These data suggested that *SORT1* expression might be affected by *cis*-regulation through the neighboring haplotype block [26].

SORT1 is a member of the VSP10P receptor family of sorting receptors, which have been intensively studied in neuroscience and direct proteins through secretory and endocytic pathways of the cell (for review see [88,89]). In 2010, three independent groups published first mechanistic work on the role of *SORT1* in LDL-metabolism with in part paradoxical results: The first study overexpressed *SORT1* in HEK293 cells, resulting in increased uptake of LDL and LDL-receptor-related protein [25]. A second article used viral overexpression in mouse liver, demonstrating that increased *SORT1* decreased plasma LDL-cholesterol and VLDL levels by reducing hepatic VLDL secretion [26]. Inverse results were seen after *SORT1* knock-down [26]. Both studies were well in line with the observation that increased expression of *SORT1* mRNA in human liver was correlated with decreased LDL-cholesterol [26], even though the proposed mechanisms would be either through increased LDL uptake [25] or reduced

VLDL secretion [26]. Results of a third article, published virtually at the same time, were seemingly at odds with the two previous articles. Using mice on the *Ldlr*^{-/-} background, these authors demonstrated that complete *Sort1* deficiency ameliorated hypercholesterolemia and atherosclerosis [24]. Additional studies on the subcellular level indicated that SORT1 interacts with apoB100 in the Golgi apparatus, thereby facilitating formation and hepatic export of apolipoprotein B containing lipoproteins [24].

Recent work [27^{*}] has reconciled the divergent hypotheses on the function of *SORT1* in lipoprotein metabolism. These authors proposed a model in which hepatic SORT1 binds intracellular apoB100 containing particles in the Golgi as well as extracellular LDL at the plasma membrane and traffics them to lysosomal degradation. They suggested a hyperbolic relationship in which complete lack as well as increased *SORT1* would both lead to a reduction in apoB and VLDL secretion, whereas intermediate *SORT1* expression would increase secretion [27^{*}]. Although common variants in *SORT1* have subtle effects on LDL-cholesterol, a recent publication provided data speaking against a role of *SORT1* missense mutations in autosomal dominant hypercholesterolemia [90].

Until now, the majority of work on the molecular mechanism at Chr1p13.3 has clearly focused on *SORT1*. Very little is known about the functions of *PSRC1*, *CELSR2*, and *MYBPHL*, which are closer to the lead Chr1p13.3 SNPs. More work is clearly warranted to establish or firmly exclude a role of these genes in lipid metabolism and atherogenesis.

CONCLUSION

Current GWAS have added additional loci to the 'genomic landscape' of CHD and MI bringing the total count to 58 at a significance cutoff of $P < 10^{-7}$. Recent advances in functional characterization of some loci promise the discovery of hitherto unknown pathways influencing atherosclerosis risk. One such example is the most replicated locus on Chr9p21.3, which might influence atherogenesis through epigenetic chromatin modification by the long ncRNA *ANRIL*. Nevertheless, our current understanding of potential causal variants and mechanisms at most GWAS loci of atherosclerotic cardiovascular disease is very limited. Although some of these loci co-segregate with known risk factors suggesting a potential causal relation, the majority is still 'terra incognita'. This is exemplified by the second most frequently found locus on Chr6p24.1, where virtually nothing is known about its function in atherogenesis. Owing to their small

effect size, the utility of genetic variants for diagnostic purposes is limited. The major promise of identified GWAS loci therefore lies in understanding their function in atherogenesis as a prerequisite for later therapeutic targeting.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 456).

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