

Experimental Biology for the Identification of Causal Pathways in Atherosclerosis

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Abstract More than 60 genomic loci have been implicated by genome-wide association studies (GWAS) and exome-wide association studies as conferring an increased risk of myocardial infarction and coronary artery disease (CAD). However, the causal gene and variant is often unclear. Using the functional analysis of genetic variants in experimental animal models, we anticipate understanding which candidate gene at a specific locus is associated with atherosclerosis and revealing the underlying molecular and cellular mechanisms, ultimately leading to the identification of causal pathways in atherosclerosis and may provide novel therapeutic targets for the treatment of atherosclerotic cardiovascular disease.

Keywords Cardiovascular diseases · Genome-wide association studies · Arteriosclerosis · Lipids · Inflammation · Metabolism

Introduction

Atherosclerotic cardiovascular disease (AS-CVD) is the leading cause of morbidity and mortality in many industrialized countries. Multiple epidemiological studies have demonstrated genetic predisposition for atherosclerotic cardiovascular disease. In the Framingham study, a family history of coronary artery disease (CAD), stroke or peripheral arterial disease was

associated with a 2.4-fold increased risk for CAD in man and 2.2-fold in women [1]. In individuals from families with premature CAD, heritability was estimated to be 92–100 %, whereas within families with older cases the heritability ranges from 15 % to 30 % [2]. Despite the recognition of the genetic predisposition to CAD for many years, recent advancements in the genomic tools and techniques including high throughput genome-wide sequencing are leading to the identification of previously unrecognized molecular pathways. The first large genome-wide association study (GWAS) for CAD was published in 2007 and identified the association of variants on chromosome 9p21 and CAD [3–5]. In studies reported between 2007 and 2015, GWAS and exome-wide association studies have identified more than 60 significant variants strongly associated with CAD and MI, encoding more than 100 different genes [6–10].

After several years deliberate yet gradual hypothesis-driven studies, GWAS and next generation sequencing have led to identification of genetic components of CAD and quantitative traits without relying on any prior biological hypothesis. In theory, this approach offers a great potential for the discovery of new causes and mechanisms of disease. Once a putative causal gene is identified by human genetic studies, overexpression and knockout studies in animal models can be used to further establish the biological function and underlying mechanisms of such gene. Table 1 shows 61 identified loci with genome-wide significance ($P < 5 \times 10^{-8}$) by large-scale GWAS and exome-wide association studies. In the 61 CAD loci, only 15 loci were associated with the conventional risk factors and most loci harbor genes that have not yet been implicated in the pathogenesis of atherosclerosis. Identification of susceptibility variants and translational findings from genetic variants can lead to novel therapeutic treatment for CAD (Fig. 1). This

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Table 1 List of SNP associated with CAD in GWAS and exome-sequencing

SNP ID	Chromosome	Reported gene	Possible mechanism	Ref
rs11206510	1p32.3	<i>PCSK9</i>	LDL metabolism	[93]
rs17114036	1p32.2	<i>PPAP2B</i>	Lipid metabolism	[72]
rs646776	1p13.3	<i>SORT1</i>	LDL metabolism	[93]
rs4845625	1p21	<i>IL6R</i>	Inflammation	[9]
rs17465637	1q41	<i>MIA3</i>	Inhibition of inflammatory cell proliferation	[93]
rs16986953	2p24.1	<i>AK097927</i>	—	[9]
rs515135	2p24-p23	<i>APOB</i>	Cholesterol metabolism	[9]
rs6544713	2p21	<i>ABCG5-ABCG8</i>	Cholesterol metabolism	[9]
rs1561198	2p11.2	<i>VAMP5-VAMP8-GGCX</i>	—	[9]
rs2252641	2q22.3	<i>ZEB2-ACO74093.1</i>	—	[9]
rs6725887	2q33.1	<i>WDR12</i>	Ribosome Biogenesis	[9]
rs9818870	3q22.3	<i>MRAS</i>	Cell proliferation and adhesion	[87]
rs4618210	3p24.3	<i>PLCL2</i>	Inflammation	[94]
rs1878406	4q31.22	<i>EDNRA</i>	Inflammation, vasoconstriction	[9]
rs7692387	4q31.1-q31.2	<i>GUCY1A3</i>	Cell proliferation, chemotaxis	[9]
rs17087335	4q12	<i>REST-NOA1</i>	—	[7]
rs273909	5q31.1	<i>SLC22A4-SLC22A5</i>	—	[9]
rs6903956	6p24.1	<i>ADTRP-C6orf105</i>	—	[95]
rs12526453	6p24	<i>PHACTR1</i>	—	[96]
rs17609940	6p21.31	<i>ANKS1A</i>	—	[72]
rs10947789	6p21	<i>KCNK5</i>	Renal potassium transport	[9]
rs12190287	6q23.2	<i>TCF21</i>	Transcription Factor	[72]
rs2048327	5p15.3	<i>SLC22A3-LPAL2-LPA</i>	Lipoprotein(a) metabolism	[97]
rs3798220	6q25.3	<i>LPA</i>	Lipid metabolism	[98]
rs4252120	6q26	<i>PLG</i>	Inflammation	[9]
rs2023938	7p21.1	<i>HDAC9</i>	Hematopoiesis	[9]
rs10953541	7q22.3	<i>BCAP29</i>	Immune system	[10]
rs11556924	7q32.2	<i>ZC3HC1</i>	Cell proliferation	[72]
rs4731702	7q32	<i>KLF14</i>	HDL metabolism	[41]
rs3918226	7q36	<i>NOS3</i>	—	[7]
rs264	8p22	<i>LPL</i>	Lipid synthesis	[9]
rs2954029	8q24.13	<i>TRIB1</i>	Lipid metabolism	[41]
rs1333049	9p21.3	<i>CDKN2A,B/ANRIL/IFNW1</i>	Cell proliferation, inflammation	[3–5]
rs579459	9q34.2	<i>ABO</i>	Thrombogenesis	[71]
rs2505083	10p11.23	<i>KIAA1462</i>	Endothelial cell function	[10]
rs501120	10q11.21	<i>CXCL12</i>	Inflammation, lipid metabolism	[10, 96]
rs1412444	10q23.2-q23.3	<i>LIPA</i>	Lipid related	[10]
rs12413409	10q24.32	<i>CYP17A1-CNNM2-NT5C2</i>	Lipid metabolism	[72]
rs10840293	11p15	<i>SWAP70</i>	Cell proliferation, immune response	[7]
rs974819	11q22.3	<i>PDGFD</i>	Inflammation, lipid metabolism	[10]
rs964184	11q23.3	<i>ZNF259-APOA5-A4-C3-A1</i>	LDL, TG metabolism	[72]
rs7136259	12q21.3	<i>ATP2B1</i>	Cellular energy transfer, calcium homeostasis	[99]
rs3184504	12q24	<i>SH2B3</i>	Cell signal adapter protein	[100]
rs671	12q24	<i>BRAP-ALDH2</i>	Inflammation	[97]
rs11830157	12q24.22-q24.23	<i>KSR2</i>	Fatty acid oxidation	[7]

Table 1 (continued)

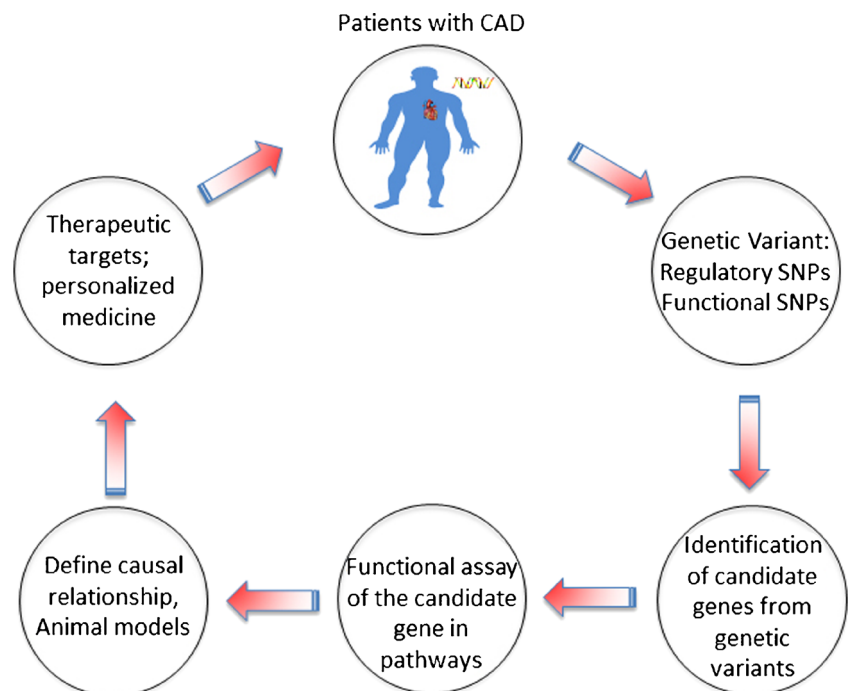
SNP ID	Chromosome	Reported gene	Possible mechanism	Ref
rs9319428	13q12	<i>FLT1</i>	Inflammation, angiogenesis	[101]
rs4773144	13q34	<i>COL4A1/A2</i>	Plaque destabilization	[72]
rs2895811	14q32.2	<i>HHIPL1</i>	Angiogenesis, vascular remodeling	[72]
rs7173743	15q25.1	<i>ADAMTS7</i>	Smooth muscle cell function	[10, 71, 72]
rs17514846	15q26.1	<i>FURIN-FES</i>	Cholesterol metabolism	[9, 72]
rs56062135	15q21.1	<i>SMAD3</i>	transforming growth factor- β signaling pathway	[7]
rs8042271	15q26.1	<i>MFG8-ABHD2</i>	Inflammation	[7]
rs216172	17q13.3	<i>SMG6</i>	mRNA editing	[72]
rs12936587	17p11.2	<i>RAI1-PEMT-RASD1</i>	—	[72]
rs46522	17q21.32	<i>UBE2Z</i>	Insulin resistance	[72]
rs7212798	17q23	<i>BCAS3</i>	Cell migration	[7]
rs663129	18q21	<i>PMAIP1-MC4R</i>	—	[7]
rs1122608	19p13	<i>LDLR</i>	LDL metabolism	[96]
rs2075650	19p13.32	<i>APOE-APOC1</i>	LDL metabolism	[102]
rs12976411	19q13.11	<i>ZNF507-LOC400684</i>	—	[7]
rs58542926	19p13.3-p12	<i>TM6SF2</i>	Cholesterol metabolism	[34]
rs9982601	21q22	<i>SLC5A3-MRPS6-KCNE2</i>	—	[96]
rs180803	22q11.23	<i>POM121L9P-ADORA2A</i>	—	[7]

review will briefly discuss current biological approaches to identify the causal genes and pathways, functional studies to unravel the underlying mechanisms of disease, potential therapeutic targets to treat atherosclerotic cardiovascular disease.

LDL-C Metabolism Pathway

It is widely recognized that high serum low-density lipoprotein cholesterol (LDL-C) levels play a critical role in the initiation and progression of atherosclerosis. Both

Fig. 1 Schematic diagram of advancing genetic variants to clinical approaches in CAD. CAD, coronary artery disease; SNP, single nucleotide polymorphism



Mendelian randomization studies and GWAS for CAD risks have indicated that LDL-C is a causal risk factor for AS-CVD [7, 11]. Evidence from clinical trials demonstrates that reducing circulating LDL-C levels by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting key enzyme in the hepatic biosynthesis of cholesterol, can result in beneficial clinical outcomes in patients at risk of CAD. The LDL-C metabolism pathway is complex, and genetic defects in any of a number of steps in this pathway can lead to increased LDL-C levels.

PCSK9 First identified by Seidah and colleagues in 2003, the serine protease proprotein convertase subtilisin/kexin type 9 (PCSK9) has been associated with mild to severe hypercholesterolaemia and an increased risk of CAD [12–14]. Sanna et al. sequenced seven genes associated with LDL-C in 256 individuals with extreme LDL-C levels, confirmed in a sample of ~10,000 Finnish and Norwegian individuals, and identified that rs11591147, which leads to a non-synonymous R46L change in exon 1 of *PCSK9*, is independently associated with LDL-C [15]. The mechanism by which *PCSK9* gene defects cause autosomal dominant familial hypercholesterolemia (FH) is likely due to gain-of-function mutations. PCSK9 is a serine protease that binds to the epidermal growth factor-like repeat A on the LDL receptor. Upon binding to the LDL receptor, PCSK9 initiates internalization and lysosomal degradation of the LDLR/PCSK9 complex, resulting in decreased LDL receptors on the surface of hepatocytes and increased LDL-C concentrations in blood. Loss-of-function mutations lead to higher levels of the LDL receptor, lower LDL-C levels, and decreased cardiovascular risk [16–18]. This phenotype has been confirmed by gain- or loss-of-function studies in mice [19–21]. Overexpression of *PCSK9* diminishes LDL receptor protein and function, causing an *Ldlr* knockout phenotype in mice [19, 20]. *Pcsk9*-deficient mice showed reduced plasma cholesterol levels by increasing LDL receptor protein expression in liver and accelerating the clearance of circulating cholesterol [19, 21]. In apolipoprotein E (*ApoE*)-deficient mice, gene inactivation of *Pcsk9* significantly reduced aortic cholesteryl esters. By comparison, overexpression of *PCSK9* induced an accumulation of aortic cholesteryl esters and accelerated development of atherosclerotic plaque [19]. In *Ldlr*-deficient mice, overexpression of *PCSK9* did not regulate the circulating cholesterol levels, aortic accumulation of cholesteryl esters and atherosclerotic plaque size, indicating that the pro-atherosclerotic effect of PCSK9 is mediated mainly through its action on the LDL receptor [19]. The *PCSK9* transgenic pigs created by transposition of a human PCSK9 D374Y gain-of-function mutation displayed reduced hepatic LDL receptor levels, impaired LDL clearance, severe hypercholesterolemia, and spontaneous development of progressive atherosclerotic lesions [22]. Although PCSK9 gain-of-

function mutations are rare, comprising only a small portion (up to 3 %) of cases of FH, the established mechanism has generated significant interest in the gene as a drug target. Both alirocumab and evolocumab, humanized monoclonal antibodies causing inactivation of PCSK9, result in large reductions in LDL cholesterol levels, as compared with placebo (39 to 62 % reduction for alirocumab and 47 to 56 % for evolocumab) [23, 24]. Although the potential cardiovascular benefits of these agents need to be further established, this newly confirmed pathway identified by human genetics has become a novel therapeutic target to lower cholesterol in patients with familial hypercholesterolemia and clinical atherosclerotic cardiovascular disease.

SORT1 In addition to Mendelian randomization studies, GWA studies have identified novel loci associated with CAD and LDL-C. One of the most compelling findings is a locus on chromosome 1p13, which is strongly associated with both plasma LDL-C and myocardial infarction (MI) in humans [25–27]. This region harbors seven genes, seryl tRNA synthetase (*SARS*), cadherin EGF lag seven-pass G type receptor 2 (*CELSR2*), proline serine rich coiled coil protein 1 (*PSRC1*), myosin binding protein H-like (*MYBPHL*), sortilin-1 (*SORT1*), proteasome (prosome, macropain) subunit, alpha type 5 (*PSMA5*), and synaptophysin-like 2 (*SYPL2*). Using expression quantitative trait loci (eQT) data, several groups confirmed that the minor allele for the SNP rs646776 and the major allele for SNP rs599839, were associated with low mRNA levels for *SORT1*, *PSRC1* and *CELSR2* in the liver [25, 28, 29]. *Celsr2* is highly expressed in cells lining all cerebral ventricles and the central canal of the spinal cord [30]. Results from *Celsr2*-deficient mice indicated impaired cilia function and hydrocephalus, but there was not informative data regarding any effects in cholesterol level [30]. Musunuru et al. demonstrated that *Sort1* overexpression led to a 40 % reduction in plasma cholesterol, whereas *Psrc1* overexpression had no effect on plasma cholesterol using adeno-associated viruses (AAVs)-mediated overexpression of *Psrc1* and *Sort1* in hyperlipidemic *Ldlr*-deficient mice [29]. *Sort1* knockdown in mouse liver using siRNAs was associated with a 20–40 % increase in plasma cholesterol [29]. These results strongly implicated *SORT1* as the causal gene at the 1p13 locus whose elevated expression confers reduction in LDL-C. However, Kjolby et al. reported different results using adenovirus-mediated overexpression of *SORT1* and *Sort1*-deficient mice, in which *Sort1*-deficient mice show decreased cholesterol levels and reduced atherosclerotic plaque area [31]. Recently, Mortensen et al. demonstrated that transfer of *Sort1*-deficient bone marrow into irradiated atherosclerotic mice reduced atherosclerosis and systemic markers of inflammation without concomitant changes in total cholesterol or LDL-C, indicating *SORT1* plays an additional inflammatory role in lymphocytes and macrophages potentially

contributing to overall atherogenesis [32]. Although the SORT1 locus represents a promising target for the treatment of atherosclerosis identified by human genetic studies, these obviously conflicting findings in animals and the yet unsettled mechanisms behind the phenotype raises some questions on their immediate translation into the therapeutic strategies.

TM6SF2 Both GWA studies and exome-wide association studies have identified that a nonsynonymous variant (rs58542926, encoding p.Glu167Lys) in transmembrane 6 superfamily member 2 (*TM6SF2*) gene is associated with blood lipid levels, nonalcoholic fatty liver disease (NAFLD) and MI [33–35]. The specific biological function of *TM6SF2* is not clear. Kozlitina et al. demonstrated knockdown of *Tm6sf2* resulted in decreased very low density lipoprotein (VLDL) secretion and increased liver triglyceride (TG) content, which is consistent with human genetics findings in the DHS, Dallas Biobank and Copenhagen Study cohorts [33, 35]. Holmen et al. demonstrated that this variant in *TM6SF2* influences total cholesterol levels and is associated with myocardial infarction (MI) [34]. In mice, transient overexpression of human wildtype *TM6SF2* resulted in high total cholesterol, LDL-C and TG, whereas knockdown of mouse *Tm6sf2* resulted in decreased serum total cholesterol levels [34]. Our results demonstrate that *TM6SF2* is a critical regulator of cholesterol metabolism and liver-specific *Tm6sf2* knockout mice challenged to high fat diet did not show increased liver triglyceride content [36]. Recently, a systematic meta-analysis in 101,326 individuals showed that carriers of the minor T allele are protected from cardiovascular disease, showing lower levels of total cholesterol, LDL-C and triglyceride. In contrast, carriers of the T allele showed a moderate increased risk for NAFLD [37]. Based on the opposing roles of *TM6SF2* on both CVD and NAFLD, it is necessary to dissect the two functions of *TM6SF2* before it becomes a therapeutic target to reduce the risk of myocardial infarction via lowering blood lipids.

HDL Metabolism Pathway

The existence of a strong inverse relationship between plasma HDL and CAD has been largely confirmed in numerous epidemiological studies. HDLs display a wide spectrum of activities including reverse cholesterol transport, anti-inflammatory and anti-oxidative effects and maintenance of endothelial function. Although most of the HDL-raising agents, such as niacin, fibrates, and cholesteryl ester transfer protein (CETP) inhibitors, have yielded convincing results to increase HDL-C levels, those clinical trials have failed to show a decrease in CAD events. Further evidence from genetic studies has largely failed to support a strong causal association

between genetically raised plasma HDL-C levels and CAD risks using HDL-associated variants at cholesteryl ester transfer protein (*CETP*), lecithin-cholesterol acyltransferase (*LCAT*), ATP-binding cassette transporter A1 (*ABCA1*), and hepatic triglyceride lipase (*LIPC*). It has been questioned whether the association between HDL-C levels and the risk of atherosclerotic events represents a causal relationship. GWA studies and Mendelian randomization studies have suggested that genetic mechanisms that raise plasma HDL-C do not seem to lower risk of MI [38]. However, based on family and twin studies, HDL-C levels have shown a heritability ranging from 40 to 60 % [39, 40]. Recent GWA studies have shown that only 10–12 % of the heritability is due to common variants [41, 42], suggesting that a large proportion of the molecular mechanisms underlying HDL-C functions and metabolism is still unknown.

KLF14 More than 10 HDL-associated loci with significant effect on CAD risk have been identified—*CETP*, *LPL*, *LIPG*, *LPL*, *TRIB*, *APOA5-APOA4-APOC3-APOA1* cluster, *PPP1R3B*, *TTC39B*, *GALNT2*, *KLF14*, *IRS*, *ANKS1A*, and *SH2B3*. Among these genes only *KLF14* and *ANKS1A* were solely associated with HDL-C levels and CAD. Teslovich et al. reported that variants at the locus containing *KLF14* and *TSGA13*, which encode Krüppel-like factor 14 and testis-specific gene A13, were associated with both HDL-C and CAD [41]. These variants were maternally restricted cis-eQTLs for *KLF14* expression and strongly correlated with expression of several other genes in adipose tissue in trans, suggesting that *KLF14* may be a master regulator of gene expression in adipose tissue and a key player in human metabolism [43]. Guo et al. demonstrated that adenovirus-mediated overexpression of *KLF14* markedly increased HDL-C levels and cholesterol efflux capacity through regulation of hepatic ApoA-I production. Studies of *Klf14* knockout mouse models have confirmed the relationship between *Klf14* and HDL-C levels [44]. Furthermore, Guo and colleagues identified perhexiline, an approved therapeutic small molecule presently in clinical use to treat angina and heart failure, as a *KLF14* activator. Treatment with perhexiline increased HDL-C levels and cholesterol efflux capacity and reduced atherosclerotic lesion development in *ApoE*-deficient mice [44]. This finding was directionally consistent with the human genetics data showing that the T allele of rs4731702 located near the *KLF14* gene was associated with a decreased risk of myocardial infarction and the T allele carriers have higher ApoA-I levels in the Mulao population in China [45]. These data were crucial in establishing *KLF14* as a causal contributor to HDL-C levels and risk of CAD and led to a rapid effort to understand its function and the therapeutic potential of targeting this protein.

Triglyceride Metabolism Pathway

For decades, the independent association between increased plasma triglyceride levels and cardiovascular risk had been largely controversial. Several randomized controlled clinical trials targeting triglyceride lowering such as fibrates failed to show beneficial effects on cardiovascular events. However, growing evidence from human genetic studies has shown a strong association between triglyceride and CAD risk. Do and colleagues analyzed 185 SNPs in 188,577 individuals from 60 studies and found that SNPs' effect on triglyceride levels is associated with risk of CAD, even after accounting for effects on LDL-C and/or HDL-C levels [46]. Furthermore, a multiple SNP Mendelian randomization analysis in over 62,000 participants (including 12,000 CAD events) demonstrated a causal relationship between triglycerides and CAD events [11].

APOC3

A locus on chromosome 11 is significantly associated with triglyceride, LDL-C, HDL-C and CAD risk identified by GWA studies. This complex cluster includes the apolipoprotein genes *APOA1*, *APOC3*, *APOA4*, and *APOA5*. ApoC-III inhibits lipoprotein lipase, promotes VLDL production and slows hepatic VLDL uptake [47, 48]. The Exome Sequencing Project performed an exome sequencing of 18,666 genes in 3734 individuals and conducted an association assay for the rare variants with triglyceride levels [49]. They identified three loss-of-function mutations in *APOC3*. The mutation carriers have 39 % lower in triglyceride levels and 40 % reduced risk of CAD than noncarriers [49]. Similar findings were confirmed by resequencing the coding regions and consensus splice sites of *APOC3* in the general population in Denmark [50]. Although the effect of ApoC-III on VLDL production in mice is somewhat controversial [51–54], antisense inhibition of apoC3 has been developed and administration of the apoC3 antisense oligonucleotide ISIS 304801 significantly decreased levels of apoC3 and triglycerides [55, 56]. ISIS 304801 treatments also resulted in increased HDL-C levels and reduced VLDL levels. Long-term outcome clinical trials targeted at lowering ApoC-III are required to establish the causality and to evaluate whether ApoC-III may serve as a CAD therapeutic target.

APOA5 GWA studies reveal the importance of ApoA-V as a regulator of plasma triglyceride homeostasis. ApoA-V accelerates lipoprotein lipase (LPL)-mediated hydrolysis of triglyceride-rich VLDL, inhibits VLDL production and facilitates VLDL clearance [57, 58]. *APOA5* transgenic mice have significantly reduced serum triglyceride levels and *Apoa5*-deficient mice have 18-fold higher VLDL levels compared to control mice [59]. Recently, an exome sequencing study

with 6721 subjects with early-onset myocardial infarction and controls identified that carriers of rare non-synonymous mutations on *APOA5* had higher plasma triglyceride levels and had a 2.2-fold increased risk for MI [60]. Those findings indicate that apoA5 may become a promising therapeutic target for triglyceridemia and CAD.

TRIB1 Lead SNPs located at chromosome 8p24 are associated with all major lipid traits and CAD in both GWAS and gene-centric array association studies, in which the minor allele of rs2954029 is associated with reduced triglycerides, decreased LDL-C, increased HDL-C, and reduced risk of CAD [41]. Adenovirus-mediated hepatic overexpression of *Trib1* resulted in reduced plasma cholesterol and triglyceride levels, accompanied by reduced expression of key genes involved in lipogenesis, such as fatty acid synthase, stearoyl-coenzyme A desaturase 1 and acetyl-CoA carboxylase 1. In contrast, *Trib1* deficient mice showed increased plasma cholesterol and triglyceride levels [61]. Recently, carbohydrate-responsive element-binding protein (ChREBP) and Sin3A (Swi-independent 3 A)-associated protein emerged as TRIB1 binding partners [62, 63], which may aid discovery of novel therapeutic targets for the management of dyslipidemia. Not known animal experimental results are available to illustrate whether *TRIB1* is a causal gene for atherosclerosis at this time.

Chromosome 9p21.3 Locus

In the initial Ottawa population of 322 cases versus 312 controls, a locus on chromosome 9p21 was identified to be associated with CAD [3–5]. This association with CAD was confirmed in diverse ethnic groups except for Africans. The 9p21 genetic risk variant is extremely common, with 75 % of humans having one or more risk alleles (50 % heterozygous and 25 % homozygous). The presence of a single copy or two copies of the 9p21 risk allele conferred a 15–20 % or 30–40 % increased risk for CAD respectively. In individuals with premature CAD, 9p21 homozygosity is associated with a 2-fold increased risk for CAD [4]. The 9p21 risk is independent of conventional risk factors such as cholesterol, diabetes, or hypertension, indicating that 9p21 exerts its effect via some novel unknown pathway, potentially leading to new therapeutic targets. However, the mechanism whereby the 9p21 locus confers increased CAD risk remains obscure. Whole 9p21 region deletion mice confirmed that the 9p21 region is a regulator of the Cyclin-dependent kinase inhibitors, but the mice do not develop atherosclerosis [64, 65]. Fine mapping of the genetic risk locus at 9p21 shows that it consists of a cluster of 59 linked SNPs over a 53 kb region. The nearest protein coding genes are the cyclin-dependent kinase inhibitors *CDKN1A*, *CDKN2B* and methylthioadenosine phosphorylase

(*MTAP*), which are abundantly expressed in atherosclerotic tissues and regulate cell cycle and apoptosis [66]. Deletion of the same region in the murine genome was associated with severe reduced expression of *Cdkn2a* and *Cdkn2b* mRNAs. In experimental atherosclerotic models, *Cdkn2a*-deficient mice have smaller lesions and loss of *Cdkn2b* is associated with advanced atherosclerotic lesions [67, 68]. *Mtap*-knockdown mice also have a significant increase in atherosclerotic lesions [67]. Another proposed underlying mechanism rests in a long noncoding RNA in the INK4 locus (*ANRIL*), which overlaps *CDKN2B*. Unlike *CDKN1A* and *CDKN2B*, *ANRIL* expression is highly associated with the 9p21 haplotype and decreased *ANRIL* is directly correlated with severity of common carotid artery stenosis [69]. *In vitro* studies have demonstrated that *ANRIL* regulates *CDKN2B* expression in cultured human fibroblasts. A significant limitation of the mouse atherosclerosis models to investigate the biological functions of *Anril* is the fact that mice lack a clear ortholog of *ANRIL* [70]. Non-human primates may be needed to illustrate the susceptibility to cardiovascular disease at the 9p21 locus. Recently, Harismendy and colleagues have identified 33 enhancers in 9p21 locus and two SNPs within the 9p21 locus which disrupt the binding for signal transducer and activator of transcription 1 (*STAT1*) to the enhancer that physically interacts with the *CDKN2A/B* locus and *MTAP* gene [73]. As *STAT1* is a downstream inflammatory effector, these findings indicate that the 9p21 locus may play a role in inflammatory signaling in the arterial wall. Ongoing fundamentally translational research on the 9p21 locus will reveal the new underlying mechanisms and may drive development of novel therapeutic approaches for atherosclerosis.

Vascular Wall Remodeling

ADAMTS-7, a member of the disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, was recently identified to be significantly associated with human CAD, restenosis or arterial calcification [9, 10, 71, 72]. As an enzyme, ADAMTS-7 affects the extracellular matrix of vascular smooth muscle cells, but this locus has no relationship to traditional risk factors. Overexpression of *ADAMTS7* induced vascular smooth muscle cells migration and promoted neointima formation following artery injury through the degradation of cartilage oligomeric matrix protein (COMP) in rats [73]. Kessler and colleagues demonstrated that *Adamts7* inhibits endothelial cell proliferation and migration via degradation of thrombospondin-1 in Comp-independent manner [74]. *Adamts7*-deficient mice showed enhanced vessel re-endothelialization and reduced neointima formation after wire injury [74]. Bauer and colleagues addressed the effect of *Adamts7* on atherosclerosis development. Deletion of *Adamts7* significantly reduced atherosclerotic lesion

formation in mice on both *ApoE* knockout and *Ldlr* knockout background without significant changes in plasma lipid profiles [75]. *Adamts7* deletion impaired VSMC migration and developed plaques with a larger fibrous cap, indicating targeting ADAMTS7 may result not only in decreased atherosclerosis but also in more stable plaques.

Inflammatory Pathway

Over the last few decades, it is well accepted that inflammatory processes not only promote initiation of atherosclerosis, but also contribute to acute cardiovascular events, such as triggering MI. Network analysis demonstrates that some genes, such as *APOA-I* and Interleukin-6 receptor (*IL-6R*), involved in lipid metabolism pathways also involved in inflammation, contributing to CAD pathogenesis [9]. Increased Interleukin-6 (IL-6) expression has been associated with CAD. IL-6 binds to its receptor and plays a central role in propagating the downstream inflammatory response during atherosclerosis progression. A non-synonymous *IL-6R* variant is associated with increased circulating levels of IL-6 and decreased CAD risk [76, 77], suggesting a causal relationship between IL-6R-related pro-inflammatory pathways and CAD. Tocilizumab, a monoclonal antibody that blocks both membrane-bound and circulating IL6R, has anti-inflammatory actions and is licensed for treatment of rheumatoid arthritis. However, rheumatoid arthritis patients treated with an IL-6R monoclonal antibody, tocilizumab, show increased circulating triglycerides and LDL-C levels [78], suggesting that blockage of IL-6R pathway to protect against atherosclerosis need to be reconsidered. The chemokine CXCL12 and its receptor, CXCR4, play an important protective role in atherosclerosis. Circulating levels of CXCL12 are decreased in CAD patients compared with healthy control subjects [79]. Rs1746048, at chromosome 10q11 near the *CXCL12* gene, is associated with CAD and plasma levels of CXCL12 [80]. The CXCL12/CXCR4 axis is critical for retention and release of hematopoietic cells from the bone marrow and *Cxcr4* deficient mice showed increased atherosclerotic lesions due to hyperactivation of circulating neutrophils [81, 82]. Other GWAS-identified signal for CAD includes *ABO* locus on chromosome 9q34 and the major histocompatibility complex (*MHC*) locus on chromosome 6p21.3 [71, 83, 84]. Further studies are required to confirm the association and to identify the causative allele.

NO Pathway

The NO-sGC-cGMP pathway is a major pathway controlling vascular smooth-muscle relaxation, vascular tone, and vascular remodeling. Under normal conditions, NO binds to soluble

guanylate cyclase (sGC) and activates sGC to generate cGMP, and thus stimulates cGMP-dependent protein kinase I, which inhibits VSMC constriction, proliferation and migration, and reduces platelet adhesion and activation as well as vascular inflammation [85]. Lu et al. performed a two-stage GWAS of CAD in a sample including 33,000 Han Chinese individuals, and identified that rs1842896 at 4q32.1 is associated with CAD [86]. *GUCY1A3*, encoding α 1-sGC, is the only gene in this CAD-associated locus. Erdmann et al. reported the segregation of two private, heterozygous mutations in *GUCY1A3* and *CCT7*, in an extended myocardial infarction family [87]. *CCT7* encodes CCT η , a member of the tailless complex polypeptide 1 ring complex, which stabilizes α 1-sGC. *In vitro* studies demonstrated that mutations in both *GUCY1A3* and *CCT7* severely reduce α 1-sGC as well as β 1-sGC protein content, and impaired α 1-sGC activity. Moreover, platelets from mutation carriers contained less α 1-sGC protein and consequently displayed reduced nitric-oxide-induced cGMP formation. Mice deficient in α 1-sGC protein displayed accelerated thrombus formation in the microcirculation after local trauma [87]. These genetic and functional studies identified a link between impaired α 1-sGC-dependent NO signaling and MI risk, possibly through accelerated thrombus formation. Studies are ongoing to reveal the role of Gucy1a3 in atherosclerosis as well as lipid metabolism.

Perspectives

GWA studies, exome sequencing, linkage analyses, and targeted resequencing have successfully identified potential causal variants. However, the understanding of causality is still rudimentary and the task of validation of causal genes and determining causal variants remains challenging. The most widely used *in vivo* model for atherosclerosis study is currently the mouse with *Ldlr* knockout or *ApoE* knockouts at the forefront. However, most of the GWAS identified susceptibility variants are found within noncoding regions, making it difficult to define disease associations with specific transcripts. In addition, the regulatory variants may not be evolutionarily conserved, for example, the mouse's lack of *Cetp* and a clear ortholog of *ANRIL* in the 9p21 region. Some large animal models, such as pigs, rabbits, and nonhuman primates, have particular advantages for certain types of studies because they have shown an atherosclerotic pattern comparable to that of humans [88]. Others and our group have generated transgenic rabbits and pigs with altered expression of human genes and specific gene knockout rabbits and pigs for cardiovascular disease studies [22, 89–92].

Several groups have shown that induced pluripotent stem cells (iPS cells) can be generated from human monocytes by the introduction of defined sets of transcription factors. These iPS cells resemble embryonic stem cells in many respects and

will provide a powerful *in vitro* model for the study of gene function. Rader's group used iPS cell-derived hepatocytes from the SR-BI P376L mutation homozygous subject to study the effects of the endogenous mutation in human hepatocyte-like cells (AHA2015 presentation). Engineered gene editing such as zinc finger nucleases and CRISPR-Cas9 systems can facilitate precise modification of the genome in iPS cells to obtain potential causal DNA variants. Directed differentiation of iPS cells makes it possible to establish cellular models with specific variants and to investigate relationships between the variants and specific metabolic pathways in appropriate human cell types.

Conclusions

In the past decade, GWA studies, exome sequencing, and targeted resequencing have offered a rapid increase in the number of CAD susceptibility loci. The truly established causal associations between genetic variation and CAD, such as *PCSK9*, have been becoming novel therapeutic targets. However, the mechanisms underlying the association of most of these loci to CAD remain unknown. By combination data from genetic variants, functional studies, genetic animal models, and drug development, ongoing studies are likely to accelerate our understanding of mechanisms linking identified loci to atherosclerosis, facilitate personalized medicine and lead to the development of novel therapeutic strategies for prevention and treatment of CAD.

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