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Leading Edge
Review

Genetics of Common, Complex Coronary Artery Disease

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Coronary artery disease represents the leading cause of death worldwide, sparing no nation, ethnicity, or economic stratum. Coronary artery disease is partly heritable. While enormous effort has been devoted to understanding the genetic basis of coronary artery disease and other common, complex cardiovascular diseases, key challenges have emerged in gene discovery, in understanding how DNA variants connect to function, and in translation of genetics to the clinic. We discuss these challenges as well as promising opportunities to bring the work closer to fruition.

Discovery Studies

Progress to Date

The most prevalent cardiovascular diseases—including coronary artery disease (CAD), atrial fibrillation, heart failure, hypertension, and stroke—are complex, reflecting the interplay of genetic and environmental factors. In contrast to monogenic cardiovascular diseases such as inherited cardiomyopathies, rhythm disorders, and vascular disorders, for which rare DNA variants with large effects are responsible, the complex diseases are influenced by common and rare DNA variants at numerous loci distributed throughout the genome. Because these variants typically have small effects and do not drive classic Mendelian inheritance patterns within families, they require large-scale, population-based association studies for discovery.

In order to connect genotype with phenotype, these studies have utilized two designs—common variant association studies (CVASs), also termed genome-wide association studies (GWASs), or rare variant association studies (RVASs) (Zuk et al., 2014) (Figure 1). In CVASs, the DNA sequence variant is observed in enough copies that it is practical to test each variant individually to estimate its frequency in disease cases versus controls. In contrast, rare variants occur too infrequently to allow association tests of individual variants. RVASs thus require aggregating rare variants into sets and comparing the aggregate frequency distribution in cases versus controls—so-called burden tests. In CVASs, the unit of analysis is the individual variant, whereas in RVASs, the unit of analysis is variation that collectively occurs within a functional genetic unit such as all exons within a gene.

CVASs of CAD, the largest of which have included hundreds of thousands of people, have been successful in the sense that they have collectively identified more than 150 associated loci at the commonly accepted genome-wide statistical significance threshold of $p < 5 \times 10^{-8}$ to account for multiple testing of millions of DNA variants (Howson et al., 2017; Nelson et al., 2017; Klarin et al., 2017; van der Harst and Verweij, 2018). Yet each novel CVAS discovery marks the potential beginning of an even more herculean effort, to understand the biological and clinical relevance of the locus, how genotype connects to phenotype (Lin and Musunuru, 2018). For each locus, the lead variant, which is usually a noncoding single nucleotide polymorphism (SNP), defines a set of variants in linkage disequilibrium that can span throughout the locus; any of them has the potential to be the causal variant, or one of multiple causal variants, responsible for the association with the disease. The causal variant(s) in turn must act upon one or more genes, either through a local effect on a gene within the locus or action at a distance on a more remote gene, in order to mediate the effect on disease risk.

Finally, the mechanism(s) by which the causal gene(s) affect disease pathogenesis warrant elucidation if new biological or therapeutic insights are to be achieved. For the thousands of discovered CVAS loci for cardiovascular diseases, in only a handful of cases have variants provisionally been connected to function. Yet broad new insights into disease can be achieved even without a detailed molecular understanding of variant-to-function relationships. For CAD, cataloging of the genes present in the plurality of the more than 150 CVAS loci identified to date defines several broad risk factors: low-density lipoprotein (LDL) cholesterol, triglyceride-rich lipoproteins, insulin resistance, thrombosis, inflammation/cell adhesion/transendothelial migration, cellular proliferation/vascular remodeling, and vascular tone/nitric oxide signaling (Klarin et al., 2017). Still, these generalizations make assumptions about the identity of causal genes in the CAD loci, and the majority of the loci defy easy characterization.

RVASs have the advantage of directly pointing to causal genes—the genes marked by the coding variants interrogated in the studies. Yet RVASs of complex cardiovascular diseases have not been nearly as successful as CVASs. This is not surprising, since the rare nature of the variants under study means that hundreds of thousands of individuals will need to undergo exome sequencing or exome chip analysis in order for an RVAS of a disease to be adequately powered to detect a statistically significant association. Another limitation of RVASs is that many of the rare variants found in genes are neutral and do not

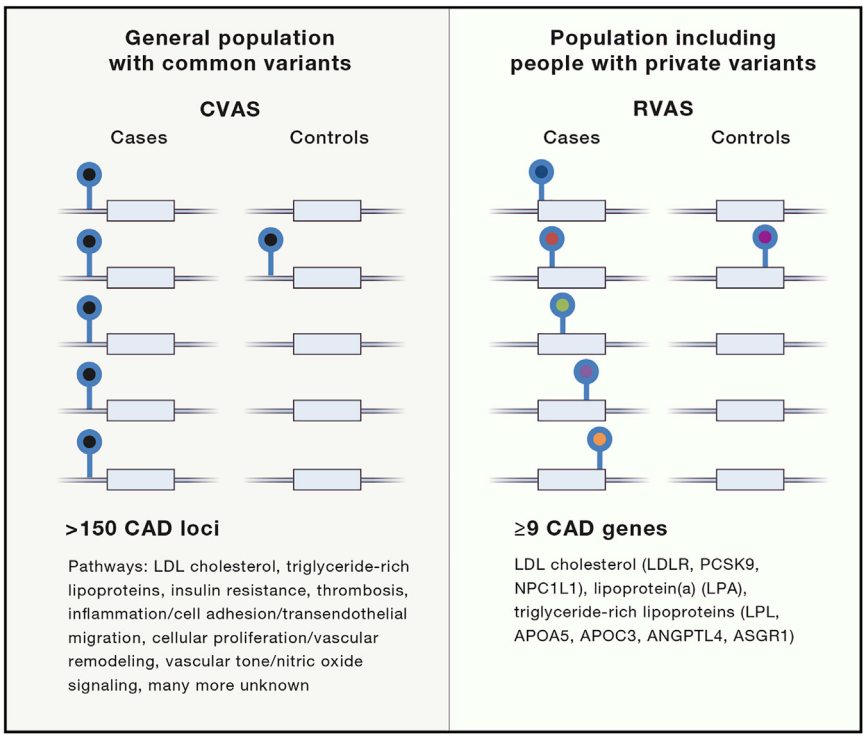

Figure 1. Contrasting Designs of CVASs and RVASs

In a CVAS, the frequencies of an individual common variant, usually noncoding, differ between cases and controls, establishing an association with a disease phenotype. The study design does not typically identify the causal variant(s) or gene(s). In a RVAS, the aggregate frequencies of collections of rare variants in a gene differ between cases and controls, establishing the gene as being causal for a disease phenotype. The results of these study designs for CAD are presented.

affect protein function; their inclusion in burden tests actually weakens the power of RVASs, since neutral variants would occur at equal frequencies in cases and controls and dilute any association signals. This can be addressed with functional assays to determine which of the variants are neutral and which are loss-of-function, with only the latter used for burden tests (Thormaehlen et al., 2015; Stitziel et al., 2017).

Despite these limitations, a few early results have emerged for CAD. Aggregated coding variants (in burden tests) in the following genes have been found to be significantly different in their frequencies between CAD cases and control individuals: *LDLR* (LDL receptor), *APOA5* (apolipoprotein A-V), *APOC3* (apolipoprotein C-III), *LPA* [lipoprotein(a), or Lp(a)], *PCSK9* (proprotein convertase subtilisin/kexin type 9), *ANGPTL4* (angiopoietin-like 4), *LPL* (lipoprotein lipase), and *ASGR1* (asialoglycoprotein receptor 1) (Do et al., 2015; TG and HDL Working Group of the Exome Sequencing Project, National Heart, Lung, and Blood Institute, 2014; Myocardial Infarction Genetics and CARDIoGRAM Exome Consortia Investigators, 2016; Nioi et al., 2016; Emdin et al., 2016; Khera et al., 2017). Sizable numbers of additional RVAS discoveries for CAD await future, larger studies.

Estimates of the heritability of CAD—the contribution to CAD from genetic variation inherited from the parents—are in the range of 40% to 50% (Marenberg et al., 1994; Zdravkovic et al., 2002; Won et al., 2015). The common variants identified by CVASs account for about 38% of the heritability (Nikpay et al., 2015). The provenance of the unaccounted heritability remains unclear, although it is possible that a substantial portion inheres in common variants that have yet to be linked to CAD. Due to their rarity, variants identified in RVASs are unlikely to account for much heritability, even in aggregate. Nonetheless, there is a strong rationale to continue to pursue the RVAS approach since any identified genes might yield novel biological insights into CAD.

The Value of Large-Scale Biobank Cohorts

Future genetic studies will be empowered by emerging large-scale biobank cohorts that have recruited or are recruiting hundreds of thousands of participants, all of whom will have genome-wide genotype data available as well as a variety of clinical phenotypes accessible via links to electronic health records. These cohorts include efforts such as UK Biobank, Million Veteran Program, China Kadoorie Biobank, and the *All of Us* Research Program from the US Precision Medicine Initiative—all intended to have comprehensive data for 500,000 to 1 million individuals. Additional national-scale cohorts include deCODE Genetics (Iceland), the Danish National Biobank, and FinnGen (Finland). Private healthcare systems have also been mounting efforts to assemble biobank cohorts, including Vanderbilt University's BioVu Biorepository, Geisinger Health System's MyCode Community Health, Kaiser Permanente Research Bank, University of Pennsylvania's Penn Medicine BioBank, and Partners Healthcare Biobank. UK Biobank stands out as an exemplar in that it has made genetic and clinical phenotype data from ~500,000 participants available to any interested researchers. Upon the initial batches of large-scale data from UK Biobank becoming available in 2017, researchers instantly were able to add hundreds of thousands of people to their genetic studies, resulting in hundreds of scientific publications (https://www.ukbiobank.ac.uk/published-papers/). Indeed, the latest wave of published CVASs on CAD and other complex cardiovascular diseases all benefited from inclusion of data from UK Biobank, Million Veterans Program, or other biobank cohorts.

To date, exome or genome sequencing data from participants in large-scale biobank cohorts are relatively sparse. For many cohorts, plans are underway to add these data in the near future; for example, it is anticipated that exome sequencing of DNA samples from the ~500,000 individuals in UK Biobank will be completed and available to academic researchers by 2020. These data will greatly empower RVASs, perhaps taking them

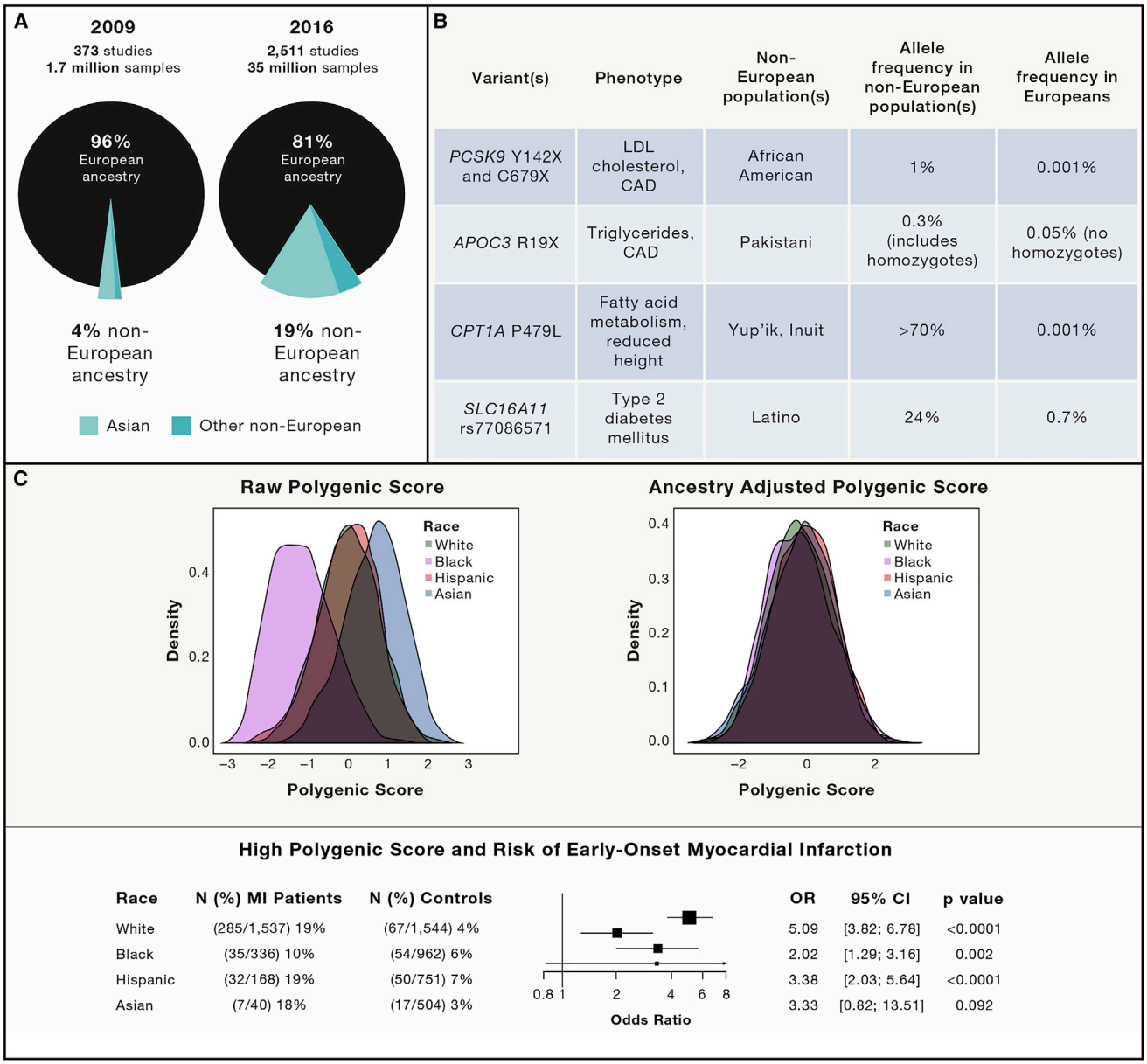

Figure 2. Differences in Genetic Studies and Findings Among World Populations

(A) The vast majority of genetic studies have been performed with individuals of European descent. Other world populations are severely underrepresented. Figure adapted from Popejoy and Fullerton, 2016.

(B) Examples of key disease-relevant genetic findings that were identified in non-European populations. They were not identified in European-based studies due to the rarity of the variants.

(C) The distributions and predictive power of polygenic scores calibrated in European-based studies vary widely among world populations. Figure adapted from Khera et al., 2018b.

to a scale that will finally result in a host of novel findings that directly implicate genes involved in CAD and other complex cardiovascular diseases.

The Need for Non-European Ancestry Genetic Studies

Despite the enormous progress in generating genome-wide genotyping data and, more lately, exome sequencing and exome chip data on very large numbers of individuals, these efforts have largely focused on people of European descent (Figure 2). In 2009, it was estimated that just 4% of the samples with genome-wide genotyping were from non-European ancestry; by 2014, the proportion had increased to 19%, an incremental improvement at best, with most of the increased proportion attributable to East Asian ancestry rather than peoples from Africa, South Asia, Latin America, South America, the Middle East, or the Pacific Islands (Popejoy and Fullerton, 2016). CVASs or RVASs performed with non-European groups might identify important

influences on disease that are not apparent in studies with Europeans, if the variants in question are rare or absent in Europeans (Skotte et al., 2017). Conversely, genotype-phenotype associations that are ascertained in Europeans might have less relevance in non-Europeans. Disparities in data can have direct clinical implications; in one example, African Americans with cardiomyopathy were informed that they had pathogenic variants in certain genes, due to the rarity of variants in the available collection of exome sequences at the time (almost exclusive from European ancestry); it later emerged that the variants were common in African American exomes and thus were benign (Manrai et al., 2016).

Unless special efforts are devoted to enriching for or even exclusively recruiting non-European participants, the rise of large-scale biobank cohorts will only exacerbate the disparity. With the exception of China Kadoorie Biobank, the largest biobanks are all dominated by European ancestry. Broader representation of all the world's populations is essential to fully understand the genetic basis of CAD and other complex cardiovascular diseases—especially since these diseases together now represent the leading cause of death worldwide, even in low-income or middle-income countries.

Variants to Function

Lessons from Forays into Biology

Despite more than a decade having passed since the first CVASs for CAD were reported, connecting the variants identified by genetic association studies to function remains an ongoing challenge. Reviewing the work that has unfolded for four of the top CVAS loci is instructive in this regard.

The highest-ranking CAD CVAS locus by strength of association lies on chromosome 9p21 (25% to 30% increase in risk per risk allele) (Samani et al., 2007; McPherson et al., 2007; Helgadottir et al., 2007; Myocardial Infarction Genetics Consortium, 2009). As defined by linkage disequilibrium in individuals of European descent, the locus is ~58 kb in size and harbors no protein-coding genes. The locus harbors some of the exons of a primate-specific long noncoding RNA termed *ANRIL* (antisense noncoding RNA in the INK4 locus); the nearest protein-coding genes, *CDKN2A* (cyclin-dependent kinase Inhibitor 2A) and *CDKN2B* (cyclin-dependent kinase Inhibitor 2B), express regulators of the cell cycle, are implicated in a variety of cancers, and lie > 100 kb from lead CAD-associated SNPs in the locus. Experimental modulation of *Cdkn2a* or *Cdkn2b* in mice yielded atherosclerotic phenotypes via macrophage or vascular smooth muscle cell activity (Kuo et al., 2011; Kojima et al., 2014). However, deletion of the orthologous ~70-kb locus in the mouse genome resulted in altered *Cdkn2a* and *Cdkn2b* expression but not in an atherosclerotic phenotype, raising doubt as to whether the human 9p21 CAD mechanism is conserved in mice (Visel et al., 2010). Long-range chromatin conformation capture studies in human vascular endothelial cells implicated enhancers within the ~58-kb locus as interacting with *CDKN2A*, *CDKN2B*, *MTAP* (S-methyl-5′-thioadenosine phosphorylase), and *IFNA21* (interferon alpha 21), with the interactions modified by exposure to interferon-γ (Harismendy et al., 2011). However, a role for interferon signaling in transcriptional regulation via genetic variation in the 9p21 locus was refuted by subsequent studies (Almontashiri et al., 2013; Erridge et al., 2013). Various SNPs in the 9p21 locus were reported to modulate local transcription factor binding (Almontashiri et al., 2015), and experimental modulation of *ANRIL* in various types of human cells altered apoptosis and proliferation phenotypes (Holdt et al., 2016), but the connections to CAD remain unclear. Most recently, deletion of the ~58-kb locus in induced pluripotent stem cells (iPSCs) derived from two individuals homozygous for the risk haplotype and an individual homozygous for the non-risk haplotype, followed by differentiation into vascular smooth muscle-like cells, suggested the risk haplotype to be related to adhesion, contraction, and proliferation phenotypes that were partly recapitulated by heterologous expression of *ANRIL* in non-risk haplotype cells (Lo Sardo et al., 2018). Despite all of this work over the past decade, the causal variant(s) and causal gene(s) remain to be conclusively defined, as does the relevant cell type(s) responsible for mediating the effect of the 9p21 locus on CAD.

Rather more progress has been achieved with the *SORT1* (sortilin 1) locus, although the work on this locus also demonstrates the challenges of using imperfect model systems to attempt to connect variants to function. Harbored in a locus on chromosome 1p13 strongly associated with both CAD (15% to 20% increase in risk per risk allele) and blood LDL cholesterol (10% to 15% increase in levels per risk allele), *SORT1* is a strong expression quantitative trait locus (eQTL) gene whose expression in liver or primary hepatocytes is increased up to > 10-fold in carriers of the non-risk allele of the lead SNP compared to homozygotes for the risk allele (Musunuru et al., 2010b; Wang et al., 2018). Of note, two genes lying closer to the lead SNP, *PSRC1* (proline/serine-rich coiled-coil 1) and *CELSR2* (cadherin, EGF LAG seven-pass G-type receptor 2), also have strong hepatic eQTLs. Identification of rs12740374 as the likely causal variant for the hepatic eQTLs entailed a series of experiments including luciferase expression assays, electrophoretic mobility shift assays, chromatin immunoprecipitation experiments, and overexpression/knockdown experiments demonstrating binding of the SNP non-risk allele sequence (but not the risk allele sequence) by C/EBP (CCAAT-enhancer-binding protein) transcription factors, resulting in transcriptional activation of *SORT1* (Musunuru et al., 2010b). Rigorous proof, i.e., genome editing of the SNP to demonstrate a direct effect on *SORT1* expression in hepatocytes, proved elusive. iPSC-derived hepatocyte-like cells failed to replicate the strong *SORT1* eQTL (Warren et al., 2017; Wang et al., 2018), perhaps due to the immaturity of the differentiated cells, making them poorly suited to interrogate the SNP's effect on *SORT1* expression. Attempted genome editing of the SNP directly in primary human hepatocytes was inefficient due to the fragility of the cells in culture, although small changes in *SORT1* were evident (Wang et al., 2018). The most definitive experiment to date entailed introduction of the entire human *SORT1* locus via bacterial artificial chromosome transgenesis into the mouse genome (locus-humanized mouse), followed by *in vivo* genome editing of the non-risk allele of rs12740374 in the mouse liver, resulting in substantial reduction of hepatic *SORT1* expression (Wang et al., 2018).

The evidence for *SORT1* as the causal gene and, in particular, for the mechanisms by which it influences CAD risk has proven to be murkier. Viral-mediated hepatic overexpression of *Sort1* in

various mouse models suggested an inverse relationship with very-low-density lipoprotein (VLDL) secretion and a direct relationship with LDL clearance from the bloodstream, consistent with the human CVAS data in which the non-risk allele conferred increased hepatic *SORT1* expression, decreased blood LDL cholesterol levels, and decreased risk of CAD (Musunuru et al., 2010b; Strong et al., 2012). (Similar experiments with *PSRC1* and *CELSR2* ruled them out as causal genes.) However, siRNA-mediated hepatic *Sort1* knockdown in some mouse models increased VLDL secretion (Ai et al., 2012), whereas in other mouse models it decreased VLDL secretion (Strong et al., 2012). Subsequent studies in independently generated whole-body *Sort1* knockout mouse models suggested that complete *Sort1* deficiency results in decreased VLDL secretion and LDL clearance, consistent with a more nuanced model of *SORT1* action in lipid metabolism (Kjolby et al., 2010; Strong et al., 2012; Gustafsen et al., 2014). It later emerged that *SORT1* has different effects at different expression levels and in different tissue types—whereas its role in hepatocytes appears to be solely atheroprotective at high *Sort1* expression levels, and it has opposing effects at low expression levels, it is atherogenic in other CAD-relevant tissues, promoting LDL uptake, foam cell formation, atherosclerosis, and vascular calcification when expressed in macrophages and smooth muscle cells (Mortensen et al., 2014; Patel et al., 2015; Goettsch et al., 2016). The balance of these various roles of *SORT1* on CAD remains to be fully defined, and in light of the complexity of its biology, *SORT1* has become a less attractive therapeutic target for CAD.

A third CAD-associated locus lies on chromosome 6p24 (10% to 15% increase in risk per risk allele), harboring at least two candidate causal genes, *PHACTR1* (phosphatase and actin regulator 1) and *EDN1* (endothelin-1). That the causal variant is rs9349379, which lies in an intron of *PHACTR1*, seems fairly unambiguous; fine mapping of the locus reveals it to be the SNP most strongly associated with CAD, with no other SNP in strong linkage disequilibrium with it (Beaudoin et al., 2015; Gupta et al., 2017). Endothelin-1 is a well-characterized protein with various roles in vascular biology, whereas any role for *PHACTR1* in tissues relevant to CAD has yet to be identified. *EDN1* has biological plausibility, but eQTL studies in vascular tissues have shown a strong eQTL for rs9349379 with *PHACTR1*, not *EDN1*; various experiments with iPSC-derived vascular-like cells genome-edited at rs9349379 yielded conflicting results with respect to *PHACTR1* versus *EDN1* expression changes (Beaudoin et al., 2015; Gupta et al., 2017; Wang and Musunuru, 2018). Further work will be needed to clarify which of the two genes, or whether both genes, are causal for the CAD association and in which tissue(s) the responsible mechanism operates, though a vascular tissue seems likely.

Finally, a CAD-associated locus on chromosome 15q25 (~10% increase in risk per risk allele) harbors the gene *ADAMTS7* (A disintegrin and metalloproteinase with thrombospondin motifs-7) (Reilly et al., 2011; Schunkert et al., 2011). A coding variant in *ADAMTS7* is associated with reduced protein maturation, proteinase activity, and migration of vascular smooth muscle cells *in vitro* (Pu et al., 2013). Support for *ADAMTS7* as the causal gene has come from knockout mice, which have reduced atherosclerosis and display enhanced endothelial cell repair and decreased cell proliferation in response to vascular injury (Kessler et al., 2015; Bauer et al., 2015). These observations suggest that inhibition of ADAMTS7 might have therapeutic value.

The travails with these four CAD loci are emblematic of human variant-to-function studies for which the causal variants are typically noncoding, meaning lack of conservation in standard model organisms such as mice; the identity and even the number of causal genes linked to a locus are uncertain; the specific tissues of relevance are uncertain; the human model systems (iPSCs, primary cells, transformed/immortalized aneuploid cultured cells) all have shortcomings; and the effect sizes associated with the variants are typically small. More sophisticated human model systems such as organoids and organs-on-a-chip hold promise but have not yet proven useful for the study of CAD loci. In contrast, coding variants have the virtue of directly marking the causal genes—which are much more likely to be conserved in structure and function in model organisms, and so are more amenable to productive study in those organisms—and typically do not cause distinct and even opposing effects in different tissues.

Increasing the Throughput of Variant-to-Function Studies

As described in the previous section, each individual novel locus identified in a CVAS or RVAS requires painstaking experimentation to fully define the variant-to-function relationship. Medium-to-high throughput functional assays could facilitate this work (Musunuru et al., 2018) (Figure 3). Such assays to rapidly ascertain the consequences of coding variants in genes linked to cardiovascular phenotypes have been established: *LDLR* in HeLa cells for CAD (Thormaehlen et al., 2015), *ANGPTL3* (angiopoietin-like 3) in mice for CAD (Stitziel et al., 2017), *TNNT2* (troponin T) in iPSC-derived cardiomyocyte-like cells for cardiomyopathy (Lv et al., 2018), and *KCNQ1* (potassium voltage-gated channel subfamily Q member 1) in Chinese hamster ovary cells for long QT syndrome (Vanoye et al., 2018). Saturation gene editing of the *BRCA1* gene was able to accurately classify thousands of coding variants in this cancer gene (Findlay et al., 2018), and a similar approach should be entertained for cardiovascular disease genes. Besides being useful for distinguishing whether patients' variants of uncertain significance in these genes are benign or pathogenic, these assays can improve the power of RVASs by allowing for ascertainment and inclusion of bona fide loss-of-function missense variants in the analyses (Thormaehlen et al., 2015; Stitziel et al., 2017).

However, these assays for medium-to-high throughput characterization of coding variants do not easily extend to the study of noncoding variants. Many if not most CVAS noncoding variants are believed to act via long-range transcriptional regulatory effects on genes, e.g., they lie in enhancer or silencer elements. As such, eQTL analyses might reveal causal genes modulated by causal variants—as described in the previous section, *SORT1* is a case in point. Linkage disequilibrium signifies that any of a number of variants in a locus could be causal for an eQTL. Computational or trans-omic approaches can help to prioritize certain variants as being more likely to be causal (e.g., the site of a variant is predicted to be a transcription factor binding site or bears epigenomic marks), but these approaches are

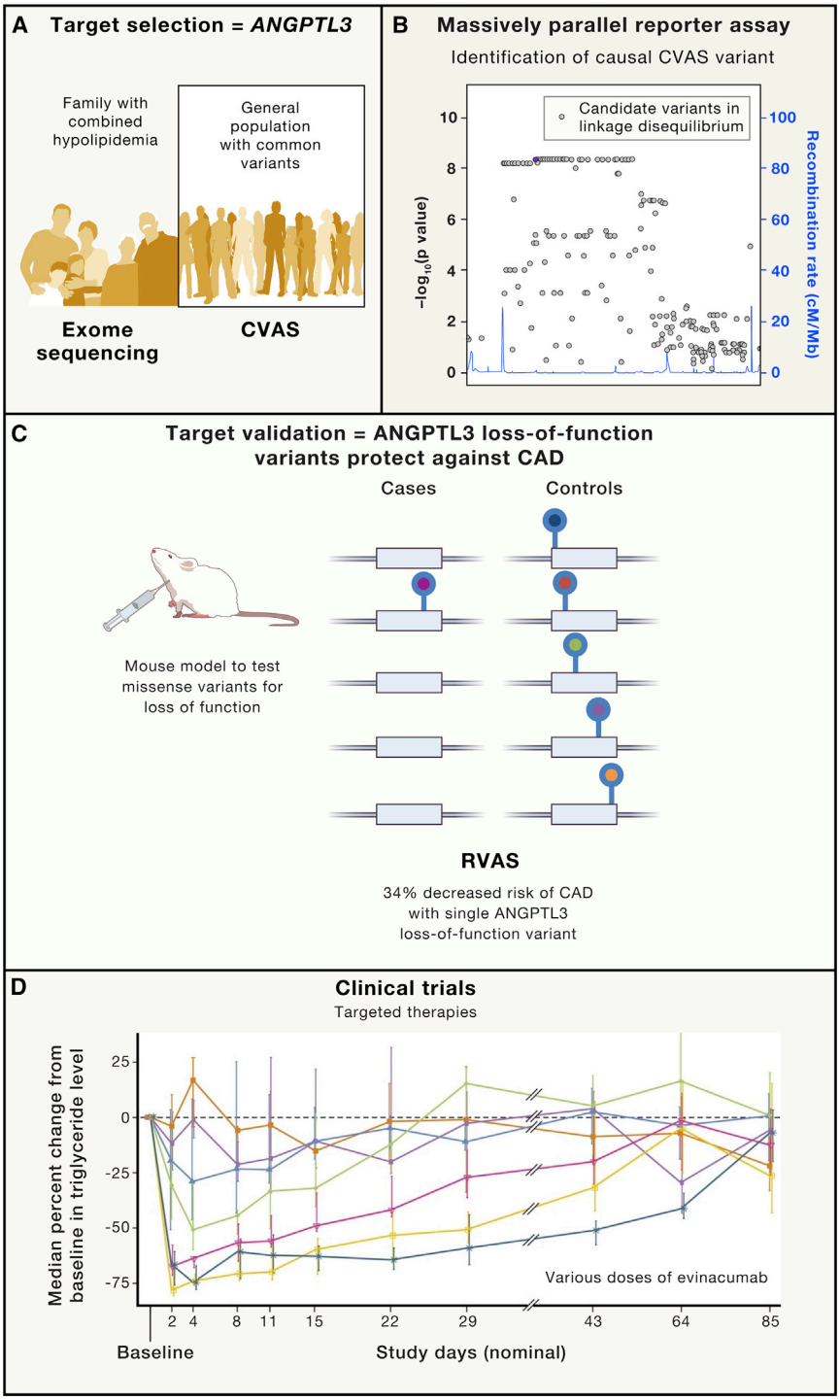

Figure 3. A Genetic Research Cycle, With *ANGPTL3* as an Example

(A) *ANGPTL3* was identified as a potential therapeutic target by parallel study designs (Musunuru et al., 2010a; Teslovich et al., 2010).

(B) A massively parallel reporter assay was used to screen the candidate causal variants in the *ANGPTL3* CVAS locus (Pashos et al., 2017).

(C) A RVAS study design, facilitated by ascertainment of loss-of-function missense variants in a mouse model, validated ANGPTL3 as a target for the prevention of CAD (Stitziel et al., 2017).

(D) Clinical trials with a monoclonal antibody against ANGPTL3 and an antisense oligonucleotide targeting *ANGPTL3* in the liver demonstrated reduced LDL cholesterol and triglyceride levels (Dewey et al., 2017; Graham et al., 2017). Figure adapted from Dewey et al. 2017.

hypothesis-generating only and require validation by functional studies (Musunuru et al., 2018). One example of a productive functional approach is the massively parallel reporter assay (MPRA). MPRAs generate high-complexity pools of reporter constructs; each candidate regulatory element with each variant allele is linked to a synthetic reporter gene carrying an identifying barcode (Melnikov et al., 2012; Patwardhan et al., 2012). The reporter construct pools are introduced into cells *in vitro* or *in vivo*, and the relative transcriptional activities of the individual elements or variants are measured by sequencing the transcribed reporter mRNAs and counting their specific barcodes. Thus, MPRAs can simultaneously profile hundreds or even thousands of variants.

In one application, analysis of population cohorts of undifferentiated iPSCs and iPSC-derived hepatocyte-like cells yielded eQTLs that colocalized with blood lipid CVAS loci. MPRAs were used to interrogate hundreds of candidate variants across three loci for regulatory activity. Three high-priority candidate variants, one for each locus (one of which harbored *ANGPTL3*), were established to be causal for the eQTLs via genome editing in iPSCs and iPSC-derived hepatocyte-like cells; this work further served to identify likely causal genes that were then validated by experiments in mouse models (Pashos et al., 2017).

Translation to the Clinic

Fruit Salad or Smoothies?

For a complex disease such as CAD, a key question is whether in any given individual the disease is driven by a single predominant pathway or whether it comprises a quantitative blend of causal pathways (Figure 4). In patients with familial hypercholesterolemia, a monogenic disorder caused by *LDLR*, *APOB*, or *PCSK9* mutations (Lehrman et al., 1985; Soria et al., 1989; Abifadel et al., 2003), early-onset CAD is unambiguously driven by elevated LDL cholesterol levels. Appropriately, clinical management for these patients is largely centered on reduction of LDL cholesterol. In another example, a family in which multiple

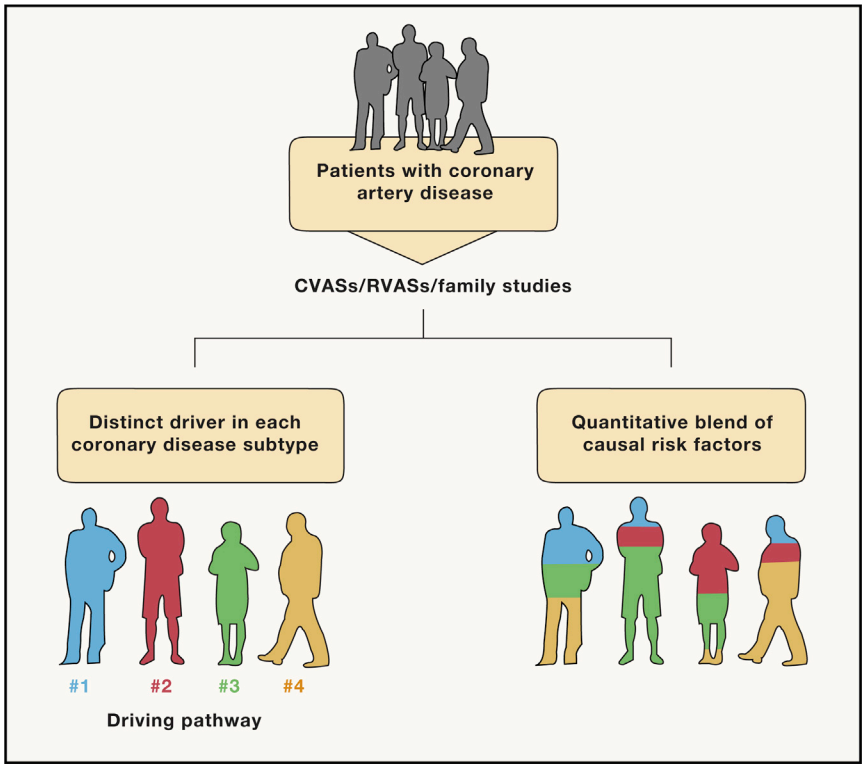

Figure 4. Competing Models of Common Complex Disease

In the "fruit salad" model (left), in each patient, disease is driven by a distinct, single pathway, and a key goal is to identify these pathways in order to categorize disease into subtypes. In the "smoothie" model (right), in each patient, disease is driven by a quantitative blend of causal driver pathways. For coronary artery disease, the accumulated evidence supports a smoothie model rather than a fruit salad model of disease. Figure adapted from Khera et al., 2017.

members experienced early-onset CAD harbored rare variants in *GUCY1A3* (guanylate cyclase soluble subunit alpha-3), which is linked to nitric oxide signaling (Erdmann et al., 2013). In principle, therapy directed at this signaling pathway would be the most effective treatment strategy for these family members. One paradigm for CAD management, then, would be based on a "fruit salad" model of disease in which CAD represents a collection of subtypes, where in any given patient there is a single distinct driver, and treatment would be based on that driver in each individual patient.

An alternative paradigm would be based on a "smoothie" model in which for any given patient with CAD, the disease is driven by a blend of different causal pathways. In this paradigm, a primary value of human genetics is to clarify the distinct driver pathways. While LDL cholesterol might not be the dominant factor in most cases of CAD, it is nonetheless a contributor and accounts for the broadly protective effects of statin therapy or naturally occurring mutations that reduce LDL cholesterol levels. One implication of this model is that on a population-wide basis, universally treating a few broadly relevant causal factors might be more productive than an attempt to identify specific drivers and to hyper-personalize treatment regimens for individual patients. When considered in aggregate, the evidence accumulated for CAD favors the smoothie model—a blend of causal pathways—rather than the fruit salad model of a distinct driver in each patient. In any given patient, CAD seems to be a blend of several causal pathways including low-density lipoproteins, triglyceride-rich lipoproteins, lipoprotein(a), blood pressure, inflammation, and clotting, among others.

Polygenic Scores: Ready for Prime Time?

Even if the causal variants and genes underlying CVAS associations are unknown, knowledge of just the lead SNPs for the associations is still valuable in that it allows for calculation of polygenic scores to improve risk prediction for complex cardiovascular diseases. These scores incorporate a handful of SNPs to as many as millions of SNPs, i.e., genome-wide polygenic scores, by adding the number of risk alleles of each variant weighted by the effect size of the variant's association with the disease. While polygenic scores have been studied for more than a decade, the availability of larger CVAS datasets and large-scale biobank cohorts are making it possible to better construct, validate, and test the scores (Kathiresan et al., 2008; Ripatti et al., 2010; Tada et al., 2016). For CAD, polygenic scores ranging from hundreds of SNPs to 6.6 million SNPs and validated/tested with hundreds of thousands of UK Biobank participants have demonstrated that the top ~5% of the general population has equivalent CAD risk to individuals with familial hypercholesterolemia, despite having relatively normal cholesterol levels; furthermore, a polygenic score improves risk prediction to a greater degree than many single traditional risk factors (Khera et al., 2018a; Thériault et al., 2018; Inouye et al., 2018; Levin et al., 2018).

Among patients with early-onset myocardial infarction, a much larger proportion have very high polygenic scores compared to familial hypercholesterolemia mutations (Khera et al., 2018b; Thériault et al., 2018). For every 100 patients with early-onset myocardial infarction (defined as a heart attack at age $\leq$ 55 years of age), roughly two harbor a rare coding mutation in a Mendelian familial hypercholesterolemia gene (*LDLR*, *PCSK9*, or *APOB*) whereas about 17 have a high genome-wide polygenic score; either monogenic familial hypercholesterolemia or a high genome-wide polygenic score confers a similar degree of relative risk (roughly 4- to 5-fold) (Khera et al., 2018a; Khera et al., 2018b) (Figure 5). This finding suggests that assessment for familial hypercholesterolemia mutations and assessment of genome-wide polygenic scores would offer complementary information useful for risk prediction, especially when combined with traditional risk factors for CAD. Conveniently, both types

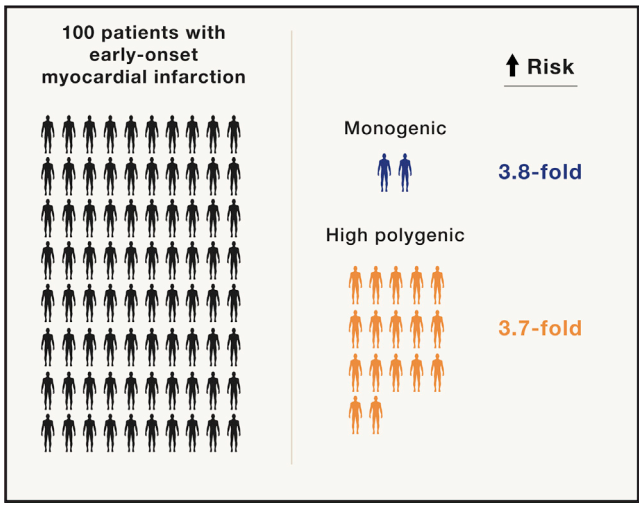

Figure 5. Relative Contributions of Monogenic Risk and Polygenic Risk for Early-Onset Myocardial Infarction

For every 100 patients with early-onset myocardial infarction, roughly two harbor a rare coding mutation in a monogenic familial hypercholesteremia gene, whereas about 17 have a high genome-wide polygenic score (Khera et al., 2018a; Khera et al., 2018b). The increases in risk for early-onset myocardial infarction conferred by rare coding mutations versus a high polygenic score are equivalent.

of information can be obtained from a single test, namely whole-genome sequencing.

Although further work is needed to assess whether polygenic scores will be useful in the primary prevention setting, the fact that the scores can be calculated as early as birth, paired with observations that both statin therapy and healthy lifestyle can substantially reduce CAD risk in patients with high polygenic scores (i.e., high genetic risk) (Mega et al., 2015; Khera et al., 2016), suggests that routine, early risk assessment and intervention could be warranted. At the same time, it must be noted that existing polygenic scores were calculated with data from individuals of European descent and so are less relevant in non-Europeans (Figure 2). Avoiding disparity with respect to polygenic scores is yet another reason to aggressively pursue genetic association studies across all world populations.

Lessons from Mendelian Randomization

Mendelian randomization uses DNA variants to assess whether an epidemiologic association between a risk factor and disease reflects a causal influence of the former on the latter. It parallels a prospective randomized controlled trial in that randomization occurs during the genesis of gametes during meiosis—a 50% chance of either of the two alleles at the site of a DNA variant ending up in a gamete and in the resulting zygote and then human being, impervious to confounding and reverse causation. If the DNA variant in question directly influences an intermediate risk factor, and the risk factor is itself causal for a disease, then the DNA variant should associate with the disease to the extent predicted by (1) the size of the effect of the DNA variant on the risk factor and (2) the size of the effect of the risk factor on the disease. While there are shortcomings to Mendelian randomization that need to be considered—most notably, the possibility of the DNA variant affecting more than one intermediate risk factor that might influence the disease in question (termed pleiotropy)—it has the potential to reliably identify causal genes and risk factors that might serve as novel therapeutic targets.

Mendelian randomization has been applied to single genes as well as sets of genes that influences blood lipid traits to explore their relationships to CAD. Variants in *LDLR*, *APOB*, and *PCSK9* have been well established to modulate blood LDL cholesterol levels via effects on cellular LDL uptake, as have their causal relationships to premature CAD in familial hypercholesterolemia patients (Lehrman et al., 1985; Soria et al., 1989; Abifadel et al., 2003). Variants in genes that modulate blood LDL cholesterol by other mechanisms, including *APOE* (apolipoprotein E), *HMGCR* (HMG-CoA reductase, targeted by the statin drugs), *LPA* [apolipoprotein(a), a component of the LDL-like Lp(a) particle], and *NPC1L1* (Niemann-Pick C1-like 1, targeted by the drug ezetimibe) have also been definitively linked to CAD (Voight et al., 2012; Clarke et al., 2009; Kamstrup et al., 2009; Myocardial Infarction Genetics Consortium Investigators, 2014; Ference et al., 2015). A Mendelian randomization analysis using a genetic score comprising 13 SNPs in loci primarily associated with LDL cholesterol found that a 1-standard deviation (SD) increase in LDL cholesterol (~35 mg/dL increase) because of genetic score conferred a 113% increase in CAD risk, even greater than the 54% increase in CAD risk predicted by observational epidemiological studies (Voight et al., 2012). This argues that any mechanism that decreases LDL cholesterol should reduce the risk of CAD.

In contrast, studies with *LIPG* (endothelial lipase) and *ABCA1* (ATP-binding cassette, sub-family A, member 1), which act on high-density lipoprotein (HDL) cholesterol but not on other lipid traits, showed no association with CAD (Voight et al., 2012; Frikke-Schmidt, 2010). A Mendelian randomization analysis using a genetic score comprising 14 SNPs in loci primarily associated with HDL cholesterol found that a 1-SD increase in HDL cholesterol (~15 mg/dL increase) because of genetic score conferred a non-significant 7% decrease in CAD risk, in contrast to the 38% decrease in CAD risk predicted by observational epidemiological studies (Voight et al., 2012). This suggests that most if not all mechanisms that increase HDL cholesterol do not reduce the risk of CAD. This has been borne out by the failure of all HDL-cholesterol-raising drugs tested in large randomized clinical trials to improve cardiovascular outcomes, with the exception of anacetrapib, which showed a modest reduction of coronary events that might be entirely to due to the drug's LDL-cholesterol-reducing effect (HPS3/TIMI55–REVEAL Collaborative Group et al., 2017). Similarly, Mendelian randomization studies of variants linked to inflammatory biomarkers have failed to show protective effects, as have drugs intended to target inflammation. One notable exception is *IL6R* (interleukin-6 receptor), with a strong association with CAD, and clinical trial results are consistent with this observation—canakinumab, which targets the interleukin-1β/interleukin-6 pathway, improved cardiovascular outcomes in a randomized clinical trial (Ridker et al., 2017).

Variants linked to at least 7 genes that influence blood triglyceride-rich lipoprotein levels have been found to be associated with CAD: *LPL*, *APOC3*, *APOA5*, *ANGPTL4*, *ASGR1* (asialoglycoprotein receptor 1), *ANGPTL3*, and *TRIB1* (tribbles homolog 1) (Triglyceride Coronary Disease Genetics Consortium and

Emerging Risk Factors Collaboration, 2010; Voight et al., 2012; TG and HDL Working Group of the Exome Sequencing Project, National Heart, Lung, and Blood Institute, 2014; Jørgensen et al., 2014; Do et al., 2015; Myocardial Infarction Genetics Consortium Investigators, 2016; Dewey et al., 2016; Nioi et al., 2016; Stitziel et al., 2017; Dewey et al., 2017). Furthermore, a Mendelian randomization analysis with a large set of triglyceride-associated SNPs that accounted for the pleiotropy commonly seen with these SNPs affirmed triglyceride-rich lipoproteins as a causal risk factor for CAD (Do et al., 2013). In this light, the substantial reduction of cardiovascular events by icosapent ethyl (purified omega-3 fatty acid with a prominent triglyceride-lowering effect) in a randomized clinical trial is intriguing (Bhatt et al., 2019). It is also noteworthy that almost all of the aforementioned genes influence the hydrolysis of triglycerides in triglyceride-rich lipoproteins (VLDL particles and chylomicrons) by lipoprotein lipase: *LPL* encodes the lipase; *APOC3*, *ANGPTL4*, and *ANGPTL3* encode inhibitors of the lipase; and *APOA5* encodes an activator. Thus, the lipoprotein lipase pathway represents a compelling therapeutic opportunity.

Human Knockouts and Phenome-wide Association Studies

Besides being useful for distinguishing causal risk factors for disease from non-causal risk factors, Mendelian randomization serves to identify individual genes as potential therapeutic targets. Further analyses can help to assure efficacy and, especially, safety before drug development programs begin in earnest.

Perhaps the most compelling evidence of safety of inhibition of a gene target is the existence of healthy individuals who entirely lack gene activity, i.e., human knockouts. *PCSK9* has become one of the most celebrated examples of genomic medicine—discovery in 2003 (Abifadel et al., 2003), subsequent finding of a link between loss-of-function variants and reduced CAD risk (Cohen et al., 2006), two monoclonal antibodies targeting the gene product approved for clinical use in 2015, and large prospective randomized clinical trials shortly thereafter pointing to improved cardiovascular outcomes and even a possible mortality benefit (Sabatine et al., 2017; Schwartz et al., 2018). Early in the drug development process, strong reassurance was provided by the identification of individuals with two loss-of-function *PCSK9* alleles who suffered from no apparent adverse health consequences (Zhao et al., 2006; Hooper et al., 2007).

Additional genes have been observed in the knockout state in humans, with implications for drug development. Four siblings with familial combined hypolipidemia (low LDL cholesterol, HDL cholesterol, and triglycerides) and no health issues were compound heterozygotes for nonsense mutations in *ANGPTL3*, ascertained by exome sequencing (Musunuru et al., 2010a) (Figure 3). In a population cohort of ~10,000 exome-sequenced individuals with a high rate of consanguinity, an entire family (mother, father, 9 children) with homozygosity for a nonsense mutation in *APOC3* was identified. They were observed to be healthy and to have reduced blood triglyceride levels, increased HDL cholesterol levels, and marked blunting of the rise in blood triglycerides normally observed after an oral fat load (Saleheen et al., 2017). As large-scale biobank cohorts undergo exome sequencing, we can expect additional human knockouts for genes relevant to complex cardiovascular diseases to emerge.

The growth of large-scale biobank cohorts with genetic data connected to electronic health record data has enabled a study design termed the phenome-wide association study (PheWAS), in which a genetic variant of interest is tested for associations with hundreds of clinical phenotypes spanning all the organ systems (Denny et al., 2010) (Figure 6). With the availability of very large cohorts such as UK Biobank, it is feasible to perform a PheWAS to assess for desirable clinical outcomes as well as undesired adverse outcomes linked to loss-of-function variants of a given gene—the ultimate "experiment of nature." In a PheWAS with the common variant rs11591147 in *PCSK9* using data from >300,000 individuals in UK Biobank, loss of gene function was correlated with a protective effect on hyperlipidemia and coronary heart disease (already well-established) and ischemic stroke (not established). It was also associated with increased risk of type 2 diabetes mellitus (previously reported) but not with any other adverse phenotypes, including those previously implicated with other lipid-modifying medications: cataracts, heart failure, atrial fibrillation, and cognitive dysfunction (Rao et al., 2018). Notably, both of the large clinical outcomes trials with PCSK9 monoclonal antibodies showed a protective effect against ischemic stroke, although no effect on new-onset diabetes (Sabatine et al., 2017; Schwartz et al., 2018). A PheWAS performed with the common variant rs9349379, the lead SNP in the *PHACTR1/EDN1* CVAS locus, using data from UK Biobank and published CVAS summary results, showed that the risk allele for CAD also associated with several other vascular diseases including fibromuscular dysplasia, hypertension, migraine headaches, cervical artery dissection, and coronary artery calcification (Gupta et al., 2017).

Genetics-guided Therapies

Favorable outcomes of "experiments of nature" can nominate genes as therapeutic targets; as described in the previous section, *PCSK9*, *ANGPTL3*, and *APOC3* are three such genes (Figure 3). *PCSK9* has already provided fertile ground for drug development. Two monoclonal antibodies targeting PCSK9 are approved for clinical use and have been validated in clinical trials (Sabatine et al., 2017; Schwartz et al., 2018). An siRNA inhibitor of hepatic *PCSK9* expression is in clinical trials (Ray et al., 2017), and a number of other agents, including traditional small molecule drugs, are under development. A monoclonal antibody targeting ANGPTL3 and an antisense oligonucleotide targeting hepatic *ANGPTL3* expression are in clinical trials, displaying substantial reductions of blood LDL cholesterol and triglyceride levels (Dewey et al., 2017; Graham et al., 2017). An antisense oligonucleotide targeting hepatic *APOC3* expression has been in clinical trials, although it was not approved by the US Food and Drug Administration in light of apparent safety concerns, and a monoclonal antibody targeting *APOC3* is in development (Khetarpal et al., 2017).

Gene editing in adult humans—so-called somatic gene editing—has the potential to offer once-and-done treatments to permanently modify one's risk of complex cardiovascular diseases. Such a strategy is attractive in light of observations that less than half of patients adhered to statin therapy prescribed during the year following a myocardial infarction, even when provided at no cost to the patient (Choudhry et al., 2011), as well as the high costs of PCSK9 monoclonal antibodies that are

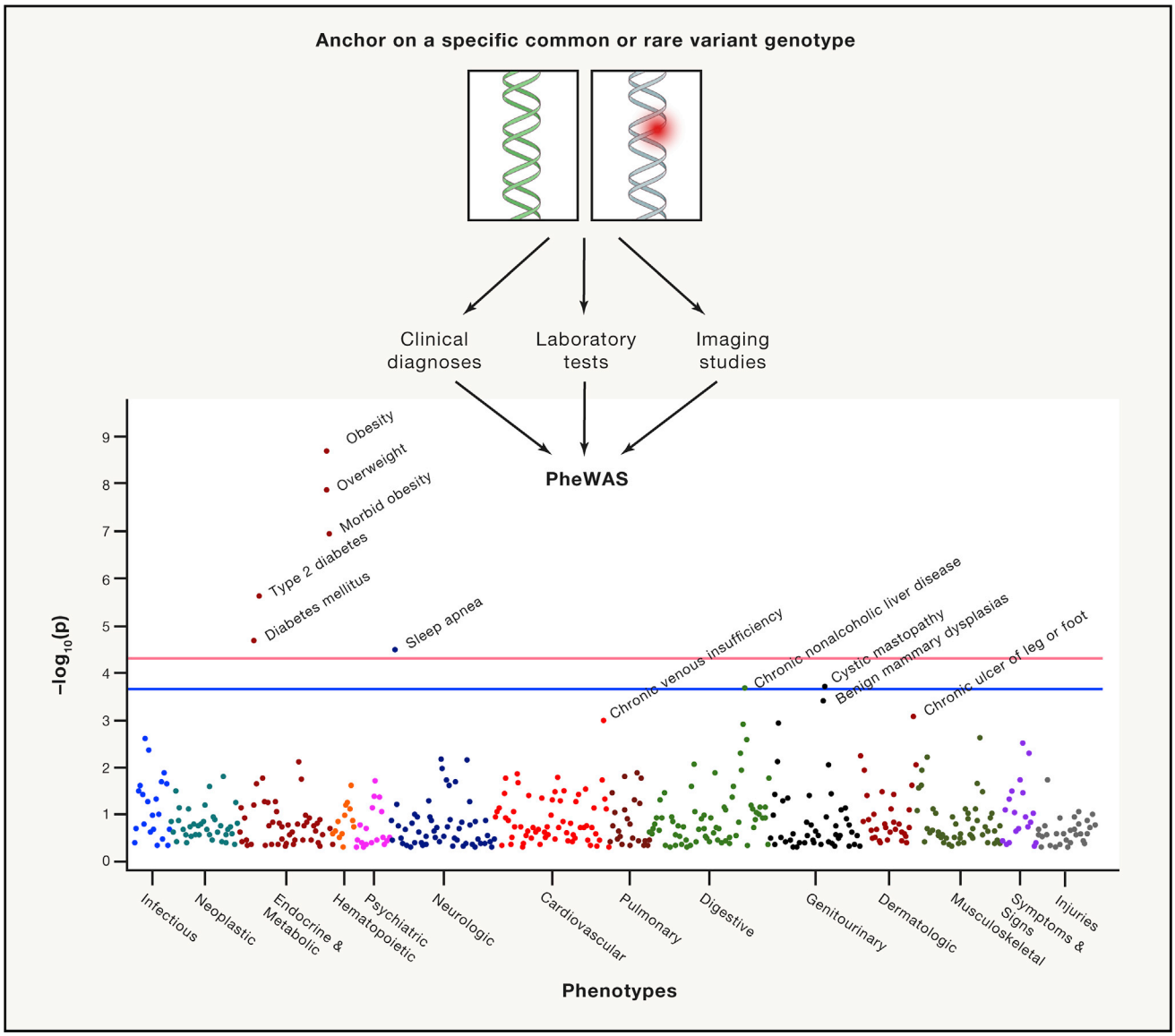

Figure 6. The Utility of the Large-Scale Biobank
A genetic variant ascertained by a CVAS or RVAS is used to interrogate the rich phenotype database available in a biobank linked to electronic health records. This enables an unbiased phenome-wide association study, or PheWAS, to identify novel genotype-phenotype associations.

taken every few weeks, for the lifetime, making it difficult for providers to obtain authorization from insurers for coverage of prescriptions. When physicians and scientists were polled about the gene-editing strategy, the majority were supportive and indicated they would take a therapy themselves (Musunuru et al., 2017).

Preclinical studies have established the viability of gene-editing approaches. Standard CRISPR-Cas9 gene editing of *PCSK9* in the liver proved effective at substantially reducing blood PCSK9 levels with a concomitant decrease in blood cholesterol levels, in wild-type mice and liver-humanized mice (mouse hepatocytes replaced with transplanted primary human hepatocytes) (Ding et al., 2014; Wang et al., 2016). The newer form of gene editing termed base editing, with which nonsense mutations can be specifically introduced into genes with high efficiency, was effective at targeting either *PCSK9* or *ANGPTL3* in the liver in mice; in hypercholesterolemic mice, base editing of *ANGPTL3* reduced both LDL cholesterol levels and triglyceride levels by > 50% (Chadwick et al., 2017; Chadwick et al., 2018). Establishing the safety of each gene-editing approach will be paramount before clinical use; initial preclinical data with *PCSK9* are encouraging (Akcakaya et al., 2018).

An alternative approach centers on long-term targeting of the protein products of genes relevant to CAD such as *APOB* and *PCSK9*. Immunological vaccines against epitopes in the apoB-100 protein have been demonstrated to elicit both antibodies and T cell-mediated responses and to reduce atherosclerotic plaques in mice (Tse et al., 2013; Kimura et al.,

2017). Vaccines against epitopes in the PCSK9 protein resulted in the production of antibodies and reduction of blood lipid levels in both mice and monkeys and a reduction in atherosclerosis in mice (Crossey et al., 2015; Landlinger et al., 2017). Similar vaccines targeting ANGPTL3 and apoC-III are being actively investigated. Like gene editing, immunological vaccines offer the possibility of once-and-done treatments that provide enduring and possibly lifelong protection against CAD.

Future Outlook

While it is challenging to predict what will happen just 5 to 10 years into the future—keeping in mind that the use of CRISPR-Cas9 in mammalian cells was first reported just 6 years ago—we foresee the following possibilities. First, further investigation of CVAS loci and increasingly larger RVASs will better define non-lipid determinants of CAD. Second, treatments aimed at causal factors other than LDL cholesterol, including triglyceride-rich lipoproteins, lipoprotein(a), and specific inflammatory mechanisms such as the interleukin-1β/interleukin-6 pathway, will be further explored and validated for use in patients, especially those at high risk for future myocardial infarctions despite being on aggressive LDL cholesterol-lowering therapy. Particularly intriguing is recent research demonstrating that somatic mutations in hematopoietic stem cells—a process termed clonal hematopoiesis—are associated with risk for coronary heart disease and this risk may be mediated by heightened inflammation involving the interleukin-1β/interleukin-6 pathway (Natarajan et al., 2018). Finally, gene editing and immunological vaccines will provide the means to bring to completion a virtuous genetics research cycle in which naturally occurring disease-protective mutations are identified in the population, and the same mechanisms are deployed in patients who are ascertained to be at high genetic risk for CAD early in their lives. These advances will be needed if we are to eliminate CAD as the leading cause of death worldwide.

DECLARATION OF INTERESTS

S.K. is a founder of Maze Therapeutics, Verve Therapeutics, and San Therapeutics. He holds equity in Catabasis and San Therapeutics. He is a member of the scientific advisory boards for Regeneron Genetics Center and Corvidia Therapeutics. He has served as a consultant for Acceleron, Eli Lilly, Novartis, Merck, Novo Nordisk, Novo Ventures, Ionis, Alnylam, Aegerion, Haug Partners, Noble Insights, Leerink Partners, Bayer Healthcare, Illumina, Color Genomics, MedGenome, Quest, and Medscape. S.K. reports patents related to a method of Identifying and treating a person having a predisposition to or afflicted with a cardiometabolic disease (20180010185) and a genetics risk predictor (20190017119).

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