JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY
© 2017 BY THE AMERICAN COLLEGE OF CARDIOLOGY FOUNDATION
PUBLISHED BY ELSEVIER

VOL. 69, NO. 16, 2017
ISSN 0735-1097/$36.00
http://dx.doi.org/10.1016/j.jacc.2017.02.030

ANGPTL3 Deficiency and Protection Against Coronary Artery Disease

Nathan O. Stitziel, MD, PhD,[a] Amit V. Khera, MD,[b,c,d] Xiao Wang, PhD,[e] Andrew J. Bierhals, MD, MPH,[f] A. Christina Vourakis, BA,[g] Alexandra E. Sperry, BA,[g] Pradeep Natarajan, MD,[b,c,d] Derek Klarin, MD,[b,c,h] Connor A. Emdin, DPhil,[b,c,d] Seyedeh M. Zekavat, BSc,[d] Akihiro Nomura, MD,[b,c,d] Jeanette Erdmann, PhD,[i,j] Heribert Schunkert, MD,[k,l] Nilesh J. Samani, MD,[m,n] William E. Kraus, MD,[o] Svati H. Shah, MD, MPH,[o] Bing Yu, PhD,[p,q] Eric Boerwinkle, PhD,[p,q] Daniel J. Rader, MD,[e,r] Namrata Gupta, PhD,[d] Philippe M. Frossard, PhD,[s] Asif Rasheed, MBBS,[s] John Danesh, DPhil,[t,u,v] Eric S. Lander, PhD,[d] Stacey Gabriel, PhD,[d] Danish Saleheen, MBBS, PhD,[s,w] Kiran Musunuru, MD, PhD, MPH,[e] Sekar Kathiresan, MD,[b,c,d] for the PROMIS and Myocardial Infarction Genetics Consortium Investigators

ABSTRACT

BACKGROUND Familial combined hypolipidemia, a Mendelian condition characterized by substantial reductions in all 3 major lipid fractions, is caused by mutations that inactivate the gene angiopoietin-like 3 (*ANGPTL3*). Whether ANGPTL3 deficiency reduces risk of coronary artery disease (CAD) is unknown.

OBJECTIVES The study goal was to leverage 3 distinct lines of evidence—a family that included individuals with complete (compound heterozygote) ANGPTL3 deficiency, a population based-study of humans with partial (heterozygote) ANGPTL3 deficiency, and biomarker levels in patients with myocardial infarction (MI)—to test whether ANGPTL3 deficiency is associated with lower risk for CAD.

METHODS We assessed coronary atherosclerotic burden in 3 individuals with complete ANGPTL3 deficiency and 3 wild-type first-degree relatives using computed tomography angiography. In the population, *ANGPTL3* loss-of-function (LOF) mutations were ascertained in up to 21,980 people with CAD and 158,200 control subjects. LOF mutations were defined as nonsense, frameshift, and splice-site variants, along with missense variants resulting in <25% of wild-type ANGPTL3 activity in a mouse model. In a biomarker study, circulating ANGPTL3 concentration was measured in 1,493 people who presented with MI and 3,232 control subjects.

RESULTS The 3 individuals with complete ANGPTL3 deficiency showed no evidence of coronary atherosclerotic plaque. *ANGPTL3* gene sequencing demonstrated that approximately 1 in 309 people was a heterozygous carrier for an LOF mutation. Compared with those without mutation, heterozygous carriers of *ANGPTL3* LOF mutations demonstrated a 17% reduction in circulating triglycerides and a 12% reduction in low-density lipoprotein cholesterol. Carrier status was associated with a 34% reduction in odds of CAD (odds ratio: 0.66; 95% confidence interval: 0.44 to 0.98; $p = 0.04$). Individuals in the lowest tertile of circulating ANGPTL3 concentrations, compared with the highest, had reduced odds of MI (adjusted odds ratio: 0.65; 95% confidence interval: 0.55 to 0.77; $p < 0.001$).

CONCLUSIONS ANGPTL3 deficiency is associated with protection from CAD. (J Am Coll Cardiol 2017;69:2054–63)

Listen to this manuscript's audio summary by *JACC* Editor-in-Chief Dr. Valentin Fuster.

From the [a]Cardiovascular Division, Department of Medicine, Department of Genetics, and McDonnell Genome Institute, Washington University School of Medicine, St. Louis, Missouri; [b]Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; [c]Cardiovascular Research Center and Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; [d]Program in Medical and Population Genetics, Broad Institute, Cambridge, Massachusetts; [e]Cardiovascular Institute, Division of Cardiovascular Medicine, Department of Medicine, and Department of Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; [f]Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri; [g]Harvard College, Harvard University, Cambridge, Massachusetts; [h]Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; [i]Institute for Integrative and Experimental Genomics, University of Lübeck, Lübeck, Germany; [j]DZHK (German Centre for Cardiovascular Research), partner site Hamburg/Lübeck/Kiel, Lübeck, Germany; [k]Deutsches Herzzentrum München, Technische

Loss-of-function (LOF) mutations leading to complete deficiency of angiopoietin-like 3 (ANGPTL3) cause familial combined hypolipidemia, a Mendelian disorder characterized by low circulating concentrations of low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides (TG) (1). ANGPTL3 is a hepatically secreted protein first identified via positional cloning of a hypolipidemic mouse strain (2). ANGPTL3 acts as a potent inhibitor of lipoprotein lipase (LPL), the primary mechanism by which triglyceride-rich lipoproteins are cleared from the circulation (3). In addition, ANGPTL3 is an endogenous inhibitor of endothelial lipase (EL) (4). Loss of ANGPTL3 function appears to decrease triglyceride-rich lipoprotein and HDL cholesterol concentrations through loss of LPL and EL inhibition, respectively. The mechanism by which ANGPTL3 regulates LDL cholesterol metabolism remains unclear (5). The seemingly favorable implications of ANGPTL3 deficiency in reducing TG concentrations and circulating LDL cholesterol catalyzed drug development programs aiming to inhibit ANGPTL3 with either a monoclonal antibody (5) or an antisense oligonucleotide (6).

SEE PAGE 2064

Although decreased atherosclerotic burden was observed in *Angptl3* knockout mice (7), the relationship of ANGPTL3 deficiency to coronary artery disease (CAD) in humans remains uncertain. Individuals who carry LOF mutations in *ANGPTL3* have lifelong reductions of circulating ANGPTL3 (8); as such, the clinical phenotypes of these individuals may inform the potential therapeutic efficacy of pharmacological ANGPTL3 inhibition.

Here, we tested the hypothesis that ANGPTL3 deficiency reduces risk of CAD in humans. We compared coronary atherosclerotic plaque burden in individuals who had complete ANGPTL3 deficiency (caused by compound heterozygous LOF

ABBREVIATIONS AND ACRONYMS

ANGPTL3 = angiopoietin-like 3
AU = Agatston units
CAD = coronary artery disease
CCTA = coronary computed tomography angiography
CI = confidence interval
EL = endothelial lipase
HDL = high-density lipoprotein
LDL = low-density lipoprotein
LOF = loss of function
LPL = lipoprotein lipase
MI = myocardial infarction
OR = odds ratio
TG = triglyceride

Universität München, Munich, Germany; [l]DZHK (German Centre for Cardiovascular Research), partner site Munich Heart Alliance, Munich, Germany; [m]Department of Cardiovascular Sciences, University of Leicester, Leicester, United Kingdom; [n]NIHR Leicester Cardiovascular Biomedical Research Unit, Glenfield Hospital, Leicester, United Kingdom; [o]Duke Molecular Physiology Institute and the Division of Cardiology, Department of Medicine, Duke University, Durham, North Carolina; [p]Human Genetics Center, The University of Texas Health Science Center at Houston, Houston, Texas; [q]Human Genome Sequencing Center, Baylor College of Medicine, Houston, Texas; [r]Institute of Translational Medicine and Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; [s]Center for Non-Communicable Diseases, Karachi, Pakistan; [t]Cardiovascular Epidemiology Unit, Department of Public Health and Primary Care, University of Cambridge, Cambridge, United Kingdom; [u]Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom; [v]National Institute of Health Research Blood and Transplant Research Unit in Donor Health and Genomics, University of Cambridge, Cambridge, United Kingdom; and the [w]Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania. This study was supported by grants from the National Heart, Lung, and Blood Institute (NHLBI) (R01HL131961 and K08HL114642 to Dr. Stitziel; R01 HL118744 to Dr. Musunuru; and R01 HL127564 and R21 HL120781 to Dr. Kathiresan); the National Human Genome Research Institute (U54HG003067 to Dr. Gabriel and Dr. Lander, UM1HG008895 to Dr. Kathiresan Dr. Gabriel, and Dr. Lander, and UM1HG008853 to Dr. Stitziel); the Barnes-Jewish Hospital Foundation (Dr. Stitziel); the Fannie Cox Prize for Excellence in Science Teaching, Harvard University (Dr. Musunuru); and the MGH Research Scholar Award (Dr. Kathiresan). PROMIS fieldwork has been supported through grants awarded to Dr. Saleheen, Dr. Danesh, and Dr. Frossard. Biomarker assays in PROMIS have been funded through grants awarded by the NHLBI (RC2HL101834 and RC1TW008485) and Fogarty International (RC1TW008485). This work was funded by the National Institutes of Health (NIH), which had no involvement in the design and conduct of the study, in the collection, analysis, and interpretation of the data, or in the preparation, review, and approval of the manuscript. Dr. Stitziel has received a research grant from AstraZeneca; and consulting fees from Aegerion Pharmaceuticals. Dr. Khera is supported by an American College of Cardiology Foundation/Merck Cardiovascular Research Fellowship, a John S. Ladue Memorial Fellowship at Harvard Medical School, and a KL2/Catalyst Medical Research Investigator Training award from Harvard Catalyst funded by the NIH (TR001100); and has received consulting fees from Merck and Amarin. Dr. Klarin is supported by the NHLBI (T32 HL007734). Dr. Samani is supported by the British Heart Foundation and is a National Institute for Health Research Senior Investigator. Dr. Rader has received consulting fees from Aegerion Pharmaceuticals, Alnylam Pharmaceuticals, Eli Lilly and Company, Pfizer, Sanofi, and Novartis; is an inventor on a patent related to lomitapide that is owned by the University of Pennsylvania and licensed to Aegerion Pharmaceuticals; and is a cofounder of Vascular Strategies and Staten Biotechnology. Dr. Danesh has received funding from the UK Medical Research Council, British Heart Foundation, UK National Institute of Health Research (NIHR), NIHR Cambridge Comprehensive Biomedical Research, European Commission Framework Programme, European Research Council, GlaxoSmithKline, Merck, NHLBI, NHS Blood and Transplant, Novartis, Pfizer, Wellcome Trust, and UK Biobank. Dr. Saleheen has received grants from Pfizer, Regeneron, Eli Lilly, and Genentech. Dr. Kathiresan has received grants from Bayer Healthcare, Aegerion Pharmaceuticals, and Regeneron Pharmaceuticals; and consulting fees from Merck, Novartis, Sanofi, AstraZeneca, Alnylam Pharmaceuticals, Leerink Partners, Noble Insights, Quest Diagnostics, Genomics PLC, and Eli Lilly and Company; and holds equity in San Therapeutics and Catabasis Pharmaceuticals. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose. Drs. Stitziel, Khera, Wang, Saleheen, Musunuru, and Kathiresan contributed equally to this work.

Manuscript received December 27, 2016; revised manuscript received February 3, 2017, accepted February 7, 2017.

mutations in *ANGPTL3*) with wild-type first-degree relatives. Next, we examined the coding regions of *ANGPTL3* in up to 180,180 individuals, identified those who carried LOF mutations in this gene with the aid of mouse models, and determined whether those mutations were associated with a lower risk of CAD. Finally, we measured circulating ANGPTL3 concentrations in individuals presenting with a first-ever myocardial infarction (MI) and compared them to concentrations in individuals without MI.

METHODS

The original kindred used to map *ANGPTL3* as a cause of familial combined hypolipidemia was recruited in the Lipid Research Clinic at the Washington University School of Medicine between 1994 and 1997. We recontacted all individuals from the kindred who inherited compound heterozygous LOF mutations in *ANGPTL3* and invited them to participate in the current study. Three of the 4 compound heterozygous carriers (individuals II-1, II-2, and II-4 in Online Figure 1) were available to participate and were matched to 3 first-degree relatives who did not carry any *ANGPTL3* LOF mutation (individuals II-8, II-7, and II-10 in Online Figure 1, respectively). Fasting laboratory values, including plasma lipids, were measured in all participants with standard clinical assays. Coronary computed tomography angiography (CCTA) was used to quantify coronary artery calcification and atherosclerotic plaque burden. Details of the image acquisition and post-acquisition processing are included in the Online Appendix. The Washington University School of Medicine Institutional Review Board approved all study protocols.

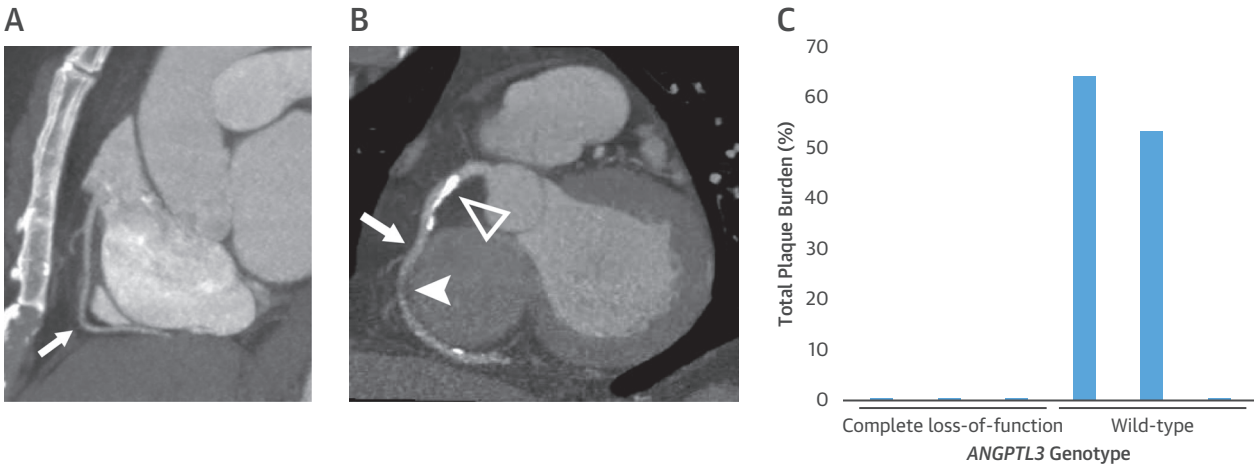
ASCERTAINMENT OF LOF MUTATIONS IN *ANGPTL3*. We identified carriers of an LOF mutation in *ANGPTL3* using previously generated exome sequencing data from 9 case-control studies of the Myocardial Infarction Genetics Consortium. These included the ATVB (Italian Atherosclerosis Thrombosis and Vascular Biology) study (9), the ESP-EOMI (Exome Sequencing Project Early-Onset Myocardial Infarction) study (10), the South German Myocardial Infarction study (11), the OHS (Ottawa Heart Study) (12), PROCARDIS (Precocious Coronary Artery Disease Study) (13), PROMIS (Pakistan Risk of Myocardial Infarction Study) (14), the Registre Gironi del COR (Gerona Heart Registry or REGICOR) study (15), the BHF-FHS (British Heart Foundation Family Heart Study) (16), and the Lubeck Myocardial Infarction study (16). Furthermore, we extracted *ANGPTL3* sequence data from exome sequencing performed in the Jackson Heart Study (17), the BioImage study (18), and the ARIC (Atherosclerosis Risk In Communities) population-based cohort study (19) in addition to targeted sequencing in the Duke CATHGEN case-control study (20). LOF mutations included those leading to truncation via a premature stop codon (nonsense), insertions or deletions that scramble protein translation beyond the variant site (frameshift), or point mutations at sites of pre-messenger ribonucleic acid splicing that alter the splicing process (splice site). Additional data for rs372257803, an intronic splice region variant in *ANGPTL3* previously linked to significantly reduced circulating TG levels (21), was obtained by high-quality genotype imputation in the United Kingdom Biobank (22), the PennCath study (23), and the Wellcome Trust Case Control Consortium Coronary Artery Disease study (24). All variant positions were based on the *ANGPTL3* canonical transcript (ENST00000371129). Additional details on gene sequencing, the imputation of rs372257803, and study cohorts are included in the Online Methods and Online Table 1.

FUNCTIONAL VALIDATION OF MISSENSE VARIANTS IN *ANGPTL3* LEADING TO LOF. Beyond the mutations that lead to LOF due to nonsense, frameshift, or splice-site disruption, studies based on evolutionary conservation have suggested that approximately 20% of all missense mutations lead to severe decrements in protein function (25,26). We sought to experimentally define such variants in *ANGPTL3* using a mouse model. Rare (minor allele frequency <1%) missense variants were prioritized if they were 1) predicted to be damaging or possibly damaging by each of 5 in silico prediction algorithms (LRT [likelihood ratio test] score, MutationTaster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, and Sorting Intolerant From Tolerant) and 2) present in at least 2 sequenced individuals of the Myocardial Infarction Genetics Consortium cohorts.

For the set of *ANGPTL3* missense variants identified previously, the functional significance of each variant was determined with adenoviral vectors developed to reconstitute the expression of the human *ANGPTL3* ortholog in the livers of *Angptl3* knockout mice. Vectors were engineered to contain either the wild-type *ANGPTL3* gene or the missense variant of interest. Missense variants were annotated as LOF if they conferred <25% of wild-type activity as assessed by percent change in circulating TG levels and percent change in circulating cholesterol levels induced by expression. Additional details are described in the Online Appendix.

ANGPTL3 PLASMA CONCENTRATION. Using a previously validated enzyme-linked immunosorbent

FIGURE 1 Coronary Computed Tomography Angiography in Humans With and Without Complete ANGPTL3 Deficiency



Representative sagittal computed tomography angiogram images show the right coronary artery **(arrow)** in **(A)** an individual with complete angiopoietin-like 3 (ANGPTL3) deficiency and **(B)** a matched first-degree relative without an ANGPTL3 mutation demonstrating calcified **(open triangle)** and noncalcified **(arrowhead)** plaque. **(C)** Total plaque burden representing the percentage of the coronary system affected by atherosclerosis is plotted by ANGPTL3 deficiency status.

assay (BioVendor, Prague, Czech Republic) (27), plasma ANGPTL3 concentrations were measured in individuals from PROMIS (14), a study that included cases presenting with a first-ever MI and control subjects free of MI.

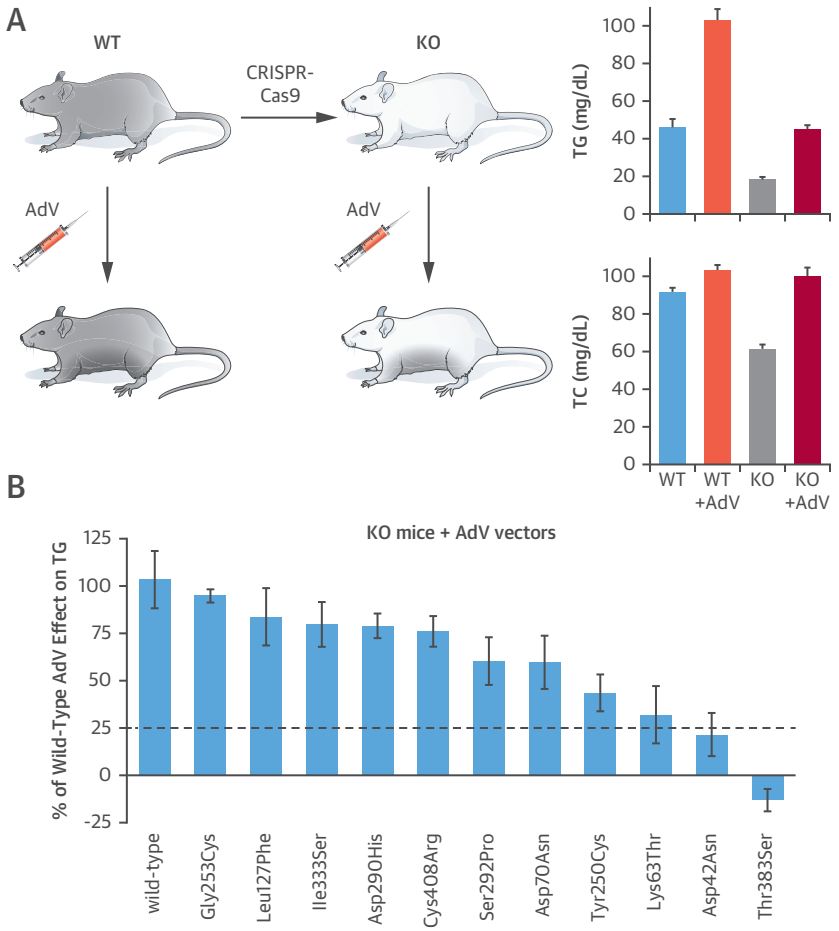
STATISTICAL ANALYSES. For changes in circulating TG and total cholesterol levels in the mouse models, normality of data was assessed with the Shapiro-Wilk test and equality of variance was assessed with the *F* test; p values were calculated with 2-sided paired-samples Student *t* tests. The association of *ANGPTL3* LOF mutations, analyzed in aggregate, with total cholesterol, LDL cholesterol, HDL cholesterol, and log-transformed TGs was assessed by linear regression with adjustment for the covariates of age, age squared, sex, study cohort, CAD status, and the first 5 principal components of ancestry. We accounted for the effect of lipid-lowering therapy in participants reporting such use at the time of lipid measurement by dividing the measured total cholesterol and LDL cholesterol by 0.8 and 0.7, respectively (28,29); HDL cholesterol and TG values were not adjusted. The association of *ANGPTL3* mutations with risk of CAD was determined via meta-analysis with Cochran-Mantel-Haenszel statistics for stratified 2-by-2 tables as implemented previously (30). In calculating the study-specific odds ratio of disease, an adjustment of 0.5 was added to all counts in studies with zero mutation carriers in cases or controls. The association of circulating plasma ANGPTL3 concentration with MI was determined by multivariable logistic regression after stratification of the population into tertiles of ANGPTL3 concentration. Statistical analyses were performed with R version 3.2.2 software (The R Project for Statistical Computing, Vienna, Austria).

RESULTS

From the original kindred used to map this gene as a cause of familial combined hypolipidemia (1), we studied 3 individuals with complete ANGPTL3 deficiency due to compound heterozygous LOF mutations in *ANGPTL3* and 3 matched first-degree relatives without an LOF *ANGPTL3* mutation. As shown in Online Table 2, participants with complete ANGPTL3 deficiency continued to exhibit very low plasma lipid concentrations nearly 20 years after the initial report. An updated medical history was obtained. One participant with complete ANGPTL3 deficiency reported a history of type 2 diabetes mellitus, hypertension, and past tobacco use. Other characteristics and laboratory values are listed in Online Table 2. We performed CCTA in all 6 individuals. The coronary calcium score was 0 Agatston units (AU) for all participants with complete ANGPTL3 deficiency (**Figure 1A**). By contrast, 2 of the 3 matched control subjects had positive coronary calcium scores (6 AU for individual II-8 and 610 AU for individual II-7) (**Figure 1B**).

We next calculated total plaque burden (a combination of both calcified and noncalcified plaque) for each participant. The total plaque burden was lower in the participants with complete ANGPTL3 deficiency (mean = 0%) than in control subjects (mean = 39%)

FIGURE 2 Mouse Model to Functionally Annotate *ANGPTL3* Missense Variants



(A) This schematic illustrates the design of the mouse study and circulating triglyceride (TG) and total cholesterol (TC) levels of wild-type (WT) and *Angptl3* (angiopoietin-like 3) knockout (KO) mice generated with CRISPR-Cas9, before and after treatment with adenovirus (AdV) expressing WT human *ANGPTL3* (11 WT mice, 18 KO mice). **(B)** The effects of mutant *ANGPTL3* alleles expressed from AdV in KO mice (5 to 6 in each group) on TG levels are shown as percentages of the effect of AdV expressing the WT *ANGPTL3* allele on TG. Mutant *ANGPTL3* alleles that had <25% of the WT allele effect on TG levels were annotated as loss-of-function alleles. (For additional details, including TC levels of mice before and after AdV injection, see Online Figure 3.)

(**Figure 1C**, Online Table 2, Online Figure 2). The small number of phenotyped individuals precluded robust statistical comparisons between groups.

ASCERTAINMENT OF *ANGPTL3* LOF MUTATIONS. We next sought to characterize the clinical effects of *ANGPTL3* LOF mutations in the population. Sequence data for *ANGPTL3* were available in 13,914 individuals with CAD and 26,198 control subjects free of CAD. From these data, 21 LOF variants were identified, including 7 variants leading to premature stop codons, 2 variants predicted to disrupt splicing, and 12 frameshift indels (Online Table 3). Eleven rare missense variants underwent functional validation in a mouse model, of which 2 (p.Asp42Asn and p.Thr383Ser) were additionally included as validated LOF variants (**Figure 2**).

In aggregate across all sequencing studies, an *ANGPTL3* LOF mutation was identified in 130 of 40,112 participants (0.32%; 95% confidence interval [CI]: 0.27% to 0.39%). One homozygote was identified with a Gln192ArgfsTer5 frameshift mutation, a 56-year-old woman of African ancestry free of clinical CAD with LDL cholesterol of 112 mg/dl, HDL cholesterol of 44 mg/dl, and TGs of 56 mg/dl.

Among sequenced individuals of European ancestry, the most frequently observed inactivating variant was the intronic splice region variant

rs372257803 (minor allele frequency = 0.17%). This variant was imputed in an additional 8,066 CAD case subjects and 140,068 control subjects, identifying an additional 68 heterozygous carriers of an *ANGPTL3* LOF mutation.

ASSOCIATION OF *ANGPTL3* LOF MUTATIONS WITH CIRCULATING LIPID LEVELS AND CAD RISK. Plasma lipid levels were available in up to 20,092 people in the Myocardial Infarction Genetics Consortium studies, including 60 heterozygous carriers of an *ANGPTL3* LOF mutation. We found that individuals carrying an LOF *ANGPTL3* mutation, compared with noncarriers, had 11% lower total cholesterol ($p = 0.0008$), 12% lower LDL cholesterol ($p = 0.04$), and 17% lower TGs ($p = 0.01$) (**Table 1**). HDL cholesterol was not significantly different between the groups ($p = 0.17$).

A cohort-based meta-analysis stratified by ancestry was performed to determine the relationship between LOF mutations in *ANGPTL3* and risk of CAD (**Figure 3**, Online Table 3). We observed a 34% reduced risk of CAD among carriers of an *ANGPTL3* LOF mutation compared with noncarriers (odds ratio [OR] of disease for carriers: 0.66; 95% CI: 0.44 to 0.98; $p = 0.04$). This effect estimate was similar in a sensitivity analysis restricted to individuals in whom complete gene sequencing (as opposed to rs372257803 imputation) was performed (OR: 0.70; 95% CI: 0.46 to 1.06; $p = 0.09$).

CIRCULATING PLASMA ANGPTL3 AND RISK OF MI. Protection from CAD among carriers of a rare LOF mutation in *ANGPTL3* led to the hypothesis that individuals with lower levels of circulating ANGPTL3 protein might similarly have reduced coronary risk. Plasma ANGPTL3 concentrations were measured in 1,493 case subjects presenting with a first-ever MI and 3,231 control subjects free of CAD from the PROMIS study. Consistent with our expectations, individuals in the lowest tertile of ANGPTL3 concentrations had significantly reduced risk of MI compared with those in the highest tertile (adjusted OR: 0.65; $p = 2.2 \times 10^{-7}$) (**Table 2**). This finding was modestly attenuated after additional adjustment for observed plasma LDL cholesterol and TGs (adjusted OR: 0.71; $p = 0.0001$).

TABLE 1 Association of LOF Mutations in ANGPTL3 With Plasma Lipid Concentrations*

Lipid Fraction	Number of Participants	% Difference†	95% CI, %	p Value
Total cholesterol	19,783	−10.9	−17.3 to −4.5	0.0008
LDL cholesterol	18,231	−11.8	−21.5 to −2.1	0.04
HDL cholesterol	20,092	−5.2	−12.8 to 2.3	0.17
Triglycerides	19,282	−17.3	−31.1 to −3.4	0.01

*Association results are from an analysis of 20,092 individuals of the Myocardial Infarction Genetics Consortium. †Linear regression was used to test for an association between LOF *ANGPTL3* mutations and plasma lipid concentrations using age, age squared, sex, study cohort, coronary artery disease, and the first 5 principal components of ancestry as covariates. Triglyceride concentrations were natural log-transformed before analysis.

ANGPTL3 = angiopoietin-like 3; CI = confidence interval; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LOF = loss of function.

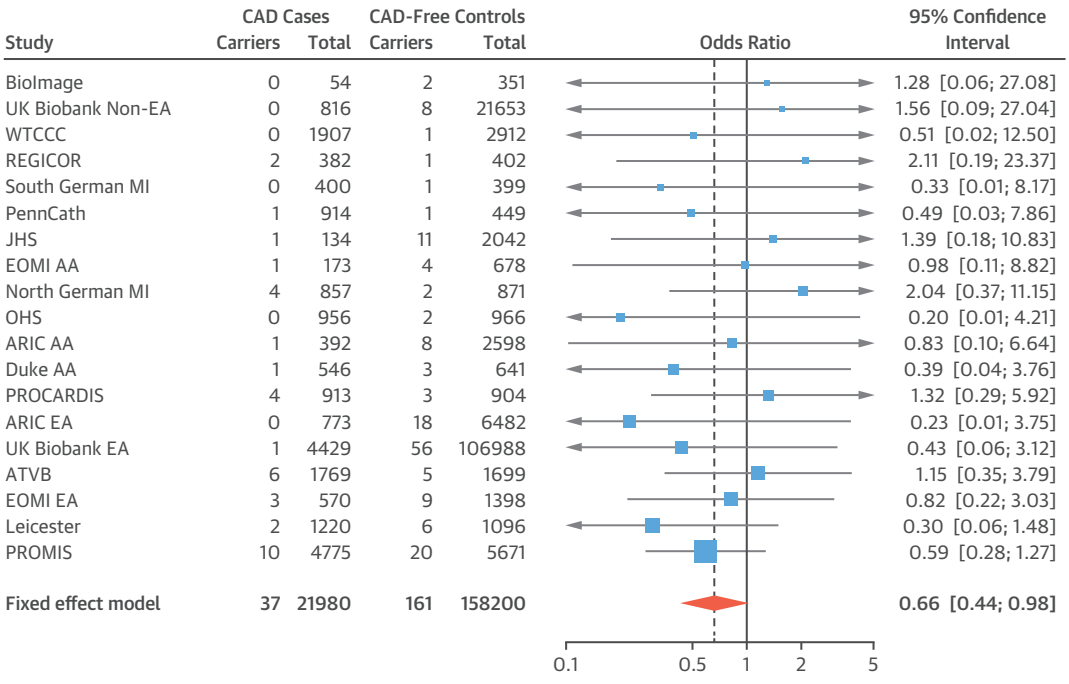
DISCUSSION

We have provided multiple lines of evidence suggesting that ANGPTL3 deficiency is associated with protection from CAD (**Central Illustration**). Detailed atherosclerotic phenotyping demonstrated an absence of coronary atherosclerotic plaque in individuals with complete ANGPTL3 deficiency. Genomic analysis of *ANGPTL3* LOF variants, including functionally validated missense variants in up to 180,180 people, showed a 34% reduction in risk of CAD among heterozygous carriers. Finally, circulating ANGPTL3 concentrations were lower in healthy control subjects than in those presenting with MI.

These results permitted several conclusions. First, identifying families with extreme phenotypes of interest could facilitate both gene discovery and hypothesis-based phenotyping. Multiple independent groups have confirmed the impact of inactivating mutations in *ANGPTL3* on decreasing lipid levels using family-based study designs (31-34). Here, we extended these observations by demonstrating that individuals with complete deficiency due to 2 inactivating mutations in the gene (effectively "human knockouts" for *ANGPTL3*) tended to have less coronary atherosclerosis as assessed by CCTA. This apparent protection from coronary atherosclerosis extended to a middle-aged participant (Online Table 2, individual II-1) with significant cardiovascular risk factors of type 2 diabetes mellitus, hypertension, and a history of cigarette smoking. Although suggestive, these results were based on a small number of family members. Large-scale gene sequencing in the population as presented here was used to confirm this observation.

Additionally, these findings lend support to ongoing drug development efforts focused on ANGPTL3 inhibition as a therapeutic strategy. Beyond a significant reduction in plasma LDL cholesterol and TG concentrations, heterozygous *ANGPTL3* LOF mutation carriers had a 34% decreased risk of CAD. Similar results were noted in a preliminary report from Dewey and colleagues (35). We also found that individuals with circulating plasma ANGPTL3 concentrations in the lowest tertile of the population (in a sense, mimicking the effect of pharmacological inhibition of

FIGURE 3 Association of *ANGPTL3* Loss-of-Function Mutations With Risk of CAD



The association of *angiopoietin-like 3* (*ANGPTL3*) mutations with risk of coronary artery disease (CAD) was determined via meta-analysis using Cochran–Mantel–Haenszel statistics for ethnicity-specific stratified 2-by-2 tables. A reduced risk of CAD was noted among carriers of an *ANGPTL3* loss-of-function variant (odds ratio of disease for carriers: 0.66; 95% confidence interval: 0.44 to 0.98; p = 0.04). AA = African ancestry; ARIC = Atherosclerosis Risk in Communities; ATVB = Italian Atherosclerosis Thrombosis and Vascular Biology; EA = European ancestry; ESP-EOMI = Exome Sequencing Project Early-Onset Myocardial Infarction; JHS = Jackson Heart Study; MI = myocardial infarction; OHS = Ottawa Heart Study; PROCARDIS = Precocious Coronary Artery Disease; PROMIS = Pakistan Risk of Myocardial Infarction Study; REGICOR = Registre Gironi del COR (Gerona Heart Registry); WTCCC = Wellcome Trust Case Control Consortium.

ANGPTL3) had a 35% reduced risk of MI. This study adds *ANGPTL3* to the list of therapeutic targets for coronary disease, which includes *ANGPTL4* (16,36), *APOC3* (11,37), *LPA* (38), *NPC1L1* (30), and *PCSK9* (39), that have been validated by finding LOF mutations that associate with protection from disease, highlighting the promise and potential of human genetic studies in identifying such targets (40).

Furthermore, these data add to a growing body of human genetics evidence linking regulation of lipoprotein lipase activity, the major mechanism by which circulating TG-rich lipoproteins are hydrolyzed, with atherosclerosis. Studies in both mice and humans have demonstrated ANGPTL3 to be a potent inhibitor of LPL, particularly in the post-prandial state (41). Consistent with effects of rare mutations in 3 additional endogenous regulators of LPL, *APOA5* (10), *APOC3* (11,37), and *ANGPTL4* (16,36), loss of *ANGPTL3* function appears to result in gain of LPL activity, reduced TG-rich lipoproteins, and protection from coronary disease. Beyond effects on TGs and LDL cholesterol, ANGPTL3 might affect glucose homeostasis and remodeling of HDL cholesterol particles by EL (4,42). The relative contributions of each of these mechanisms, as they relate to risk of CAD, warrant additional investigation.

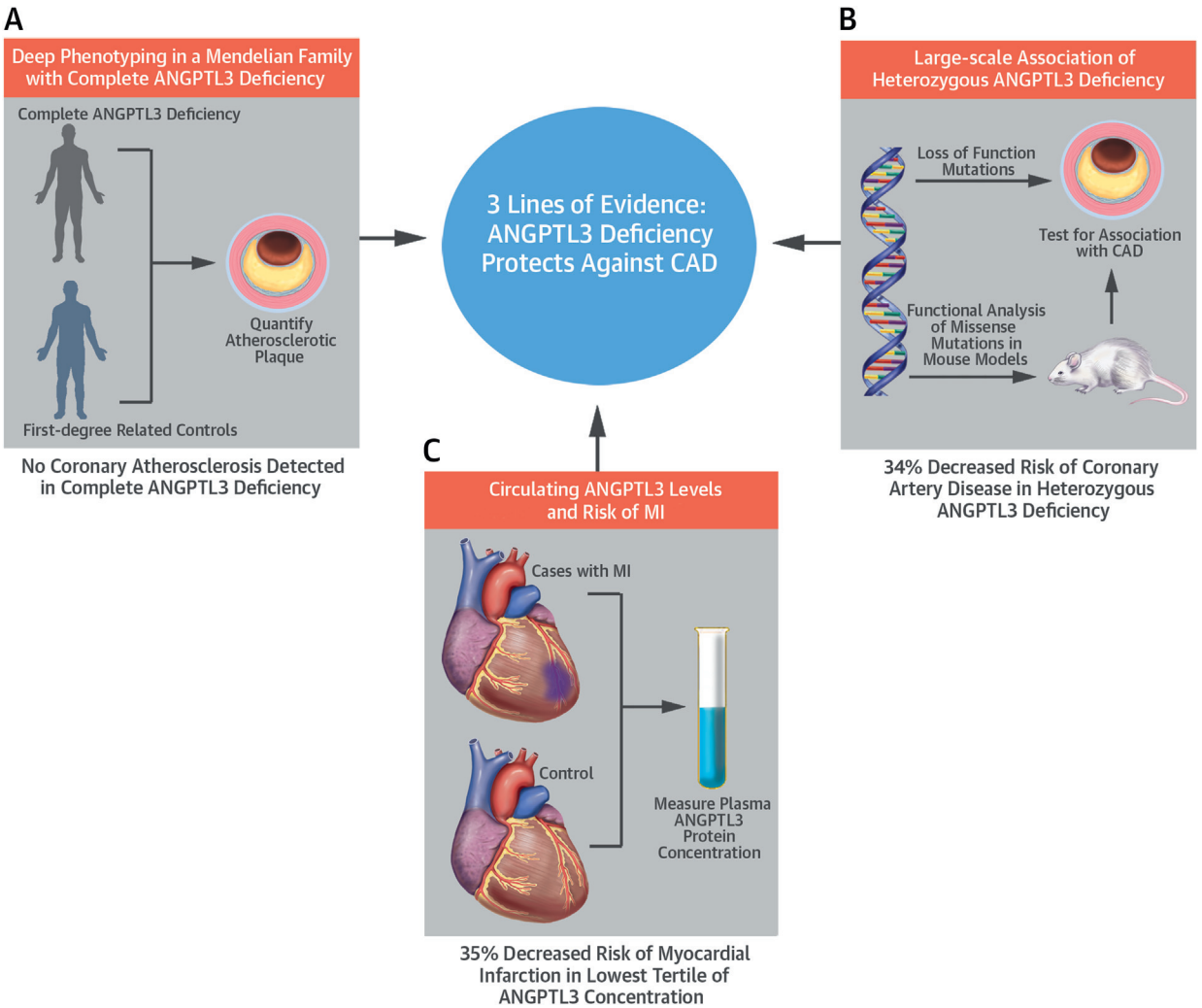
TABLE 2 Association of ANGPTL3 Concentration With MI Risk in PROMIS

	ANGPTL3 Concentration (ng/ml)	Adjusted Odds Ratio for MI (95% CI)	
		Model 1*	Model 2†
Tertile 1 (n = 1,575)‡	379-1,375	1.00	1.00
Tertile 2 (n = 1,574)	272-378	0.75 (0.64-0.88)	0.79 (0.66-0.93)
Tertile 3 (n = 1,575)	18-271	0.65 (0.55-0.77)	0.71 (0.60-0.85)
p Value for trend		2.2×10^{-7}	0.0001

*Sex, current smoking, diabetes, and hypertension as covariates. †Model 1 plus LDL cholesterol and log-transformed triglycerides as covariates. ‡Individuals with the highest tertile of ANGPTL3 concentration served as the reference group for this analysis.

MI = myocardial infarction; PROMIS = Pakistan Risk of Myocardial Infarction Study; other abbreviations as in Table 1.

CENTRAL ILLUSTRATION **ANGPTL3 Deficiency and Protection From Coronary Artery Disease**



Stitziel, N.O. et al. J Am Coll Cardiol. 2017;69(16):2054–63.

Multiple lines of evidence suggest that angiopoietin-like 3 (ANGPTL3) deficiency is associated with protection from coronary artery disease (CAD). **(A)** A genotype-guided callback study of human "knockouts" for *ANGPTL3*, which used detailed atherosclerotic phenotyping, demonstrated an absence of coronary atherosclerotic plaque in individuals with complete ANGPTL3 deficiency. **(B)** Genomic analysis of *ANGPTL3* loss-of-function variants, including missense variants that were experimentally found to disrupt ANGPTL3 function, found in up to 180,180 individuals showed a 34% reduction in risk of CAD among loss-of-function variant carriers. **(C)** Circulating ANGPTL3 protein concentrations were lower in healthy control subjects than in those presenting with a myocardial infarction. MI = myocardial infarction.

Finally, we have provided proof-of-concept for rare *ANGPTL3* missense variant prioritization using a combination of bioinformatics tools and experimental characterization in vivo. Any given *ANGPTL3* missense variant might perturb protein function via numerous potential mechanisms, including decreased expression, impaired hepatic secretion, or inability to bind and inhibit LPL. We developed an *Angptl3* knockout mouse that exhibited a phenotype of very low TGs. We attempted to rescue this phenotype using adenoviral vectors producing either wild-type ANGPTL3 or a protein that included the missense variant of interest. This proved useful in determining that only 2 of 11 screened missense variants led to near complete loss of ANGPTL3 protein function

(i.e., <25% of wild-type activity). The ability to confidently annotate the functional consequences of rare missense variants in a gene of interest remains one of the biggest hurdles to rare variant gene discovery efforts (43). We present one approach to address this challenge.

STUDY LIMITATIONS. First, CCTA was performed in only a subset of the original family used to identify *ANGPTL3* as the cause of familial combined hypolipidemia. Second, the functional characterization of missense variants was performed in only 11 variants, and alternative thresholds for defining LOF are possible. Third, genotype imputation was used to identify carriers of an *ANGPTL3* splice-site imputation in some cohorts; however, a sensitivity analysis that was restricted to those studies in which complete sequencing of *ANGPTL3* was available yielded similar results.

CONCLUSIONS

Deep phenotyping in a family, gene sequencing in the population, and biomarker analysis in case and control subjects showed ANGPTL3 deficiency to be associated with a reduced risk of CAD. Whether pharmacological inhibition of ANGPTL3 function will prove useful in the treatment or prevention of CAD remains to be determined.

ACKNOWLEDGMENTS The authors are very grateful to the family presented here for participating in this study and for their ongoing contributions to furthering biomedical research. The authors also thank Ms. Teresa Roediger for coordinating the clinical imaging portion of the study.

ADDRESS FOR CORRESPONDENCE: Dr. Nathan Stitziel, Cardiovascular Division, Washington University, 660 South Euclid Avenue, Campus Box 8086, Saint Louis, Missouri 63110. E-mail: nstitziel@wustl.edu. OR Dr. Kiran Musunuru, University of Pennsylvania, 3400 Civic Center Boulevard, Bldg 421, 11-104 Smilow Center for Translational Research, Philadelphia, Pennsylvania 19104. E-mail: kmus@mail.med.upenn.edu. OR Dr. Sekar Kathiresan, Center for Genomic Medicine, Massachusetts General Hospital, Simches Research Center, 185 Cambridge Street, CPZN 5.830, Boston, Massachusetts 02114. E-mail: skathiresan@partners.org.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Hereditary combined hypolipidemia is caused by mutations that inactivate the gene *ANGPTL3* and is characterized by low blood levels of all 3 major lipid fractions. Individuals with a loss-of-function *ANGPTL3* mutation have reduced odds of CAD, which suggests that ANGPTL3 deficiency protects against CAD.

TRANSLATIONAL OUTLOOK: Interventions targeting ANGPTL3 should be considered for potential clinical application.

REFERENCES

1. Musunuru K, Pirruccello JP, Do R, et al. Exome sequencing, ANGPTL3 mutations, and familial combined hypolipidemia. N Engl J Med 2010;363:2220–7.

2. Koishi R, Ando Y, Ono M, et al. Angptl3 regulates lipid metabolism in mice. Nat Genet 2002;30:151–7.

3. Shimizugawa T, Ono M, Shimamura M, et al. ANGPTL3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase. J Biol Chem 2002;277:33742–8.

4. Shimamura M, Matsuda M, Yasumo H, et al. Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase. Arterioscler Thromb Vasc Biol 2007;27:366–72.

5. Gusarova V, Alexa CA, Wang Y, et al. ANGPTL3 blockade with a human monoclonal antibody reduces plasma lipids in dyslipidemic mice and monkeys. J Lipid Res 2015;56:1308–17.

6. Sehgal A, Vaishnaw A, Fitzgerald K. Liver as a target for oligonucleotide therapeutics. J Hepatol 2013;59:1354–9.

7. Ando Y, Shimizugawa T, Takeshita S, et al. A decreased expression of angiopoietin-like 3 is protective against atherosclerosis in apoE-deficient mice. J Lipid Res 2003;44:1216–23.

8. Minicocci I, Santini S, Cantisani V, et al. Clinical characteristics and plasma lipids in subjects with familial combined hypolipidemia: a pooled analysis. J Lipid Res 2013;54:3481–90.

9. Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group. No evidence of association between prothrombotic gene polymorphisms and the development of acute myocardial infarction at a young age. Circulation 2003;107:1117–22.

10. Do R, Stitziel NO, Won HH, et al. Exome sequencing identifies rare LDLR and APOA5 alleles conferring risk for myocardial infarction. Nature 2015;518:102–6.

11. Crosby J, Peloso GM, Auer PL, et al. Loss-of-function mutations in APOC3, triglycerides, and coronary disease. N Engl J Med 2014;371:22–31.

12. McPherson R, Pertsemlidis A, Kavaslar N, et al. A common allele on chromosome 9 associated with coronary heart disease. Science 2007;316:1488–91.

13. Clarke R, Peden JF, Hopewell JC, et al. Genetic variants associated with Lp(a) lipoprotein level and coronary disease. N Engl J Med 2009;361:2518–28.

14. Saleheen D, Zaidi M, Rasheed A, et al. The Pakistan Risk of Myocardial Infarction Study: a resource for the study of genetic, lifestyle and other determinants of myocardial infarction in South Asia. Eur J Epidemiol 2009;24:329–38.

15. Senti M, Tomas M, Marrugat J, Elosua R, REGICOR Investigators. Paraoxonase1-192 polymorphism modulates the nonfatal myocardial infarction risk associated with decreased HDLs. Arterioscler Thromb Vasc Biol 2001;21:415–20.

16. Stitziel NO, Stirrups KE, Masca NG, et al. Coding variation in ANGPTL4, LPL, and SVEP1 and the risk of coronary disease [published correction appears in N Engl J Med 2016;374:1898]. N Engl J Med 2016;374:1134–44.

17. Peloso GM, Lange LA, Varga TV, et al. Association of exome sequences with cardiovascular

traits among blacks in the Jackson Heart Study. Circ Cardiovasc Genet 2016;9:368–74.

18. Baber U, Mehran R, Sartori S, et al. Prevalence, impact, and predictive value of detecting subclinical coronary and carotid atherosclerosis in asymptomatic adults: the BioImage study. J Am Coll Cardiol 2015;65:1065–74.

19. Li AH, Morrison AC, Kovar C, et al. Analysis of loss-of-function variants and 20 risk factor phenotypes in 8,554 individuals identifies loci influencing chronic disease. Nat Genet 2015;47:640–2.

20. Davies RW, Wells GA, Stewart AF, et al. A genome-wide association study for coronary artery disease identifies a novel susceptibility locus in the major histocompatibility complex. Circ Cardiovasc Genet 2012;5:217–25.

21. Helgadottir A, Gretarsdottir S, Thorleifsson G, et al. Variants with large effects on blood lipids and the role of cholesterol and triglycerides in coronary disease. Nat Genet 2016;48:634–9.

22. Sudlow C, Gallacher J, Allen N, et al. UK biobank: an open access resource for identifying the causes of a wide range of complex diseases of middle and old age. PLoS Med 2015;12:e1001779.

23. Reilly MP, Li M, He J, et al. Identification of ADAMTS7 as a novel locus for coronary atherosclerosis and association of ABO with myocardial infarction in the presence of coronary atherosclerosis: two genome-wide association studies. Lancet 2011;377:383–92.

24. Samani NJ, Erdmann J, Hall AS, et al. Genomewide association analysis of coronary artery disease. N Engl J Med 2007;357:443–53.

25. Boyko AR, Williamson SH, Indap AR, et al. Assessing the evolutionary impact of amino acid mutations in the human genome. PLoS Genet 2008;4:e1000083.

26. Yampolsky LY, Kondrashov FA, Kondrashov AS. Distribution of the strength of selection against amino acid replacements in human proteins. Hum Mol Genet 2005;14:3191–201.

27. Mehta N, Qamar A, Qu L, et al. Differential association of plasma angiopoietin-like proteins 3 and 4 with lipid and metabolic traits. Arterioscler Thromb Vasc Biol 2014;34:1057–63.

28. Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins [published corrections appear in Lancet 2005;366:1358 and Lancet 2008;371:2084]. Lancet 2005;366:1267–78.

29. Tobin MD, Sheehan NA, Scurrah KJ, Burton PR. Adjusting for treatment effects in studies of quantitative traits: antihypertensive therapy and systolic blood pressure. Stat Med 2005;24:2911–35.

30. Stitziel NO, Won HH, Morrison AC, et al. Inactivating mutations in NPC1L1 and protection from coronary heart disease. N Engl J Med 2014;371:2072–82.

31. Martin-Campos JM, Roig R, Mayoral C, et al. Identification of a novel mutation in the ANGPTL3 gene in two families diagnosed of familial hypobetalipoproteinemia without APOB mutation. Clin Chim Acta 2012;413:552–5.

32. Minicocci I, Montali A, Robciuc MR, et al. Mutations in the ANGPTL3 gene and familial combined hypolipidemia: a clinical and biochemical characterization. J Clin Endocrinol Metab 2012;97:E1266–75.

33. Pisciotta L, Favari E, Magnolo L, et al. Characterization of three kindreds with familial combined hypolipidemia caused by loss-of-function mutations of ANGPTL3. Circ Cardiovasc Genet 2012;5:42–50.

34. Noto D, Cefalu AB, Valenti V, et al. Prevalence of ANGPTL3 and APOB gene mutations in subjects with combined hypolipidemia. Arterioscler Thromb Vasc Biol 2012;32:805–9.

35. Dewey FE, Gusarova V, O'Dushlaine C, et al. Genetic and pharmacological inactivation of ANGPTL3 is associated with reduced atherosclerotic cardiovascular disease (abstr.). Circulation 2016;134:A16563.

36. Dewey FE, Gusarova V, O'Dushlaine C, et al. Inactivating variants in ANGPTL4 and risk of coronary artery disease. N Engl J Med 2016;374:1123–33.

37. Jorgensen AB, Frikke-Schmidt R, Nordestgaard BG, Tybjaerg-Hansen A. Loss-of-function mutations in APOC3 and risk of ischemic vascular disease. N Engl J Med 2014;371:32–41.

38. Lim ET, Wurtz P, Havulinna AS, et al. Distribution and medical impact of loss-of-function variants in the Finnish founder population. PLoS Genet 2014;10:e1004494.

39. Cohen JC, Boerwinkle E, Mosley TH Jr., Hobbs HH. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 2006;354:1264–72.

40. Stitziel NO, Kathiresan S. Leveraging human genetics to guide drug target discovery. Trends Cardiovasc Med 2016 Aug 26 [E-pub ahead of print].

41. Minicocci I, Tikka A, Poggiogalle E, et al. Effects of angiopoietin-like protein 3 deficiency on postprandial lipid and lipoprotein metabolism. J Lipid Res 2016;57:1097–107.

42. Robciuc MR, Maranghi M, Lahikainen A, et al. Angptl3 deficiency is associated with increased insulin sensitivity, lipoprotein lipase activity, and decreased serum free fatty acids [published correction appears in Arterioscler Thromb Vasc Biol 2013;33:e124]. Arterioscler Thromb Vasc Biol 2013;33:1706–13.

43. Zuk O, Schaffner SF, Samocha K, et al. Searching for missing heritability: designing rare variant association studies. Proc Natl Acad Sci U S A 2014;111:E455–64.

KEY WORDS human genetics, loss-of-function mutations, myocardial infarction

APPENDIX For a supplemental Methods section as well as supplemental figures and tables, please see the online version of this article.