ANGPTL3 Deficiency and Protection Against Coronary Artery Disease

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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; ) © 2017 by the American College of Cardiology Foundation.
loss-of-function (LOF) mutations leading to complete deficiency of angioøpoietin-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 heparically 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).

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 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 Biolmage 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
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%)

![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.](image-url)
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). LDL 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 x 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).

**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
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

<table>
<thead>
<tr>
<th>ANGPTL3 Concentration (ng/ml)</th>
<th>Adjusted Odds Ratio for MI (95% CI)</th>
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<tbody>
<tr>
<td>Tertile 1 (n = 1,575)†</td>
<td>379-1,375</td>
</tr>
<tr>
<td>Tertile 2 (n = 1,574)†</td>
<td>272-378</td>
</tr>
<tr>
<td>Tertile 3 (n = 1,575)†</td>
<td>18-271</td>
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\( \ddagger \)Sex, current smoking, diabetes, and hypertension as covariates. 1Model 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.
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.
The family presented here for participating in this study. ANGPTL3 decreases very low density lipoprotein levels and protects against atherosclerosis in apoE-deficient mice. J Lipid Res 2003;44:1216-23.


REFERENCES


STUDY LIMITATIONS. First, CCTA was performed in only a subset of the original family used to identify ANGPTL3 as the cause of familial combined hyperlipidemia. 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.

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COMPETENCY IN MEDICAL KNOWLEDGE: Hereditary combined hyperlipidemia 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.

PERSPECTIVES


