

ORIGINAL ARTICLE

Coding Variation in *ANGPTL4*, *LPL*, and *SVEP1* and the Risk of Coronary Disease

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ABSTRACT

BACKGROUND

The discovery of low-frequency coding variants affecting the risk of coronary artery disease has facilitated the identification of therapeutic targets.

METHODS

Through DNA genotyping, we tested 54,003 coding-sequence variants covering 13,715 human genes in up to 72,868 patients with coronary artery disease and 120,770 controls who did not have coronary artery disease. Through DNA sequencing, we studied the effects of loss-of-function mutations in selected genes.

RESULTS

We confirmed previously observed significant associations between coronary artery disease and low-frequency missense variants in the genes *LPA* and *PCSK9*. We also found significant associations between coronary artery disease and low-frequency missense variants in the genes *SVEP1* (p.D2702G; minor-allele frequency, 3.60%; odds ratio for disease, 1.14; $P=4.2\times 10^{-10}$) and *ANGPTL4* (p.E40K; minor-allele frequency, 2.01%; odds ratio, 0.86; $P=4.0\times 10^{-8}$), which encodes angiotensin-like 4. Through sequencing of *ANGPTL4*, we identified 9 carriers of loss-of-function mutations among 6924 patients with myocardial infarction, as compared with 19 carriers among 6834 controls (odds ratio, 0.47; $P=0.04$); carriers of *ANGPTL4* loss-of-function alleles had triglyceride levels that were 35% lower than the levels among persons who did not carry a loss-of-function allele ($P=0.003$). *ANGPTL4* inhibits lipoprotein lipase; we therefore searched for mutations in *LPL* and identified a loss-of-function variant that was associated with an increased risk of coronary artery disease (p.D36N; minor-allele frequency, 1.9%; odds ratio, 1.13; $P=2.0\times 10^{-4}$) and a gain-of-function variant that was associated with protection from coronary artery disease (p.S447*; minor-allele frequency, 9.9%; odds ratio, 0.94; $P=2.5\times 10^{-7}$).

CONCLUSIONS

We found that carriers of loss-of-function mutations in *ANGPTL4* had triglyceride levels that were lower than those among noncarriers; these mutations were also associated with protection from coronary artery disease. (Funded by the National Institutes of Health and others.)

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ALTHOUGH GENOMEWIDE ASSOCIATION studies have identified more than 56 loci associated with the risk of coronary artery disease,^{1,3} the disease-associated variants are typically common (minor-allele frequency >5%) and located in noncoding sequences; this has made it difficult to pinpoint causal genes and affected pathways. This lack of a causal mechanism has in part hindered the immediate translation of the findings of genomewide association studies into new therapeutic targets. However, the discovery of rare or low-frequency coding-sequence variants that affect the risk of coronary artery disease has facilitated advances in the prevention and treatment of disease. The most recent example of such advances is the development of a new class of therapeutic agents that is based on the discovery of the gene encoding proprotein convertase subtilisin/kexin type 9 (PCSK9) as a regulator of low-density lipoprotein (LDL) cholesterol⁴ and the discovery that low-frequency and loss-of-function variants in this gene protect against coronary artery disease.^{5,6}

Recently, low-frequency coding variation across the genome was systematically tabulated with the use of next-generation exome and whole-genome sequencing data from more than 12,000 persons of various ancestries (including a major contribution from the National Heart, Lung, and Blood Institute Exome Sequencing Project). Protein-altering variants (i.e., nonsynonymous, splice-site, and nonsense single-nucleotide substitutions) that were observed at least twice among these 12,000 persons were included in a genotyping array (hereafter referred to as the exome array). In addition, the exome array contains previously described variants from genomewide association studies, a sparse genomewide grid of common markers, markers that are informative with regard to ancestry (i.e., African American, Native American, and European), and some additional content. Additional information on the design of the exome array is provided at http://genome.sph.umich.edu/wiki/Exome_Chip_Design. In this study, we focused on the 220,231 autosomal variants that were present on the array and were expected to alter protein sequence (i.e., missense, nonsense, splice-site, and frameshift variants) and used these to test the contribution

of low-frequency coding variation to the risk of coronary artery disease.

METHODS

STUDY DESIGN AND PARTICIPANTS

We performed a discovery study involving 42,335 patients with coronary artery disease and 78,240 controls from 20 individual studies (hereafter referred to as the discovery cohort). For variants with suggestive associations, we sought replication of our findings in an independent study of 30,533 patients and 42,530 controls assembled from 8 individual studies (hereafter referred to as the replication cohort). The names of the individual studies and information on the numbers of participants and phenotypic definitions of patients and controls in the discovery cohort and the replication cohort are provided in Tables S1 and S2, respectively, in the Supplementary Appendix, available with the full text of this article at NEJM.org. The 5755 participants from the Bangladesh Risk of Acute Vascular Events (BRAVE) study and the 22,072 participants from the Pakistan Risk of Myocardial Infarction Study (PROMIS) were of South Asian ancestry; all other participants were of European ancestry.

GENOTYPING AND QUALITY CONTROL

Samples were genotyped on the Illumina HumanExome BeadChip array, version 1.0 or 1.1, or the Illumina OmniExome array (which includes markers from the HumanExome BeadChip) in accordance with the manufacturer's recommended protocol. Our genotyping methods, as well as the quality-control procedures that were used to remove low-quality samples and variants, are described in the Supplementary Appendix.

FOLLOW-UP ANGPTL4 SEQUENCING

The exon sequences of *ANGPTL4* were obtained from the exome sequences⁷ of 6924 persons who had early-onset myocardial infarction and those of 6834 persons who were free from coronary artery disease (see the Methods section in the Supplementary Appendix for details). The names of the individual studies and information on the numbers of participants and phenotypic definitions of patients and controls for *ANGPTL4* se-

quencing are provided in Table S3 in the Supplementary Appendix.

STATISTICAL ANALYSIS

Of the 220,231 variants on the exome array, 54,003 (covering 13,715 genes) were present in our study at sufficient frequency (minor-allele frequency >0.01%) to allow for individual-variant association testing as described in the Supplementary Appendix. In the discovery phase, we defined a suggestive novel association between a variant and the risk of coronary artery disease as one with a meta-analysis P value of 1×10^{-4} or lower. For variants with suggestive association, we performed association analysis in the replication studies as described in the Supplementary Appendix. We defined significant novel associations as those that were nominally significant ($P < 0.05$) in the replication cohort and that had an overall P value of less than 7.7×10^{-8} in the discovery and replication cohorts combined (a Bonferroni-corrected threshold that accounted for 54,003 markers with minor-allele frequencies >0.01% being tested initially and 12 markers being tested in the replication cohort).

To test for association between variants and risk factors for coronary artery disease, we examined the relationship between low-frequency variants that were significantly associated with disease in the combined (i.e., discovery and replication) analysis and plasma lipid levels in 10,088 samples from the discovery cohort; in order to minimize any effect of ascertainment bias, we limited the analysis to persons without coronary artery disease who had lipid measurements available. We also examined the relationships between the significantly associated low-frequency variants and blood pressure in 146,562 persons from the Cohorts for Heart and Aging Research in Genomic Epidemiology Plus (CHARGE+) consortium. Tables S4 and S5 in the Supplementary Appendix list the individual studies and numbers of participants that contributed to the analyses of plasma lipid levels and blood pressure, respectively. Finally, we queried publicly available exome-array data to explore the relationship between significantly associated low-frequency variants and type 2 diabetes (data from the Type 2 Diabetes Genetic Exploration by Next-Generation Sequencing in Multi-Ethnic Samples [T2D-GENES] Consortium, Genetics of Type 2

Diabetes [GoT2D] Consortium, and the Diabetes Genetics Replication and Meta-Analysis [DIAGRAM] Consortium; accessed in November 2015 at www.type2diabetesgenetics.org/). Additional details of the risk-factor association analyses are provided in the Methods section in the Supplementary Appendix.

From the sequencing data, we used linear regression to test the association between *ANGPTL4* loss-of-function alleles and plasma lipid levels in 8085 persons for whom lipid measurements were available, using models described in the Supplementary Appendix. We calculated the significance of the association between *ANGPTL4* loss-of-function alleles and the risk of coronary artery disease with the use of 100,000 study-stratified permutations of case-control phenotypes.

RESULTS

LOW-FREQUENCY CODING VARIANTS ASSOCIATED WITH CORONARY ARTERY DISEASE

The discovery cohort comprised 120,575 persons (42,335 patients and 78,240 controls) (Table S1 in the Supplementary Appendix). In the discovery cohort, we found significant associations between low-frequency coding variants in the *LPA* and *PCSK9* genes and coronary artery disease (Table 1). Both gene loci also harbor common noncoding variants associated with coronary artery disease that had previously been discovered through genomewide association studies. These variants were also present on the exome array and had significant associations with coronary artery disease in our study (Table 1). In a conditional analysis, the associations between coronary artery disease and the low-frequency coding variants in both *LPA* and *PCSK9* were found to be independent of the associations between coronary artery disease and the more common variants (Table 1).

In addition to the variants in *LPA* and *PCSK9*, 12 additional suggestive associations ($P < 1 \times 10^{-4}$) between low-frequency coding variants and coronary artery disease were identified in the discovery study (Table S6 in the Supplementary Appendix). We tested for associations between these variants and coronary artery disease among the 73,063 persons (30,533 patients and 42,530 controls) in the replication cohort (see Tables S2

Table 1. Low-Frequency Coding Variations Previously Associated with Coronary Artery Disease.*

Locus and SNP	Chromosome and Nucleotide Position†	Allele 1/ Allele 2	Frequency of Allele 1 %	Functional Effect	Odds Ratio‡	P Value	Conditional P Value§
<i>LPA</i>							
rs3798220	6: 160961137	C/T	1.99	p.I4399M	1.54	9.7×10 ⁻²⁴	1.9×10 ⁻¹⁸
rs2048327	6: 160863532	C/T	37.36	NA	1.09	8.1×10 ⁻²¹	1.8×10 ⁻¹⁵
<i>PCSK9</i>							
rs11591147	1: 55505647	T/G	1.52	p.R46L	0.78	8.0×10 ⁻¹⁰	1.9×10 ⁻⁷
rs11206510	1: 55496039	C/T	18.23	NA	0.93	1.7×10 ⁻⁸	4.6×10 ⁻⁶

* NA denotes not applicable, and SNP single-nucleotide polymorphism.

† Chromosome numbers and positions refer to genome build GRCh37.

‡ Odds ratios are for the development of disease in carriers of allele 1.

§ The conditional P value is the P value from an association test conditioning on the other marker listed in the locus.

Table 2. Novel Low-Frequency Coding Variations Showing Significant Association with Coronary Artery Disease.

Locus and SNP	Chromosome and Nucleotide Position*	Allele 1/ Allele 2	Frequency of Allele 1 %	Functional Effect	Stage	Odds Ratio†	P Value
<i>SVEP1</i> rs111245230							
	9: 113169775	C/T	3.60	p.D2702G	Discovery	1.14	1.1×10 ⁻⁷
					Replication	1.13	1.0×10 ⁻³
					Combined‡	1.14	4.2×10 ⁻¹⁰
<i>ANGPTL4</i> rs116843064							
	19: 8429323	A/G	2.01	p.E40K	Discovery	0.87	3.0×10 ⁻⁵
					Replication	0.86	3.4×10 ⁻⁴
					Combined‡	0.86	4.0×10 ⁻⁸

* Chromosome numbers and positions refer to genome build GRCh37.

† Odds ratios are for the development of disease in carriers of allele 1. P values for testing differences between the effects observed in the discovery and replication results were 0.73 for rs111245230 and 0.11 for rs116843064 (Cochran's Q test for heterogeneity).

‡ The combined stage refers to a meta-analysis that included 72,868 patients with coronary artery disease and 120,770 controls free from the disease.

and S6 in the Supplementary Appendix for details). Variants in two genes showed association in the replication set and reached the genome-wide level of significance ($P < 7.7 \times 10^{-8}$) in the combined data set (Table 2). A missense variant (encoding p.D2702G; minor-allele frequency, 3.60%) in the gene *SVEP1* (which encodes sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1) was associated with an increased risk of coronary artery disease, and a low-frequency missense variant (encoding p.E40K; minor-allele frequency, 2.01%) in *ANGPTL4*, which encodes angiotensin-like 4, was associated with protection against coronary artery disease (Table 2).

ASSOCIATION BETWEEN VARIANTS AND RISK FACTORS FOR CORONARY ARTERY DISEASE

We next examined the associations of the *SVEP1* p.D2702G and *ANGPTL4* p.E40K variants with plasma lipid levels, blood pressure, and type 2 diabetes to determine whether their associations with coronary artery disease could potentially be mediated through an effect on these known risk factors. To minimize any potential effect of case-control ascertainment in testing the association with plasma lipid levels, we restricted our analysis to 10,088 controls who were free from coronary artery disease and to population-based samples with available lipid data from the discovery cohort. The tests of association with

Table 3. Association between Low-Frequency Variants and Traditional Risk Factors.*

Variant and Risk Factor	Effect (95% CI)†	P Value
<i>SVEP1</i> rs111245230 C allele		
LDL cholesterol	0.011 (-0.066 to 0.088)	0.78
HDL cholesterol	0.023 (-0.054 to 0.099)	0.56
Triglycerides	0.050 (-0.025 to 0.125)	0.19
Systolic blood pressure	0.94 (0.58 to 1.3)	3.0×10^{-7}
Diastolic blood pressure	0.57 (0.35 to 0.79)	4.4×10^{-7}
Type 2 diabetes	1.13 (1.10 to 1.16)	0.0062
<i>ANGPTL4</i> rs116843064 A allele		
LDL cholesterol	-0.064 (-0.153 to 0.025)	0.16
HDL cholesterol	0.295 (0.206 to 0.384)	8.2×10^{-11}
Triglycerides	-0.335 (-0.424 to -0.246)	1.6×10^{-13}
Systolic blood pressure	-0.18 (-0.66 to 0.30)	0.46
Diastolic blood pressure	-0.13 (-0.43 to 0.16)	0.38
Type 2 diabetes	0.90 (0.87 to 0.92)	0.028

* Shown in the table are low-frequency variants associated with coronary artery disease that are outside of known loci that were previously identified in genome-wide association studies. CI denotes confidence interval, HDL high-density lipoprotein, and LDL low-density lipoprotein.

† The effect is the difference per each copy of the allele in units of standard deviation change (in LDL cholesterol level, HDL cholesterol level, and the natural logarithm of triglyceride level), millimeters of mercury (for systolic and diastolic blood pressure), or the odds ratio for disease (for type 2 diabetes) for carriers as compared with noncarriers.

blood pressure were performed on a set of 146,562 samples from the CHARGE+ consortium, and the results for tests of association with type 2 diabetes were extracted from publicly available databases. Additional details of the sources and samples for these analyses of risk factors are provided in Tables S4 and S5 in the Supplementary Appendix.

We found a significant association between *SVEP1* p.D2702G and blood pressure (Table 3, and Table S7 in the Supplementary Appendix). The allele associated with an increased risk of coronary artery disease was also associated with higher systolic blood pressure (0.94 mm Hg higher for each copy of the allele among allele carriers, $P=3.0 \times 10^{-7}$) and higher diastolic blood pressure (0.57 mm Hg higher for each copy of the allele among allele carriers, $P=4.4 \times 10^{-7}$). We did not find an association between *SVEP1* p.D2702G and any plasma lipid trait. In contrast, *ANGPTL4* p.E40K was not associated with blood pressure but instead was found to be associated

with significantly lower levels of triglycerides (approximately 0.3 standard deviation units lower for each copy of the allele among allele carriers, $P=1.6 \times 10^{-13}$) (Table 3) and with higher levels of high-density lipoprotein (HDL) cholesterol (approximately 0.3 standard deviation units higher for each copy of the allele among allele carriers, $P=8.2 \times 10^{-11}$) (Table 3). In a conditional analysis, these effects appeared to be at least partially independent of each other (Table S8 in the Supplementary Appendix). We did not observe any significant association between *ANGPTL4* p.E40K and LDL cholesterol level (Table 3). Both *SVEP1* p.D2702G and *ANGPTL4* p.E40K were nominally associated with type 2 diabetes in a direction concordant with the associated risk of coronary artery disease.

ANGPTL4 LOSS-OF-FUNCTION MUTATIONS, PLASMA LIPID LEVELS, AND CORONARY ARTERY DISEASE

The finding that a missense allele in *ANGPTL4* reduced the risk of coronary artery disease, potentially by reducing triglyceride levels, raised the possibility that complete loss-of-function variants in *ANGPTL4* may have an even more dramatic effect on triglyceride concentrations and the risk of coronary artery disease. We therefore examined sequence data for the seven protein-coding exons of *ANGPTL4* in 6924 patients with early-onset myocardial infarction and 6834 controls free from coronary artery disease (details of the patients and controls are provided in Table S3 in the Supplementary Appendix). We discovered a total of 10 variants that were predicted to lead to loss of gene function (Fig. 1A, and Table S9 in the Supplementary Appendix), carried by 28 heterozygous persons; no homozygous or compound heterozygous persons were discovered. Carriers of loss-of-function alleles had significantly lower levels of triglycerides than did noncarriers (a mean of 35% lower among carriers, $P=0.003$) (Fig. 1B, and Table S10 in the Supplementary Appendix), with no significant difference in LDL or HDL cholesterol levels. Moreover, we found a lower risk of coronary artery disease among carriers of loss-of-function alleles (9 carriers among 6924 patients vs. 19 carriers among 6834 controls; odds ratio for disease, 0.47; $P=0.04$) (Table S11 in the Supplementary Appendix). A similar investigation was performed for the 48 protein-coding exons of *SVEP1*; however, only 3 loss-of-function allele carriers were

discovered (2 carriers among 6924 patients vs. 1 carrier among 6834 controls).

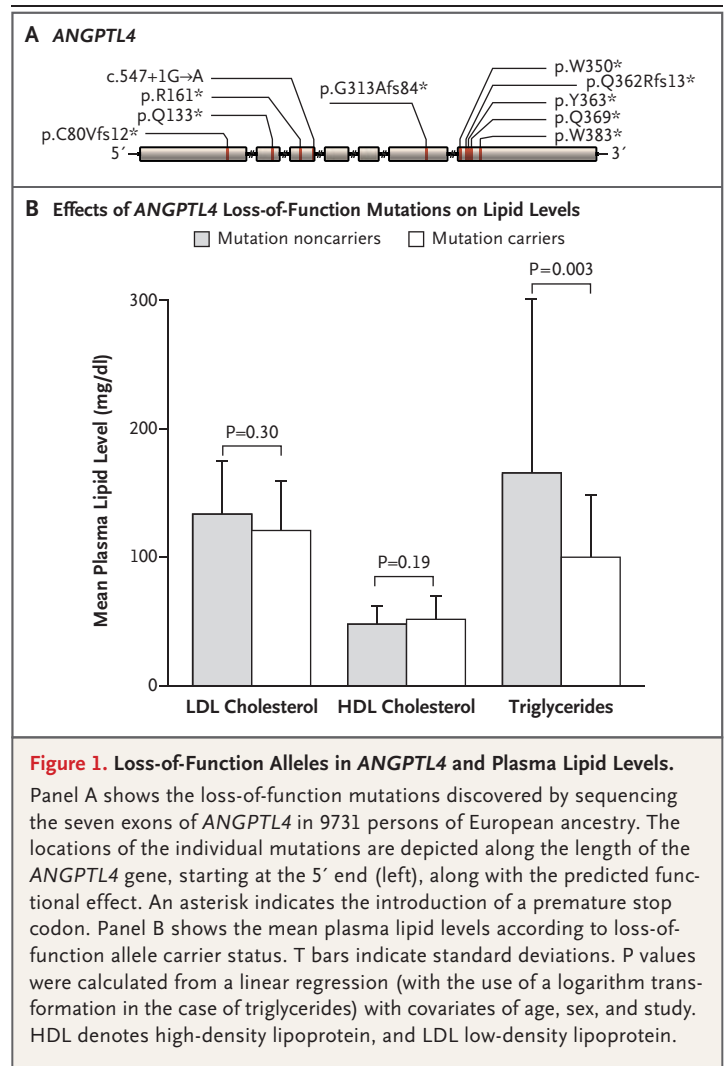
CODING VARIATION IN LPL AND THE RISK OF CORONARY ARTERY DISEASE

On the basis of the fact that a loss of ANGPTL4 function was associated with reduced risk of coronary artery disease and that ANGPTL4 inhibits lipoprotein lipase (LPL), one would expect a gain of LPL function to also be associated with a lower risk of coronary artery disease, whereas a loss of LPL function would be expected to be associated with a higher risk. In observations consistent with these expectations, we found a low-frequency missense variant in *LPL* on the exome array that was associated with an increased risk of coronary artery disease (p.D36N; minor-allele frequency, 1.9%; odds ratio for disease, 1.13; $P=2.0\times 10^{-4}$) (Table S12 in the Supplementary Appendix); previous studies have shown that this allele (also known as p.D9N) is associated with LPL activity that is 20% lower in allele carriers than in noncarriers.⁸ We also identified a nonsense mutation in *LPL* on the exome array that was significantly associated with a reduced risk of coronary artery disease (p.S447*; minor-allele frequency, 9.9%; odds ratio, 0.94; $P=2.5\times 10^{-7}$) (Table S12 in the Supplementary Appendix). Contrary to most instances in which the premature introduction of a stop codon leads to loss of gene function, this nonsense mutation, which occurs in the penultimate codon of the gene, paradoxically induces a gain of LPL function.⁹

DISCUSSION

Through large-scale exomewide screening, we identified a low-frequency coding variant in *ANGPTL4* that was associated with protection against coronary artery disease and a low-frequency coding variant in *SVEP1* that was associated with an increased risk of the disease. Moreover, our results highlight LPL as a significant contributor to the risk of coronary artery disease and support the hypothesis that a gain of LPL function or loss of ANGPTL4 inhibition protects against the disease.

ANGPTL4 has previously been found to be involved in cancer pathogenesis and wound healing.¹⁰ Previous functional studies also revealed that ANGPTL4 regulates plasma triglyceride concentration by inhibiting LPL.¹¹ The minor



allele at p.E40K has previously been associated with lower levels of triglycerides and higher levels of HDL cholesterol.¹² We now provide independent confirmation of these lipid effects. In vitro and in vivo experimental evidence suggests that the lysine allele at p.E40K results in destabilization of ANGPTL4 after its secretion from the cell in which it was synthesized. It may be that the p.E40K variant leads to increases in the enzymatic activity of LPL because of this destabilization.¹³ Previous, smaller studies produced conflicting results regarding p.E40K and the risk of coronary artery disease^{14,15}; we now provide robust support for an association between p.E40K and a reduced risk of coronary artery disease.

To provide confirmatory orthogonal evidence that a loss of ANGPTL4 function is associated

with a decreased risk of coronary artery disease, we searched for loss-of-function mutations in this gene. We found that *ANGPTL4* loss-of-function mutations were associated with substantially lower triglyceride levels (35% lower than in persons who were not carriers of a loss-of-function mutation), and we also found that these loss-of-function alleles were associated with a 53% lower risk of coronary artery disease. The identification by Dewey et al.¹⁶ of additional *ANGPTL4* inactivating mutation carriers, now reported in the *Journal*, provides further evidence of the association between a loss of *ANGPTL4* function and lower triglyceride levels and a reduced risk of coronary artery disease.

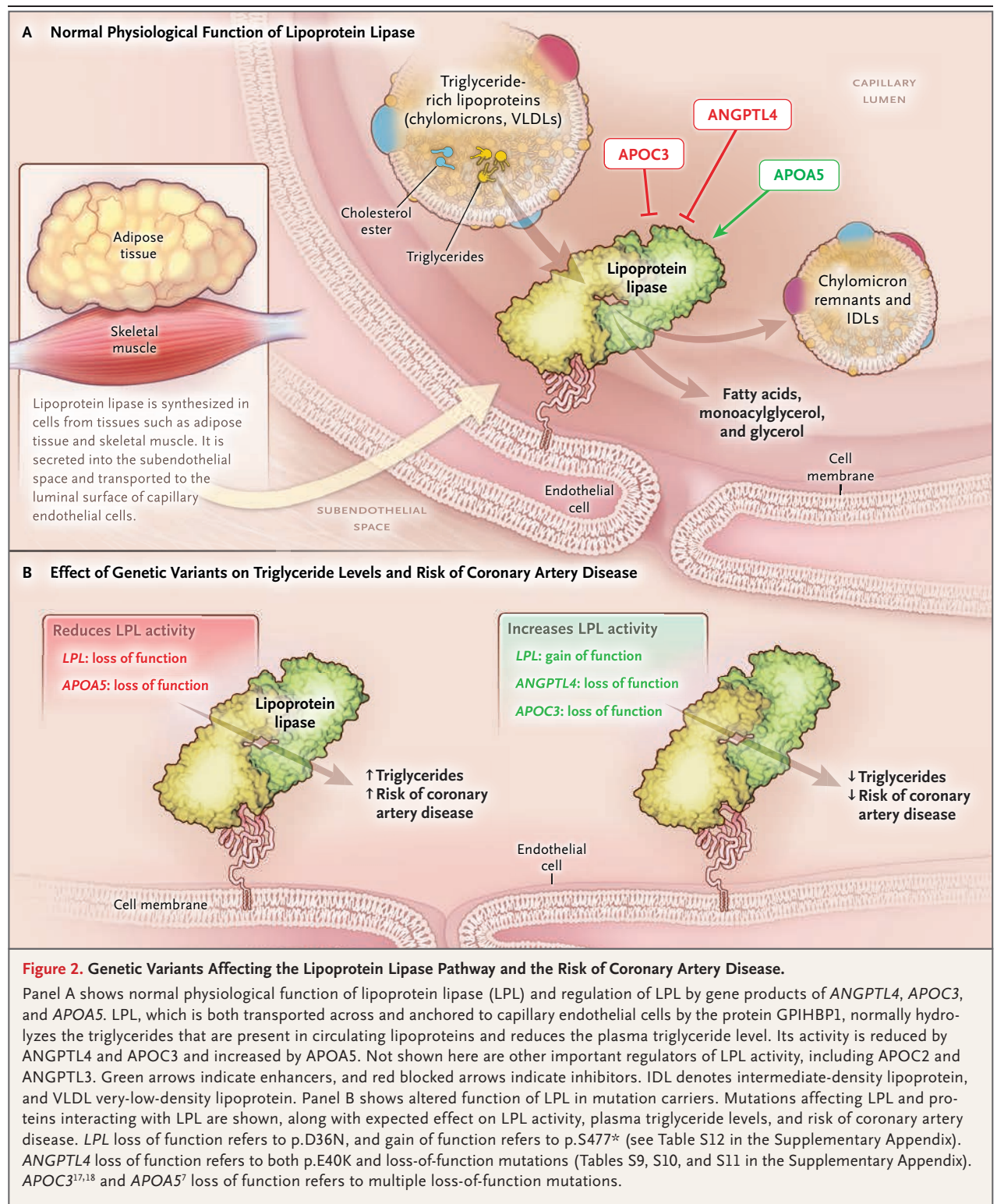
Because *ANGPTL4* inhibits LPL, we examined coding variants in *LPL* that were present on the exome array. A gain-of-function mutation in *LPL* was found to be associated with protection against coronary artery disease, whereas a partial loss-of-function mutation was associated with increased risk. These data corroborate evidence that has connected the risk of myocardial infarction to coding-sequence mutations in two other genes encoding proteins that modulate LPL activity: apolipoprotein A5⁷ and apolipoprotein C3.^{17,18} Thus, the available human genetic evidence, including our current findings, is coalescing around the conclusion that, in addition to elevated levels of LDL cholesterol, disordered metabolism of triglyceride-rich lipoproteins through the LPL pathway contributes substantially to coronary artery disease (Fig. 2). These data also support the hypothesis that therapeutic modulation of this pathway — either by direct enhancement of LPL activity or by blocking of the effects of natural LPL inhibitors, such as *ANGPTL4* (Fig. 2B) — should reduce both triglyceride levels and the risk of coronary artery disease. Studies involving inhibitors of APOC3,¹⁹ of *ANGPTL4*,^{16,20} or of other inhibitory regulators in the LPL pathway will be needed to address this possibility.

The identification of a disease-associated missense variant in *SVEP1* points to a potentially novel genetic mechanism leading to atherosclerosis. *SVEP1* is a cell-adhesion molecule that acts as a ligand for integrin $\alpha 9\beta 1$ ²¹; knockdown of *SVEP1* alters inflammatory signaling in a cellular model of sepsis.²² Although we observed that the low-frequency variant that was associated with

an increased risk of coronary artery disease was also associated with significantly higher blood pressure, the latter effect was modest as compared with that found in a previous study, in which a genetically mediated 1.6-mm Hg increase in systolic blood pressure was associated with a 10% increased risk of coronary artery disease²³ (in our study, *SVEP1* p.D2702G was associated with an approximately 1-mm Hg increase in systolic blood pressure but a 14% increased risk of coronary artery disease). Thus, the exact mechanism by which *SVEP1* contributes to coronary artery disease and the extent to which hypertension and diabetes mediate this risk remain to be clarified.

Several limitations of our study deserve consideration. In our samples, approximately three quarters of the variants on the array were either monomorphic (i.e., only the human reference allele was observed among the persons in the discovery cohort) or very rare (frequency below 0.1%); therefore, we cannot make any estimates of their contribution to the risk of coronary artery disease. Moreover, we estimate that the Illumina HumanExome BeadChip, version 1.0, covers only approximately 80% of coding variants in persons of European ancestry that have an allele frequency of 0.1% or higher (Fig. S1 in the Supplementary Appendix). Furthermore, despite a combined sample size of more than 120,000 persons in the discovery cohort, we had only 80% power to detect an odds ratio of approximately 1.5 for variants with a minor-allele frequency of 0.5% (or an odds ratio of approximately 2.0 for variants with a minor-allele frequency of 0.1%) at the required stringent level of significance (Fig. S2 in the Supplementary Appendix). Hence, analyses of even larger data sets and more comprehensive coverage of exonic variants may reveal further coding variants associated with the risk of coronary artery disease.

In summary, through large-scale exomewide screening, we identified several low-frequency coding variants that are associated with either an increased risk of or protection against coronary artery disease. Our results highlight the LPL pathway as a significant contributor to the risk of coronary artery disease and support speculation that therapeutic modulation of this pathway might be protective against coronary artery disease.



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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

APPENDIX

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