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Research Article

Genetics

Elevated plasma concentrations of HDL cholesterol (HDL-C) are associated with protection from atherosclerotic cardiovascular disease. Animal models indicate that decreased expression of endothelial lipase (*LIPG*) is inversely associated with HDL-C levels, and genome-wide association studies have identified *LIPG* variants as being associated with HDL-C levels in humans. We hypothesized that loss-of-function mutations in *LIPG* may result in elevated HDL-C and therefore performed deep resequencing of *LIPG* exons in cases with elevated HDL-C levels and controls with decreased HDL-C levels. We identified a significant excess of nonsynonymous *LIPG* variants unique to cases with elevated HDL-C. In vitro lipase activity assays demonstrated that these variants significantly decreased endothelial lipase activity. In addition, a meta-analysis across 5 cohorts demonstrated that the low-frequency Asn396Ser variant is significantly associated with increased HDL-C, while the common Thr111Ile variant is not. Functional analysis confirmed that the Asn396Ser variant has significantly decreased lipase activity both in vitro and in vivo, while the Thr111Ile variant has normal lipase activity. Our results establish that loss-of-function mutations in *LIPG* lead to increased HDL-C levels and support the idea that inhibition of endothelial lipase may be an effective mechanism to raise HDL-C.

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Loss-of-function variants in endothelial lipase are a cause of elevated HDL cholesterol in humans

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Elevated plasma concentrations of HDL cholesterol (HDL-C) are associated with protection from atherosclerotic cardiovascular disease. Animal models indicate that decreased expression of endothelial lipase (*LIPG*) is inversely associated with HDL-C levels, and genome-wide association studies have identified *LIPG* variants as being associated with HDL-C levels in humans. We hypothesized that loss-of-function mutations in *LIPG* may result in elevated HDL-C and therefore performed deep resequencing of *LIPG* exons in cases with elevated HDL-C levels and controls with decreased HDL-C levels. We identified a significant excess of nonsynonymous *LIPG* variants unique to cases with elevated HDL-C. In vitro lipase activity assays demonstrated that these variants significantly decreased endothelial lipase activity. In addition, a meta-analysis across 5 cohorts demonstrated that the low-frequency Asn396Ser variant is significantly associated with increased HDL-C, while the common Thr111Ile variant is not. Functional analysis confirmed that the Asn396Ser variant has significantly decreased lipase activity both in vitro and in vivo, while the Thr111Ile variant has normal lipase activity. Our results establish that loss-of-function mutations in *LIPG* lead to increased HDL-C levels and support the idea that inhibition of endothelial lipase may be an effective mechanism to raise HDL-C.

Introduction

Elevated levels of HDL cholesterol (HDL-C) are inversely associated with atherosclerotic cardiovascular risk, independent of LDL (1). The heritability of HDL-C is approximately 50% (2), with significant influence from environmental factors such as physical activity, alcohol consumption, and smoking (3). Based on the study of monogenic conditions, genetic causes of low HDL-C levels include mutations in *ABCA1* (4–7), *APOA1* (7), and lecithin-cholesterol acyltransferase (*LCAT*) (7, 8), whereas genetic causes of high HDL-C levels include mutations in cholesterol ester transfer protein (*CETP*) (9). Homozygous *CETP* deficiency is found predominantly in Japanese individuals, and

mutations in other genes are likely to contribute to the phenotype of elevated HDL.

Endothelial lipase (EL; *LIPG*) is an HDL candidate gene, in which loss-of-function mutations are hypothesized to result in elevated HDL-C concentration. EL is a member of the triglyceride lipase family of proteins that includes lipoprotein lipase and hepatic lipase, and it exhibits a conserved catalytic triad, heparin-binding properties, lipid-binding domains, and cysteine residues (10). EL primarily hydrolyzes phospholipids with little triglyceride lipase activity (10, 11) and hydrolyzes HDL the most efficiently of all the lipoprotein fractions (12). Overexpression of EL in mice decreases HDL-C levels (13), whereas the inhibition or deletion of EL in mice increases HDL-C levels (14). In baboons, HDL-C levels have also been associated with variation in *LIPG* expression (15).

We previously sequenced the *LIPG* gene in a limited number of individuals with high HDL-C levels and reported 4 nonsynonymous variants: the common Thr111Ile (rs2000813) variant, the low-frequency Asn396Ser variant, and the rare Gly26Ser and Thr298Ser variants (16). The Gly26Ser and Thr298Ser variants were primarily found in African Americans. Early association studies of SNPs in *LIPG* with HDL-C produced conflicting results with regard to Thr111Ile as well as other common non-coding *LIPG* SNPs (3, 17–23). Recently, SNPs near the *LIPG* gene

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: AAV, adeno-associated virus; CAD, coronary artery disease; EL, endothelial lipase; FHS, Framingham Heart Study Offspring cohort; GWAS, genome-wide association study; HDL-C, HDL cholesterol; HHDL, University of Pennsylvania High HDL Cholesterol Study; HPFS, Health Professionals Follow-Up Study; LD, linkage disequilibrium; LDL-C, LDL cholesterol; NHS II, Nurses' Health Study II; PennCATH, University of Pennsylvania Catheterization Study; Penn HDL CC, University of Pennsylvania HDL Case-Control Study; QTDT, quantitative transmission disequilibrium test; SIRCA, Study of Inherited Risk of Coronary Atherosclerosis; UKE Hamburg CC, Universitätsklinikum Hamburg-Eppendorf Case-Control Study.

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**Table 1**
Baseline characteristics

Cohort	HHDL sequencing cohort cases (<i>n</i> = 372)	LHDL sequencing cohort controls (<i>n</i> = 213)	FHS (<i>n</i> = 1,796)	NHS II/HPFS (<i>n</i> = 1,808)	PennCATH (<i>n</i> = 1,598)
Ascertainment	Physician referral, HDL ≥ 95th PCTL	Physician referral, HDL ≤ 25th PCTL	Community-based, prospective cohort	Healthy participants in cross-sectional study	Catheterization lab-based recruitment
Mean age (yr)	59.8 ± 11.4	58.3 ± 14.4	49.8 ± 9.6	57.6 ± 11.8	58.9 ± 10.0
Female (%)	61.3	58.2	51.0	25.7	34.0
HDL (mg/dl)	102.9 ± 20.0	34.3 ± 6.0	50.6 ± 13.2 ^A	55.5 ± 19.4	41.5 ± 15.8
BMI (kg/m ²)	23.4 ± 3.3	28.9 ± 5.4	26.6 ± 4.8	25.2 ± 3.6	29.0 ± 6.8
Smoker (%)	10.0	27.3	25.0	7.6	42.2
Alcohol use (%)	88.3	62.8	69.4	81.0	–

Values with “±” are mean ± SD. ^AHDL was averaged over the 7 exams of FHS. LHDL, low HDL; PCTL, percentile; –, data unavailable.

were identified in several human genome-wide association studies (GWASs) as being associated with HDL-C levels (24–29). We hypothesized that rare loss-of-function EL variants are a cause of high HDL-C and that deep medical resequencing efforts would enable us to identify rare mutations that exhibit a substantial phenotypic effect. Thus, we sequenced the 10 exons of *LIPG* in participants of mixed European ancestry with extremely high HDL-C (≥95th percentile) and low HDL-C (≤25th percentile) and assessed the functionality of the newly identified nonsynonymous variants. We also assessed the association of the common Thr111Ile variant and of the low-frequency Asn396Ser variant with HDL-C in several population-based and case-control studies, as well as their lipolytic function.

Results

Identification and functional analysis of rare nonsynonymous *LIPG* variants in participants with high HDL-C. We resequenced all 10 *LIPG* exons in 585 participants of mixed European ancestry from the extreme tails of the HDL-C phenotype distribution (Table 1), drawn from the University of Pennsylvania High HDL Cholesterol Study (HHDL) cohort; participants attending a lipid outpatient clinic, Universitätsklinikum Hamburg-Eppendorf (UKE Hamburg); and a cross-sectional community screening of healthy Canadian volunteers, including 372 individuals with high HDL-C (≥95th percentile for age and gender) and 213 individuals of mixed European descent with low HDL-C (≤25th percentile for age and gender), for a total of 1,170 chromosomes sequenced. Unique nonsynonymous variants identified through *LIPG* exon resequencing were exclusively found in the group with high HDL-C. In total,

10 rare nonsynonymous variants (7 unique) were identified in participants with high HDL-C compared with none in the participants with low HDL-C (Table 2). This represents a significant excess of nonsynonymous variants in the group with high HDL-C compared with the group with low HDL-C ($P = 0.02$; Fisher exact test). Individual fasting lipid and lipoprotein measurements for participants with rare *LIPG* variants are available in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI37176DS1), along with summary information from sequenced participants not carrying a *LIPG* variant. Additional lipoprotein measurements and NMR lipoprotein profile measurements, when available for a subset of these individuals, are available in Supplemental Table 2.

For variants affecting a single amino acid, computational prediction performed by PolyPhen (30) suggested that most of these variants are possibly or probably damaging to normal EL function (Table 2). PolyPhen was unable to perform predictions for the Fs114DelA or X501Arg variants, which produce multiple amino acid changes. The Fs114DelA variant is a single nucleotide deletion, which results in a frameshift that changes amino acids 115–117 (Asp, Ala, Asn) to Thr, Pro, Met and then truncates the protein with a premature stop codon, which results in a protein with a predicted molecular mass of 13.4 kDa (Figure 1A). This mutation results in termination of the protein well before the catalytic triad, yielding a predictably nonfunctional protein. The X501Arg variant is a mutation in the *LIPG* stop codon, which allows translation to continue for an additional 49 amino acids before reaching the next in-frame stop codon, resulting in a protein with a predicted molecular mass of 62 kDa (the predicted molecular mass of WT

Table 2
Nonsynonymous sequence variants in *LIPG* exons found exclusively in participants with high HDL over the 95th percentile

Nucleotide change ^A	Amino acid change ^B	High HDL	Low HDL	Predicted effect	HDL-C (mg/dl)
592A>*	Fs114DelA	1	0	NA	121
598G>A	Ala116Thr	1	0	Benign	124
778G>A	Gly176Arg	1	0	Probably damaging	133
968T>C	Ile239Thr	1	0	Possibly damaging	113
1276A>G	Met342Val	1	0	Possibly damaging	141
1678C>T	Arg476Trp	4	0	Possibly damaging	98–160
1753T>C	X501Arg	1	0	NA	102

The effect of each amino acid substitution on protein function was predicted with the use of PolyPhen (30). ^ARelative to transcription start site. ^BRelative to translation start site. NA, not applicable; *, deletion.

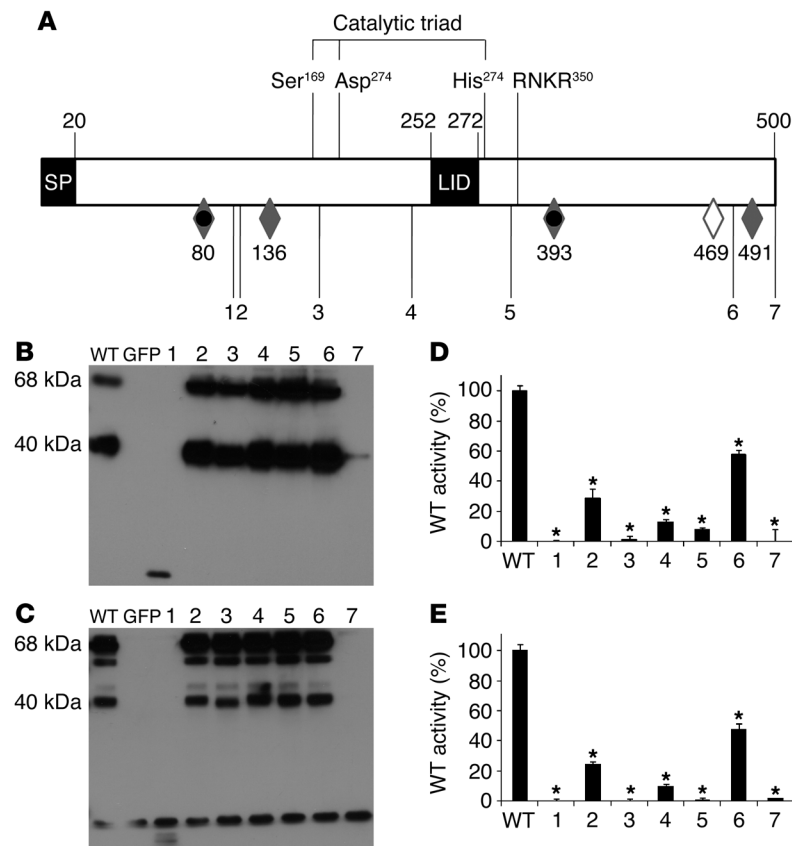


Figure 1 Functional analysis of unique nonsynonymous variants identified in participants with high HDL-C. (A) Schematic of EL protein structure. Diamonds represent potential N-linked glycosylation sites. Filled diamonds indicate validated N-linked glycosylation sites (32), and diamonds with a black circle indicate N-linked glycosylation sites conserved across lipoprotein lipase and hepatic lipase. Numbering indicates amino acid residues and differs from previous publications due to the inclusion of the 20–amino acid signal peptide (SP) in the numbering. RNKR indicates the site of proteolytic cleavage of the 68-kDa full-length EL into a 40-kDa N-terminal fragment and a 28-kDa C-terminal fragment. The “lid” domain and conserved catalytic triad are indicated. Nonsynonymous EL variants identified in participants with high HDL-C were created by site-directed mutagenesis and transiently expressed using 293 cells in the presence of heparin. (B) Expressed EL was visualized by immunoblotting of the conditioned media and (C) cell lysate. (D) Media from cells transiently expressing WT EL or variant EL in the presence of heparin were collected and assayed for the hydrolysis of dipalmitoylphosphatidyl choline and (E) isolated human HDL₃. Activity data for the EL variants were normalized to a percentage of WT EL activity. Assays were performed in triplicate and separate transfections were repeated at least 3 times. Presented results are from a representative experiment. 1, Fs114DelA; 2, Ala116Thr; 3, Gly176Arg; 4, Ile239Thr; 5, Met342Val; 6, Arg476Trp; 7, X501Arg. Error bars indicate ± SD. *P ≤ 0.001 compared with WT.

EL is 56.9 kDa). Nonsynonymous variants were also assessed by aligning the human EL amino acid sequence (AAD30434) with that from various other species, including mouse (NP_034850), rat (AAX11354), chimpanzee (XP_512126), and rhesus monkey (XP_001090086) using ClustalW (31). Four of the nonsynonymous variants were in residues conserved across all of the species and resulted in nonconservative changes (Ala116Thr, Gly176Arg, Ile239Thr, and Met342Val). The remaining variant (Arg476Trp) was in a nonconserved residue, but resulted in a nonconservative change from the amino acid found in any of the species.

To test the activities of the EL variants identified through resequencing, an EL expression plasmid (32) was modified through site-directed mutagenesis to contain each of the EL nonsynonymous variants. Each of the variants was expressed in 293 cells (Figure 1, B and C), except the X501Arg variant, which had only a faint N-terminal 40-kDa band and no detectable full-length band. The activity of each EL variant was tested against the synthetic phospholipid substrate dipalmitoylphosphatidyl choline as previously described (32) and against isolated human HDL₃ as previously described (33). Each assay was performed in triplicate and transfections were repeated at least 3 separate times. Each EL variant identified exclusively in the high HDL group exhibited a significant decrease in lipolytic activity compared with WT EL, with the majority of the variants having nearly undetectable activity in both assays of lipase function (Figure 1, D and E).

Association of common nonsynonymous LIPG variants with HDL-C and functional analysis. Not surprisingly, the common Thr111Ile variant and the low-frequency Asn396Ser variant, both of which we had previously reported in people of mixed European descent (16), were identified in both sequencing groups. Thr111Ile occurred at a similar frequency in both groups, whereas Asn396Ser was found with much greater frequency in the group with high HDL-C (P = 0.007; Table 3). Summary fasting lipid and lipoprotein measurements for sequenced participants with high HDL levels with the Asn396Ser LIPG variant are available in Supplemental Table 1. PolyPhen (30) predicted the Asn396Ser variant to be possibly damaging with regard to normal EL function but predicted the Thr111Ile variant to have a benign effect on normal EL function. Alignment analysis using ClustalW (31) revealed that Asn396 is highly conserved in all species and adjacent to an N-linked glycosylation site that is conserved across the triglyceride lipase family (Figure 2A). In contrast, Thr111 is not conserved across species, with the Ile111 variant found in rodent LIPG genes.

We formally tested for an association of these 2 LIPG variants with HDL-C in the Framingham Heart Study Offspring cohort (FHS; n = 1,796) (Table 4). The Thr111Ile variant was not associated with HDL-C concentration. In contrast, the Asn396Ser variant was found to be highly significantly associated with HDL-C, with the Asn396Ser minor allele associated with an approximately 8 mg/dl increase in HDL-C (P = 4 × 10⁻⁵). Asn396Ser was also significantly associated with increased HDL₂ and HDL₃ subfractions, increased HDL particle size, increased large HDL particles, and increased apoA-I levels (Table 4). The Asn396Ser variant was not associated with LDL, triglyceride, apoB, or sizes of other lipoprotein particles, nor was the Thr111Ile variant associated with any of these additional lipid and lipoprotein measures (Supplemental Table 3).

We attempted to replicate these findings in several other cohorts, including a subset of the Nurses’ Health Study II (NHS II), a subset of the Health Professionals Follow-Up Study

**Table 3**Sequence variants in *LIPG* exons occurring in participants from both extremes of the HDL phenotypic distribution

Nucleotide change ^A	Amino acid change ^B	Genotype	High HDL	Low HDL	Predicted effect	P value
584C>T	Thr111Ile (rs2000813)	Heterozygous	162 (43.5%)	107 (50.2%)	Benign	1.000
		Homozygous	35 (9.4%)	13 (6.1%)		
		MAF	0.31	0.31		
1439A>G	Asn396Ser	Heterozygous	23 (6.2%)	3 (1.4%)	Possibly damaging	0.007
		Homozygous	0	0		
		MAF	0.03	<0.01		

Values represent the numbers of sequence variants, with percentages identified through sequencing 372 high HDL cases and 213 low HDL controls in parentheses. The effect of each amino acid substitution on protein function was predicted with the use of PolyPhen (30). ^ARelative to transcription start site.

^BRelative to translation start site. MAF, minor allele frequency.

(HPFS), the University of Pennsylvania Catheterization Study (PennCATH) cohort, and case-control cohorts from Universitätsklinikum Hamburg-Eppendorf (UKE Hamburg CC) and the University of Pennsylvania HDL Case-Control Study (Penn HDL CC; Tables 1 and 5). Statistical evidence from these cohorts was summarized using a weighted z-statistic meta-analysis. In none of these cohorts was Thr111Ile associated with HDL-C levels. In contrast, Asn396Ser was strongly associated with elevated HDL-C across all cohorts studied (combined P value = 1.7×10^{-8} ; Table 6). Mean lipid values listed by genotype are available in Supplemental Table 3.

Finally, to test for association of the variants with HDL-C levels within families, multigenerational families from our HHDH cohort, with vertical transmission of the minor alleles of the Thr111Ile and Asn396Ser variants, provided DNA for geno-

typing. The Thr111Ile variant was genotyped in 136 available individuals from 32 separate families, yielding 38 informative transmissions, and the Asn396Ser variant was genotyped in 100 available individuals from 20 separate families, yielding 39 informative transmissions. The quantitative transmission disequilibrium test (QTDT) was used to test for an association of both variants with HDL-C within the families. No population stratification was detected in our samples ($P > 0.4$). There was no evidence that Thr111Ile was associated with HDL-C levels within families ($P = 0.365$). In contrast, Asn396Ser was significantly associated with an increase in HDL-C in the within-family association ($P = 0.008$). Family members with the Asn396Ser variant had an increase of approximately 11 mg/dl in HDL-C above family members without the variant (WT = 63.7 ± 17.2 mg/dl, Asn396Ser = 74.9 ± 31.0 mg/dl).

Figure 2

Functional analysis of the common Thr111Ile variant and the low-frequency Asn396Ser variant. (A) Schematic of EL protein structure as described in Figure 1. Thr111Ile and Asn396Ser were created by site-directed mutagenesis and transiently expressed using 293 cells in the presence of heparin. (B) Expressed EL was visualized by immunoblotting of the conditioned media and cell lysate. (C) Media from cells transiently expressing WT EL or variant EL in the presence of heparin were collected and assayed for the hydrolysis of dipalmitoylphosphatidyl choline and (D) isolated human HDL₃. Activity data for the EL variants were normalized to a percentage of WT EL activity. Assays were performed in triplicate, and separate transfections were repeated at least 3 times. Presented results are from a representative experiment. 1, Thr111Ile; 2, Asn396Ser. * $P \leq 0.001$ compared with WT. (E) Somatic gene transfer of WT and Asn396Ser EL into male *Lipg*^{-/-} mice was performed by administering AAV2/8, carrying the designated transgene via i.p. injection. HDL-C was measured at indicated intervals. Triangles, LacZ ($n = 4$); circles, Asn396Ser ($n = 3$); squares, WT ($n = 4$). Error bars indicate \pm SD.

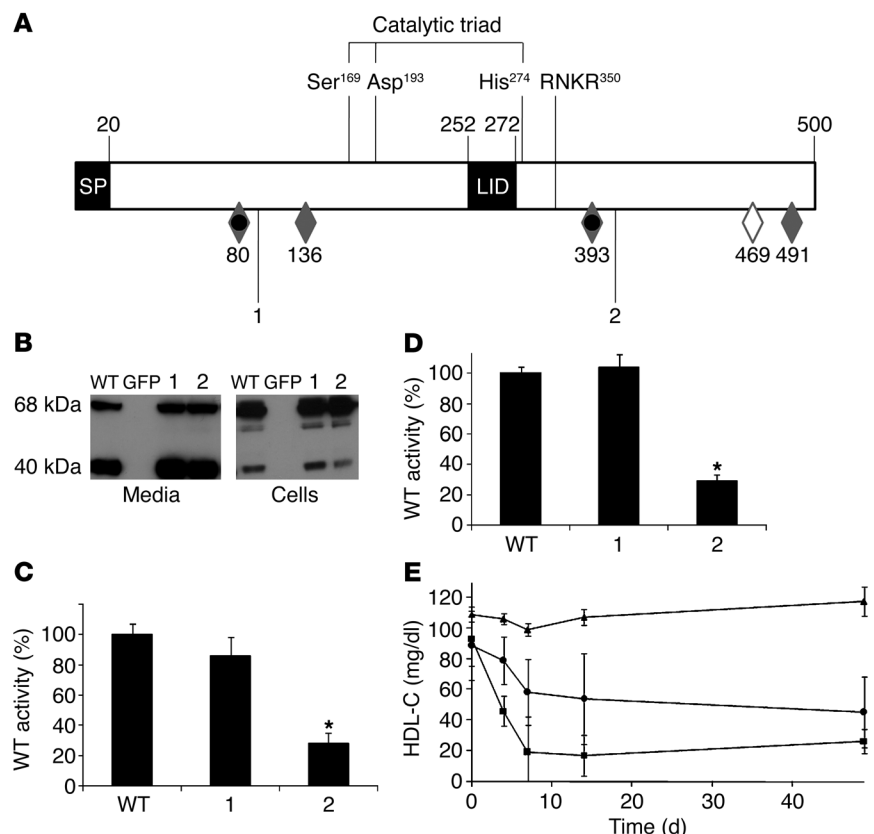




Table 4
FHS association analysis

	Thr111Ile		Asn396Ser	
	Δ SD	P value	Δ SD	P value
HDL-C	-0.003	0.92	0.58	4 × 10 ⁻⁵
HDL ₂	-0.020	0.66	0.35	0.03
HDL ₃	-0.010	0.82	0.57	6 × 10 ⁻⁴
HDL size	-0.020	0.60	0.45	8 × 10 ⁻³
HDL small particle	0.030	0.45	-0.09	0.59
HDL intermediate particle	-0.020	0.63	-0.06	0.74
HDL large particle	-0.004	0.93	0.61	4 × 10 ⁻⁴
apoA-1	-0.060	0.62	0.16	7 × 10 ⁻³

Δ SD represents the proportion of 1 SD change in standardized residual (mean = 0, SD = 1 after adjustment for age, age², BMI, alcohol intake, smoking status, menopause, and hormone replacement therapy separately by gender) per copy of the minor allele. One SD unit in FHS was 13.2 mg/dl.

In order to test their functionality, we created the Thr111Ile and Asn396Ser variants in the EL expression plasmid using site-directed mutagenesis and analyzed their lipolytic activity in vitro (Figure 2, B–D). Consistent with the epidemiologic data, Thr111Ile had lipolytic activity similar to WT EL, whereas Asn396Ser had significantly ($P < 0.001$) reduced lipolytic activity with less than 40% of WT activity. We also created adeno-associated virus (AAV) vectors encoding WT EL and the Asn396Ser variant and injected them into *Lipg*^{-/-} mice to compare their in vivo biological activity against HDL. The Asn396Ser variant was substantially less effective than WT EL at reducing HDL-C levels, consistent with reduced activity against HDL in vivo (Figure 2E).

Evaluation of linkage disequilibrium. Recently, several human GWASs have reported that correlated SNPs 40–65 kb downstream of the *LIPG* gene are associated with HDL-C levels (the most robustly associated being rs2156552 and rs4939883, which are in high linkage disequilibrium [LD] with each other). However, the causative variant is, as of yet, unidentified, and none of these correlated SNPs are in LD with known functional variants in *LIPG* (24–29). HapMap data (release 21) suggests that the Thr111Ile variant is not significantly correlated with the downstream variants (squared correlation coefficient [r^2] = 0.078 – 0.083). Genotyping of these downstream *LIPG* variants performed in the HHDL (rs4939883), Penn HDL CC (rs4939883), and PennCATH (rs2156552) cohorts confirmed that the downstream *LIPG* SNPs

from GWAS reports are not significantly correlated with either the Thr111Ile or the Asn396Ser variants ($r^2 < 0.03$ and $r^2 < 0.01$, respectively) in any of these cohorts (Supplemental Figure 1).

Discussion

While biochemical and animal studies have suggested that EL is an important modulator of HDL-C concentration, definitive evidence that it plays an important role in human HDL metabolism has been notably lacking. Recent GWAS results have suggested that common variation near the *LIPG* locus is associated with HDL-C in humans, although the causal variant or variants are still unidentified. Resequencing of candidate genes in participants at the extremes of a quantitative trait has been shown to efficiently identify both common and functional rare variants of a gene (34) and has been particularly successful at identifying genetic causes of lipid phenotypes (7, 34–36). Here, we report the discovery of rare loss-of-function mutations in *LIPG* in persons with very high HDL-C levels and that a low-frequency variant with reduced activity is significantly ($P = 1.7 \times 10^{-8}$) associated with higher HDL-C.

The first *LIPG* resequencing efforts were performed by our group in a small number ($n = 20$) of participants with high HDL, identifying 4 nonsynonymous variants of EL (16). This study lacked statistical power and did not characterize the activities of the variants. Only 1 nonsynonymous variant, Thr111Ile, was found to be common (minor allele frequency > 0.05), and thus has been the subject of several additional studies of limited power, with equivocal and inconsistent results. Several studies have attempted to determine if there is any effect of the Thr111Ile variant on a variety of cardiovascular measures. Overall, these studies have been underpowered and lack functional evidence to support their conclusions: (a) Ma et al. reported that Thr111Ile was associated with an increase in HDL-C among 372 participants (17); (b) Yamakawa-Kobayashi et al. failed to detect an association of Thr111Ile with HDL-C levels in 340 Japanese children (22); (c) Paradis and colleagues found an association of Thr111Ile with increased levels of the HDL₃ subfraction in 281 females (18); (d) Halverstadt et al. found an association of Thr111Ile with NMR measurements of HDL size in 83 healthy elderly participants but not with overall HDL-C levels (19); (e) Mank-Seymour et al. showed a weak association of Thr111Ile with increased HDL-C among 594 participants (3); (f) Hutter et al. found a weak association of Thr111Ile with HDL-C levels in 541 Japanese Americans (20); (g) Shimizu et al. failed to find an association of Thr111Ile with HDL-C in 107 Japanese acute myocardial infarction cases and controls (23); and (h) Tang et al. found a weak associa-

Table 5
Baseline characteristics

Cohort	UKE Hamburg CC cases (n = 193)	UKE Hamburg CC controls (n = 194)	Penn HDL CC cases (n = 606)	Penn HDL CC controls (n = 437)
Ascertainment	Lipid clinic, HDL ≥ 95th PCTL	Lipid clinic, HDL ≤ 5th PCTL	Physician referral, HDL ≥ 95th PCTL	Family history of premature CAD, HDL ≤ 50th PCTL
Mean age (yr)	45.6 ± 14.7	45.3 ± 12.7	58.8 ± 11.4	47.8 ± 8.1
Female (%)	59.1	38.7	72.8	39.6
HDL (mg/dl)	89.1 ± 19.1	27.3 ± 3.9	98.3 ± 17.5	38.8 ± 7.5
BMI (kg/m ²)	24.1 ± 4.3	29.2 ± 4.5	23.5 ± 11.2	28.5 ± 5.1
Smoker (%)	16.9	47.4	4.0	15.5
Alcohol use (%)	–	–	75.2	66.8

Values with “±” are mean ± SD.



Table 6
Results of association analyses

Population-based cohorts	Statistical measures	FHS	NHS II/HPFS	PennCATH	UKE Hamburg CC ^A	Penn HDL CC ^B	Combined analysis
Thr111Ile	<i>P</i>	0.920	0.18	–	0.55	0.59	0.25
	β	0.000 ^C	0.79 ^D	–	0.12	0.07	–
	SEM	0.038	0.58	–	0.21	0.14	–
Asn396Ser	<i>P</i>	4 × 10 ⁻⁵	0.03	0.020	0.06	5 × 10 ⁻³	1.7 × 10 ⁻⁸
	β	0.580 ^C	5.78 ^D	5.010 ^E	1.59	1.37	–
	SEM	0.142	2.62	2.054	0.84	0.49	–

^ACase-control status adjusted for age, age², gender, BMI, and smoking. ^BCase-control status adjusted for age, age², gender, BMI, smoking, and alcohol.

^CProportion of 1 SD change in standardized residual (mean = 0, SD = 1 after adjustment for age, age², BMI, alcohol intake, smoking status, menopause, and hormone replacement therapy when available separately by gender) per copy of the minor allele. One SD unit in FHS was 13.2 mg/dl. ^DPredicted change in HDL (mg/dl) per copy of the minor allele, adjusted for age, age², alcohol, smoking status, BMI, lab batch, and substudy. ^EPredicted change in HDL (mg/dl) per copy of the minor allele, adjusted for ascertainment group, study age, gender, BMI, and smoking status.

tion of Thr111Ile with increased HDL-C in 265 Chinese coronary artery disease (CAD) cases and controls (21). Our data from a combined sample of 3,845 participants, and functional studies of the variant definitively establish that Thr111Ile is not associated with HDL-C and in vitro studies show that it has normal lipolytic activity. Furthermore, the Thr111Ile variant is not correlated with the downstream *LIPG* SNPs identified in GWASs, $r^2 < 0.1$, according to HapMap data (37) and our own genotyping (Supplemental Figure 1), further confirming that it is not the etiological variant under the HDL-C signal detected near *LIPG* in recent GWASs.

Here, we report an expanded *LIPG* resequencing effort, resequencing the *LIPG* exons on 1,170 chromosomes in participants from both extremes of the HDL concentration distribution. Our resequencing efforts did not identify any nonsynonymous variants strongly correlated with the downstream *LIPG* SNPs identified in the HDL GWASs, and it is unlikely that further resequencing of the *LIPG* locus will reveal any coding variants that can explain this signal. This suggests that the HDL-C peak near *LIPG* in GWASs is not due to a coding variant but rather due to some type of regulatory variant. Interestingly, studies in baboons have suggested that *LIPG* promoter variants are associated with variation in HDL-C levels (15). Ongoing studies in our lab are addressing the role of promoter variants in the human *LIPG* gene and their association with plasma HDL-C levels.

Our results validate deep medical resequencing in extremes of a quantitative phenotype to identify rare mutations that exert a substantial phenotypic effect. Notably, we show that nonsynonymous *LIPG* variants are significantly more common in participants with elevated HDL-C and that these variants are true loss-of-function variants, a finding which mirrors the accumulation of rare variants in *ABCA1*, *LCAT*, and *APOA1* in participants with low HDL-C (7). Furthermore, we establish that while the common Thr111Ile variant is definitively not associated with variation in HDL-C concentration and has normal lipolytic activity, the much less common Asn396Ser is significantly associated with elevated HDL-C concentration, with a *P* value on meta-analysis considered to be significant genome wide ($P = 1.7 \times 10^{-8}$), and that it has substantially reduced lipolytic activity in vitro and in vivo. We further replicated these findings in families with elevated HDL-C, an approach that is robust to population stratification, a problem that theoretically can affect population association studies.

Our results highlight the utility of a functional assay for experimentally testing mutations found upon resequencing. Computational programs such as PolyPhen (30) or SNPs3D (38) primarily

predict changes in protein structure stability. However, they cannot predict specific functional impairment (such as ligand binding, disruption of the catalytic site, disruption of posttranslational modification, or the introduction of some allosteric effects) and may introduce a bias that can obscure the true relationship between the variants and the phenotype of interest when used exclusively. While the majority of the *LIPG* variants we found were correctly predicted by PolyPhen to have a deleterious effect on EL function, likely through decreased protein structure stability, the Ala116Thr variant was incorrectly predicted to be a benign change. It may prove particularly insightful to further characterize this variant, as its significant ($P < 0.001$) reduction in lipolytic activity may result from a specific functional impairment of a novel functional element in EL.

It will be important to use loss-of-function variants in *LIPG* as an approach to assess the effects of lifetime exposure to elevated HDL-C due to reduced EL activity, much as loss-of-function proprotein convertase subtilisin/kexin type 9 (*PCSK9*) variants that reduce LDL cholesterol (LDL-C) concentrations were found to greatly decrease cardiovascular risk (39). However, the low allele frequency of the Asn396Ser variant and its comparatively modest HDL-C effect require a very large number of participants in order to have adequate power to achieve this goal. Specifically, the low-frequency Asn396Ser variant is present in roughly 2.2% of people of mixed European descent, which is lower than the 2.6% of African American participants or the 3.2% of participants of mixed European descent harboring sequence variants of *PCSK9* shown to protect against coronary heart disease. The Asn396Ser variant also appears to have an approximate 10%–16% increase in HDL-C, lower than the 15%–28% decrease in LDL-C from *PCSK9* variants (39). As the minor allele frequency and effect size decrease, increasingly large cohorts are needed to detect a phenotypic effect on cardiovascular disease. We attempted to quantify an effect of the Asn396Ser variant on cardiovascular disease in FHS using a surrogate, namely carotid intimal medial thickness measured by carotid ultrasonography. These analyses showed no significant difference (Supplemental Table 4) but were clearly underpowered. A definitive answer will hopefully come with the widespread use of a cardiovascular candidate gene SNP array (40), being used to genotype multiple cohorts, including the National Heart, Lung and Blood Institute's Candidate-gene Association Resource.

Pharmacologic options to raise HDL-C concentration are currently limited. The recent failure of torcetrapib (41), and the ongoing debate as to whether the increased mortality was an



off-target or mechanism-based effect (42), highlights the need for the development of new pharmacological approaches to raise HDL-C. The increasing costs of developing and testing new drugs, particularly when so many fail human trials even after extensive testing in animal models, necessitates a prioritization of potential targets that are most likely to produce safe and effective drugs. Human genetic data linked to both intermediate phenotypes, such as elevated HDL-C, and outcomes, such as reduced CAD events, offers a powerful validation of a clinical target (43), particularly when individuals with loss-of-function variants appear healthy. Our results provide important human genetic evidence that inhibition of EL would be likely to raise HDL-C concentrations in humans. Whether or not the resulting increase in HDL-C due to EL inhibition will have an impact on cardiovascular outcome remains an open question.

Methods

Materials. Fatty acid-free BSA, heparin, and FBS were purchased from Sigma-Aldrich. DMEM, antibiotic/antimycotic, Lipofectamine, and NuPAGE 10% Bis-Tris gels were purchased from Invitrogen. An HRP-conjugated goat anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories Inc. A polyclonal anti-human EL antibody NB400-118 was purchased from Novus Biologicals.

Research participants for the sequencing cohorts. HHDL is a cross-sectional study of genetic factors contributing to elevated HDL-C levels. Proband with elevated HDL-C (greater than the 75th percentile for age and gender) are identified by physician referrals or through the Hospital of the University of Pennsylvania clinical laboratory. Relatives of HHDL probands are also invited to participate in the study. Participants complete a lifestyle questionnaire and provide a blood sample for the measurement of HDL and other lipid-related traits. Analytical measurements were performed as previously described (44). Plasma total cholesterol, HDL-C, and triglyceride levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems), using Sigma reagents (Sigma-Aldrich). LDL-C was calculated using the Friedewald formula. When triglyceride levels were more than 400 mg/dl, the LDL-C was not calculated. apoA-I and apoB were measured with immunoturbidimetric assays, using reagents from Diasoren Inc. The University of Pennsylvania Institutional Review Board approved the study protocol. Over 2,000 participants have been enrolled in the HHDL study to date, with recruitment ongoing. NMR lipoprotein analyses were performed by Liposcience Inc.

Patients attending the lipid outpatient clinic, UKE Hamburg CC, between 1997 and 2007 have previously been described (45). Informed consent was obtained and the study was approved by the Ethik-Kommission der Ärztekammer, Hamburg, Germany. At the patients' first visit, a detailed case history was taken and biochemical and biometric values were determined. The patients had a 30- to 60-minute session with a dietician who discussed their normal diet and gave dietary advice. Existing therapy was, where possible, discontinued, and at a second visit approximately 6 weeks later, biochemical and biometric values were again determined to provide data under diet/absence of drug therapy. This second lipid value is used in our analysis.

Cases were participants mixed European descent with HDL-C at or above the 95th percentile for age and sex from either study (females, range 75–186 mg/dl; males, range 68–131 mg/dl). Controls were participants of mixed European descent with HDL-C at or below the 25th percentile, excluding individuals with HDL-C below 20 mg/dl to eliminate participants with likely monogenic disorders of lipoprotein metabolism, leading to reduced HDL-C concentration (females, range 20–49 mg/dl; males, range 22–42 mg/dl).

Research participants for the primary community-based cohort. The FHS ($n = 5,124$ participants) was recruited in 1971; the participants have been examined approximately every 4 to 8 years. We studied genotypes in a panel of 1,809 unrelated individuals who provided blood samples for DNA extraction during the sixth examination cycle (1995–1998). HDL measurements were available at up to 7 time points for each individual. We used the HDL mean from the available measures for each individual. HDL₂, HDL₃, HDL size, HDL subfractions, and apoA-I, measured at exam 4, were determined as described previously (46–48). The Institutional Review Board at Boston Medical Center approved the study, and all participants gave written informed consent.

Research participants for the replication cohorts. Our case-control cohorts (Penn HDL CC and UKE Hamburg CC) included cases drawn from HHDL ($n = 602$), controls drawn from the University of Pennsylvania Study of Inherited Risk of Coronary Atherosclerosis (SIRCA) cohort ($n = 437$), and cases and controls drawn from the lipid outpatient clinic, UKE Hamburg CC ($n = 420$; 199 cases, 221 controls), respectively. HHDL and UKE Hamburg CC cohorts are described above. SIRCA is a cross-sectional study of factors associated with coronary artery calcification in asymptomatic participants, recruited on the basis of a family history of premature CAD. Study design and initial findings have been previously published (49).

Our population-based replication cohorts included a subset of individuals from the NHS II/HPFS ($n = 1,808$), which have been reported on previously (50, 51), and the PennCATH ($n = 1,598$). PennCATH is composed of consecutive participants undergoing coronary angiography at University of Pennsylvania Health System hospitals and has been previously described (52).

Sequencing, variant discovery, and genotyping. LIPG exons and exon-intron junctions were sequenced as previously described (16). All identified variants were confirmed by resequencing, Taqman custom genotyping assays (Applied Biosystems), or RFLP analysis. Genotyping in HHDL, SIRCA, FHS, and NHS II/HPFS was performed by Taqman custom genotyping assays. Genotyping in PennCATH was performed using the Illumina IBC Candidate Gene array, version 1 (40). Genotyping in the UKE Hamburg CC cohort was by RFLP as described previously (16).

Preparation of plasmids for in vitro analysis. The cDNA for human EL (NM006033) was inserted into the pcDNA3 mammalian expression vector (Invitrogen). Mutagenesis of the EL expression plasmid to introduce each of the nonsynonymous variants was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with previously described polymerase chain reaction conditions (32). To create the X501Arg variant expression plasmid, the cDNA for human EL (BC060825) was altered through site-directed mutagenesis and the lengthened coding region was inserted into the pcDNA3 mammalian expression vector. Plasmids were sequenced after site-directed mutagenesis to confirm the change and to rule out additional, nonspecific changes.

Cell culture. HEK293 cells were cultured in DMEM containing 10% FBS and 1% antibiotic/antimycotic. For lipase activity assays, cells were grown to 90% confluency (in 100-mm dishes), and 5.85 μ g of plasmid-expressing EL was transfected using Lipofectamine (Invitrogen), according to the manufacturer's instructions. At 24 hours after the transfection, media were removed and replaced with serum-free media containing 10 U/ml heparin. To promote lipase dissociation from cells, at 47.5 hours after the transfection, an additional 10 U/ml heparin was added to the media in each plate. At 48 hours after the transfection, media were collected and centrifuged at 100 g for 5 minutes to remove any cell debris. The supernatant was divided into aliquots and stored at -80°C .

Protein analyses. Proteins in conditioned media samples from transfected cells were separated on NuPAGE 10% Bis-Tris gels (Invitrogen), and gels were transferred to PVDF membranes. PVDF membranes were probed



using a 1:1,000 dilution of the rabbit anti-human EL polyclonal antibody and a 1:5,000 dilution of HRP-conjugated anti-rabbit IgG and visualized using ECL Western Blotting Detection Reagents (GE Healthcare).

Lipase assays. Phospholipase assays, using the glycerol-stabilized substrate dipalmitoylphosphatidyl choline, were performed as previously described (12) in triplicate. Each transfection was repeated at least 3 separate times. HDL₃ was isolated by potassium bromide density gradient ultracentrifugation (53) from pooled human plasma, and assays of lipoprotein lipid hydrolysis by recombinant lipases were performed as described previously (33) in triplicate. The free fatty acids generated by the hydrolysis of lipoproteins were measured in triplicate using a NEFA C kit (Waco Pure Chemical Industries) according to the manufacturer's instructions. All activity data were normalized to the percentage of WT lipase.

AAV vector construction. The cis-plasmids pAAV.TBG.EL.WT and pAAV.TBG.EL.Asn396Ser were created by cloning the coding region from the EL expression plasmid described above into the pAAV.TBG cis-plasmid (54). The AAV2/8-EL.WT and AAV2/8-EL.Asn396Ser viruses were produced by triple transfection into 293 cells, with an adenovirus helper plasmid and a chimeric packaging construct in which the AAV2 *rep* gene is fused with the *cap* gene of serotype 8. A control *LacZ* gene was packaged into an AAV, also of serotype 2/8. Genome copy was determined by TaqMan (Applied Biosystems) analysis.

Animals. Male 8- to 10-week-old *Lipg*^{-/-} mice on a C57BL/6 background (14) were administered AAV at 3×10^{10} genome copies in phosphate-buffered saline by i.p. injection. Blood samples were collected at several time points thereafter under anesthesia with isoflurane (Vedeco Inc.). The University of Pennsylvania IACUC committee approved this protocol.

Plasma analysis. Blood samples were collected from the retro-orbital venous plexus puncture using heparinized capillary tubes (Fisher Scientific). Plasma was separated by centrifugation at 7,000 g for 10 minutes. HDL-C was measured enzymatically on a Cobas Fara II autoanalyzer (Roche Diagnostic Systems Inc.) using Wako Chemicals reagents.

Statistics. Functional prediction of nonsynonymous variants was performed using PolyPhen (30), and protein alignments were produced using ClustalW (31). Numbers of variants identified in each sequencing group were compared using Fisher exact test.

Triglyceride values were log transformed. All genetic analyses assumed an additive model of inheritance. FHS was analyzed, using multivariable linear regression of the residuals of lipid phenotypes, separately by gender, after adjustment for means of age, age², BMI, alcohol intake, and smoking status. Additionally, in women, the proportion of exams that a woman was menopausal and on hormone replacement therapy were included as covariates. NHS II/HPFS was analyzed using multivariable linear regression of HDL-C (mg/dl), adjusted for age, age², alcohol, smoking status, BMI, lab batch, and substudy. PennCATH was analyzed using multivariable linear regression of HDL-C (mg/dl), after adjustment for ascertainment group, age, gender, BMI, and smoking status. For case-control cohorts, genotype frequencies in the respective populations were analyzed using a generalized linear model with adjustment for age, age², gender, BMI, and smoking status. The Penn HDL CC cohort also included an adjustment for alcohol use.

Each study was analyzed separately, and to summarize the data, we performed a meta-analysis as implemented in the METAL software (28). As each study was analyzed slightly differently, we chose to use a weighted z-statistic meta-analysis, which uses the *P* value and the direction of the effect to calculate a z-statistic. Individual z-statistics were combined and an

overall z-statistic was calculated as a weighted sum of each of the individual z-statistics, where weights were proportional to the square root of the number of individuals examined in each sample and were selected such that the squared weights sum to 1. The corresponding *P* value was then calculated.

The QTDT (55, 56) was used to test association of *LIPG* variants with HDL-C levels in extended pedigrees. The orthogonal model implemented in QTDT was applied in the variance component framework to describe the non-shared environment, common environment, additive, and polygenic effects that are similar among individuals in a pedigree, after having estimated identity by descent probability using simwalk2 for scoring allelic transmission that accommodates families of any size and uses all available genotype data (57). HDL-C was normally distributed throughout our pedigrees and not transformed. We used age, age², and sex as covariates. Due to the small samples sizes, *P* values for association tests were calculated based on 1,000 Monte Carlo simulations. The empirical significance level was computed from 1,000 Monte Carlo simulations and determined to be 0.018.

LD calculations and visualization were performed using the Haploview software (58). In vitro assays and comparisons between groups of mice were analyzed using an unpaired 2-tailed Student's *t* test. *P* values of less than 0.05 were considered to be statistically significant.

Statistical methods for analyzing carotid intimal medial thickness in FHS are described in the Supplemental Methods.

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