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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:41263119

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Loss-of-function variants in endothelial lipase are a cause of elevated HDL cholesterol in humans

Andrew C. Edmondson,1 Robert J. Brown,1 Sekar Kathiresan,2,3 L. Adrienne Cupples,4 Serkalem Demissie,4 Alisa Knodle Manning,4 Majken K. Jensen,5 Eric B. Rimm,5,6 Jian Wang,7 Amrith Rodrigues,1 Vaneeta Bamba,1 Sumeet A. Khetarpal,1 Megan L. Wolfe,1 Stephanie DerOhannessian,1 Mingyao Li,8 Muredach P. Reilly,1,9 Jens Aberle,10 David Evans,10 Robert A. Hegele,7 and Daniel J. Rader1,9

1Institute for Translational Medicine and Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. 2Cardiovascular Research Center and Center for Human Genetic Research, Massachusetts General Hospital, and Broad Institute Center for Genotyping and Analysis of Harvard and Massachusetts Institute of Technology, Cambridge, Massachusetts, USA. 3Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA. 4Boston University and Framingham Heart Study, Boston, Massachusetts, USA. 5Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts, USA. 6Department of Medicine, Harvard Medical School, Channing Laboratory, Brigham and Women’s Hospital, and Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA. 7Robarts Research Institute and Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada. 8Department of Biostatistics and Epidemiology and 9Cardiovascular Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. 10Endokrinologie und Stoffwechsel, Medizinische Klinik III, Zentrum für Innere Medizin, Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany.

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 lecinthin-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 noncoding 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, aden-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; HPPS, Health Professionals Follow-Up Study; LD, linkage disequilibrium; LDL-C, LDL cholesterol; NHS II, Nurses’ Health Study II; PennCATH, University of Pennsylvania Catherization 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.

Citation for this article: /J Clin. Invest. 119:1042-1050 (2009). doi:10.1172/JCI37176.
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), for a total of 1,170 chromosomes sequenced. Unique non-synonymous variants identified through 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 1**

Baseline characteristics

<table>
<thead>
<tr>
<th>Cohort</th>
<th>HHDL sequencing cohort cases (n = 372)</th>
<th>LHDL sequencing cohort controls (n = 231)</th>
<th>FHS (n = 1,796)</th>
<th>NHS II/HPFS (n = 1,808)</th>
<th>PennCATH (n = 1,598)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascertainment</td>
<td>Physician referral, HDL ≥ 95th PCTL</td>
<td>Physician referral, HDL ≤ 25th PCTL</td>
<td>Community-based, prospective cohort</td>
<td>Healthy participants in cross-sectional study</td>
<td>Catheterization lab–based recruitment</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>59.8 ± 11.4</td>
<td>58.3 ± 14.4</td>
<td>49.8 ± 9.6</td>
<td>57.6 ± 11.8</td>
<td>58.9 ± 10.0</td>
</tr>
<tr>
<td>Female (%)</td>
<td>61.3</td>
<td>58.2</td>
<td>51.0</td>
<td>25.7</td>
<td>34.0</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>102.9 ± 20.0</td>
<td>34.3 ± 6.0</td>
<td>50.6 ± 13.2A</td>
<td>55.5 ± 19.4</td>
<td>41.5 ± 15.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4 ± 3.3</td>
<td>28.9 ± 5.4</td>
<td>26.6 ± 4.8</td>
<td>25.2 ± 3.6</td>
<td>29.0 ± 6.8</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>10.0</td>
<td>27.3</td>
<td>25.0</td>
<td>7.6</td>
<td>42.2</td>
</tr>
<tr>
<td>Alcohol use (%)</td>
<td>88.3</td>
<td>62.8</td>
<td>69.4</td>
<td>81.0</td>
<td>–</td>
</tr>
</tbody>
</table>

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

**Table 2**

Nonsynonymous sequence variants in LIPG exons found exclusively in participants with high HDL-C over the 95th percentile

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>High HDL</th>
<th>Low HDL</th>
<th>Predicted effect</th>
<th>HDL-C (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>592A&gt;^T</td>
<td>Fs114DeLA</td>
<td>1</td>
<td>0</td>
<td>NA</td>
<td>121</td>
</tr>
<tr>
<td>598G&gt;A</td>
<td>Ala116Thr</td>
<td>1</td>
<td>0</td>
<td>Benign</td>
<td>124</td>
</tr>
<tr>
<td>778G&gt;A</td>
<td>Gly176Arg</td>
<td>1</td>
<td>0</td>
<td>Probably damaging</td>
<td>133</td>
</tr>
<tr>
<td>968T&gt;C</td>
<td>Ile239Thr</td>
<td>1</td>
<td>0</td>
<td>Possibly damaging</td>
<td>113</td>
</tr>
<tr>
<td>1276A&gt;G</td>
<td>Met342Val</td>
<td>1</td>
<td>0</td>
<td>Possibly damaging</td>
<td>141</td>
</tr>
<tr>
<td>1678C&gt;T</td>
<td>Arg476Trp</td>
<td>4</td>
<td>0</td>
<td>Possibly damaging</td>
<td>96–160</td>
</tr>
<tr>
<td>1753T&gt;C</td>
<td>X501Arg</td>
<td>1</td>
<td>0</td>
<td>NA</td>
<td>102</td>
</tr>
</tbody>
</table>

The effect of each amino acid substitution on protein function was predicted with the use of PolyPhen (30). *Relative to transcription start site. **Relative to translation start site. NA, not applicable; *, deletion.
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 (AAAX11354), 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 dipalmitoylphosphatidylcholine as previously described (32) and against isolated human HDLs as previously described (33). Each assay was performed in triplicate and transfec- tions 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 × 10⁻¹⁰). 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.
In contrast, Asn396Ser was strongly associated with elevated HDL-C across all cohorts studied (combined P value = $1.7 \times 10^{-8}$; 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 HHDL cohort, with vertical transmission of the minor alleles of the Thr111Ile and Asn396Ser variants, provided DNA for genotyping. 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 within the 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).

### Table 3

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Genotype</th>
<th>High HDL</th>
<th>Low HDL</th>
<th>Predicted effect</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>584C&gt;T</td>
<td>Thr111Ile (rs2000813)</td>
<td>Heterozygous</td>
<td>162 (43.5%)</td>
<td>107 (50.2%)</td>
<td>Benign</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygous</td>
<td>35 (9.4%)</td>
<td>13 (6.1%)</td>
<td>.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAF</td>
<td>0.31</td>
<td>0.31</td>
<td>.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>1439A&gt;G</td>
<td>Asn396Ser</td>
<td>Heterozygous</td>
<td>23 (6.2%)</td>
<td>3 (1.4%)</td>
<td>Possibly damaging</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Homozygous</td>
<td>0</td>
<td>0</td>
<td>.</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MAF</td>
<td>0.03</td>
<td>&lt;0.01</td>
<td>.</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

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). Relative to transcription start site.

---

**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 ≤ 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 ± SD.
variants performed in the
cohorts confirmed that the downstream
ants (squared correlation coefficient \[ r^2 \])
SNPs are in LD with known functional variants in
in high linkage disequilibrium \([LD]\) with each other). However,
robustly associated being rs2156552 and rs4939883, which are
of the
GWASs have reported that correlated SNPs 40–65 kb downstream
reduced activity against HDL in vivo (Figure 2E).
effective than WT EL at reducing HDL-C levels, consistent with
ity against HDL. The Asn396Ser variant was substantially less
vectors encoding WT EL and the Asn396Ser variant and injected
40% of WT activity. We also created adeno-associated virus (AAV)
ure 2, B–D). Consistent with the epidemiologic data, Thr111Ile
ed mutagenesis and analyzed their lipolytic activity in vitro (Fig
Asn396Ser variants in the EL expression plasmid using site-direct

In order to test their functionality, we created the Thr111Ile and
Asn396Ser variants in the EL expression plasmid using site-direct-
ed mutagenesis and analyzed their lipolytic activity in vitro (Fig-
ure 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 adenovirus-based viruses (AAV)
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 vari-
ants (squared correlation coefficient \( r^2 \) = 0.078 – 0.083). Geno-
typing 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, 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. Rese-
queencing 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 pheno-
types (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
\( P = 1.7 \times 10^{-7} \) 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, identify-
ing 4 nonsynonymous variants of EL (16). This study lacked statis-
tical power and did not characterize the activities of the variants.
Only 1 nonsynonymous variant, Thr111Ile, was found to be com-
mon (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 cardio-
vascular 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 HDL-C 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 (23); (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 4**

FHS association analysis

<table>
<thead>
<tr>
<th>Variant</th>
<th>Thr111Ile</th>
<th>Asn396Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ SD</td>
<td>( P ) value</td>
</tr>
<tr>
<td>HDL-C</td>
<td>–0.003</td>
<td>0.92</td>
</tr>
<tr>
<td>HDL-2</td>
<td>–0.020</td>
<td>0.66</td>
</tr>
<tr>
<td>HDL-3</td>
<td>–0.010</td>
<td>0.82</td>
</tr>
<tr>
<td>HDL size</td>
<td>–0.020</td>
<td>0.60</td>
</tr>
<tr>
<td>HDL small particle</td>
<td>0.030</td>
<td>0.45</td>
</tr>
<tr>
<td>HDL intermediate particle</td>
<td>–0.020</td>
<td>0.63</td>
</tr>
<tr>
<td>HDL large particle</td>
<td>–0.004</td>
<td>0.93</td>
</tr>
<tr>
<td>apoA-1</td>
<td>–0.060</td>
<td>0.62</td>
</tr>
</tbody>
</table>

\( \Delta SD \) represents the proportion of 1 SD change in standardized residual \((\text{mean} = 0, SD = 1\) after adjustment for age, age\(^2\), 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.

**Table 5**

Baseline characteristics

<table>
<thead>
<tr>
<th>Cohort</th>
<th>UKE Hamburg CC cases ((n = 193))</th>
<th>UKE Hamburg CC controls ((n = 194))</th>
<th>Penn HDL CC cases ((n = 606))</th>
<th>Penn HDL CC controls ((n = 437))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascertainment</td>
<td>Lipid clinic, HDL ≥ 95th Pctl</td>
<td>Lipid clinic, HDL ≤ 5th Pctl</td>
<td>Physician referral, HDL ≥ 95th Pctl</td>
<td>Family history of premature CAD, HDL ≥ 50th Pctl</td>
</tr>
<tr>
<td>Mean age (yr)</td>
<td>45.6 ± 14.7</td>
<td>45.3 ± 12.7</td>
<td>58.8 ± 11.4</td>
<td>47.8 ± 8.1</td>
</tr>
<tr>
<td>Female (%)</td>
<td>59.1</td>
<td>38.4</td>
<td>72.8</td>
<td>39.6</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>89.1 ± 19.1</td>
<td>27.3 ± 3.9</td>
<td>98.3 ± 17.5</td>
<td>38.8 ± 7.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.1 ± 4.3</td>
<td>29.2 ± 4.5</td>
<td>23.5 ± 11.2</td>
<td>28.5 ± 5.1</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>16.9</td>
<td>47.4</td>
<td>4.0</td>
<td>15.5</td>
</tr>
<tr>
<td>Alcohol use (%)</td>
<td>–</td>
<td>–</td>
<td>75.2</td>
<td>66.8</td>
</tr>
</tbody>
</table>

Values with “±” are mean ± SD.

http://www.jci.org
Volume 119
Number 4
April 2009
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 variant 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 pro-protein 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 the 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
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 Diasorex 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 Arzteammer, 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).

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 μg 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...
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 Diagnostics System Inc.) using Wako Chemicals reagents.

Animals. Male 8- to 10-week-old Lpg−/− mice on a C57BL/6 background (14) were administered AAV at 3 x 1010 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 Diagnostics System 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 genotypic 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 cultures 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.

Acknowledgments

We would like to acknowledge expert technical support from the London Regional Genomics Centre. The Broad Institute Center for Genotyping and Analysis is supported by grant U54 RR020278 from the National Center for Research Resources. This work was supported by an National Heart, Lung and Blood Institute Ruth L. Kirschstein National Research Service Award for Individual Predoctoral MD/PhD Fellows (F30, to A.C. Edmondson), a Research Fellowship of the Heart and Stroke Foundation of Canada (to R.J. Brown), a Doris Duke Charitable Foundation Distinguished Clinical Scientist Award (to D.J. Rader), and support from Genome Canada through the Ontario Genomics Institute (to R.A. Hegele). The FHS of the National Heart, Lung and Blood Institute of the NIH and Boston University School of Medicine is supported by the National Heart, Lung and Blood Institute’s FHS (contract no. N01-HC-25195). A portion of this research was conducted using the Linux Cluster for Genetic Analysis (LinGA-II) funded by the Robert Dawson Evans Endowment of the Department of Medicine at Boston University School of Medicine and Boston Medical Center. We would also like to thank the individuals and families from all of these studies for their participation as well as the referring physicians both in and outside the University of Pennsylvania Health System, and in particular, John Hoekstra and James Underberg for their help with recruitment of the HHDL study.

Received for publication August 18, 2008, and accepted in revised form January 28, 2009.

Address correspondence to: Daniel J. Rader, University of Pennsylvania School of Medicine, 654 BBII/III Labs, 421 Curie Blvd., Philadelphia, Pennsylvania 19104-6160, USA. Phone: (215) 573-4176; Fax: (215) 573-8606; E-mail: rader@mail.med.upenn.edu.


