The Impact of Partial and Complete Loss-of-Function Mutations in Endothelial Lipase on High-Density Lipoprotein Levels and Functionality in Humans

Roshni R. Singaraja, PhD; Suthesh Sivapalaratnam, MD, PhD; Kees Hovingh, MD, PhD; Marie-Pierre Dubé, PhD; José Castro-Perez, PhD; Heidi L. Collins, PhD; Steven J. Adelman, PhD; Meliana Riwanto, BS; Jasmin Manz, BS; Brian Hubbard, PhD; Ian Tietjen, PhD; Kenny Wong, PhD; Lyndon J. Mitnauil, PhD; Margaret van Heek, PhD; Linus Lin, PhD; Thomas A. Roddy, PhD; Jason McEwen, BS; Geesje Dalling-Thie, PhD; Leonie van Vark-van der Zee, BS; Germaine Verwoert, MS; Michael Winther, PhD; Cornelia van Duijn, PhD; Albert Hofman, MD, PhD; Mieke D. Trip, MD, PhD; A. David Marais, MD, PhD; Bela Asztalos, Phd; Ulf Landmesser, MD, PhD; Eric Sijbrands, MD, PhD; John J. Kastelein, MD, PhD; Michael R. Hayden, MD, PhD

Background—Endothelial lipase is a phospholipase with activity against high-density lipoprotein. Although a small number of mutations in LIPG have been described, the role of LIPG in protection against atherosclerosis is unclear.

Methods and Results—We identified 8 loss-of-function (LOF) mutations in LIPG in individuals with high-density lipoprotein cholesterol. Functional analysis confirmed that most rare mutations abolish lipase activity in vitro, indicating complete LOF, whereas 2 more common mutations N396S and R476W reduce activity by ≈50%, indicating partial LOF and implying ≈50% and ≈75% remaining endothelial lipase function in heterozygous complete LOF and partial LOF mutation carriers, respectively. Complete LOF mutation carriers had significantly higher plasma high-density lipoprotein cholesterol levels compared with partial LOF mutation carriers. Apolipoprotein B-depleted serum from complete LOF carriers showed significantly enhanced cholesterol efflux acceptor capacity, whereas only trends were observed in partial LOF carriers. Carriers of LIPG mutations exhibited trends toward reduced coronary artery disease in 4 independent cohorts (meta-analysis odds ratio, 0.7; P=0.04).

Conclusions—Our data suggest that the impact of LIPG mutations is directly related to their effect on endothelial lipase function and support that antagonism of endothelial lipase function improves cardioprotection. (Circ Cardiovasc Genet. 2013;6:54-62.)

Key Words: cardiovascular disease • genetics • high-density lipoprotein cholesterol • lipids

Reduction of low-density lipoprotein cholesterol (LDL-C) has long been a primary target of coronary artery disease (CAD) prevention. However, despite lowering LDL-C with statins, a significant residual risk of CAD still remains, emphasizing the need for novel therapies.1 Plasma high-density lipoprotein cholesterol (HDL-C) is an independent risk factor and is inversely associated with CAD.2 The protective function of HDL-C in CAD is postulated at least in part to arise from its role in reverse cholesterol transport, the process whereby excess cellular cholesterol is transported to the liver for fecal excretion, and through its anti-inflammatory, antioxidative, and antiapoptotic functions.3

Clinical Perspective on p 62

Endothelial lipase (EL) is a member of the triglyceride lipase family that includes lipoprotein lipase and hepatic lipase. Lipoprotein lipase primarily acts on triglyceride-rich apolipoprotein B (Apo-B)-containing lipoproteins, hepatic lipase acts on all classes of lipoproteins, and EL acts preferentially on HDL lipids.4

The online-only Data Supplement is available at http://circgenetics.ahajournals.orglookup/suppl/doi:10.1161/CIRCGENETICS.111.962613/-/DC1. Correspondence to Michael R. Hayden, MD, PhD, University of British Columbia, 950 W, 28th Ave, Vancouver, BC, V5Z 4H4, Canada. E-mail mhr@cmmt.ubc.ca

© 2012 American Heart Association, Inc.

Circ Cardiovasc Genet is available at http://circgenetics.ahajournals.org

DOI: 10.1161/CIRCGENETICS.111.962613
Studies of EL functionality in mice by overexpression and gene targeting show altered plasma HDL-C levels by affecting the fractional catabolic rate of HDL.1 One model suggests that EL hydrolyzes HDL phospholipids, generating smaller phospholipid-depleted HDL particles that are more rapidly catabolized.2 Studies in humans also suggest that mutations that reduce EL activity correlate with increased plasma HDL-C levels.3-5

The role of EL in modulating risk for CAD is unclear. Male Lipg−/− mice show increased plasma HDL-C, phospholipid, and LDL-C,9,10 as well as increased HDL particle size.7 Associated with this, aortic lesions are reduced in Lip g−/−ApoE−/− mice.9 However, aortic lesions are not changed in another model of Lipg−/−ApoE−/− or Lip g−/−Ldlr−/−.10 and the reasons for this discrepancy are not readily apparent.

It is unclear in humans whether loss of function (LOF) of LIPG leading to raised HDL-C levels confers a decreased risk for atherosclerosis.

Here we have assessed the effects of complete LOF (CLOF) and partial LOF (PLOF) LIPG mutations on HDL-C levels, HDL functionality, and risk for CAD. Individuals with heterozygous mutations in LIPG show significantly increased plasma HDL-C and improved HDL functionality. In addition, mutations in LIPG are associated with reduced atherosclerosis.

Materials and Methods

Patients
Subjects were selected from the Lipid Clinic Network and Vascular Research Network (Amsterdam, the Netherlands) and through the Center for Molecular Medicine and Therapeutics (Vancouver, Canada). The main criterion was HDL-C ≥290th percentile, adjusted for age and gender, in unrelated probands. Families of unrelated probands were expanded, and detailed pedigrees were generated. All individuals in families in which the high HDL-PL had a mutation in LIPG were genotyped to assess segregation of the high HDL-C trait with LIPG mutations. All carriers of LIPG mutations were pooled and compared with pooled noncarrier controls consisting of first-degree relatives of each mutation carrier. The ethics committees of the Academic Medical Center, Amsterdam, and the University of British Columbia, Vancouver, accepted the protocol for genetic analysis. All subjects signed informed consent forms.

Fasting blood was drawn into ethylenediaminetetraacetic acid-containing tubes and plasma and stored at −80°C. Leukocytes were isolated for DNA extraction. Lipoprotein measurements were performed as described.16

DNA Sequencing
DNA primers were designed to flank LIPG exons and adjacent intron boundaries as defined in the UCSC (hg18) release of the human genome. Reference DNA/protein sequences used for sequencing and data analysis include NM_006033.2 and NP_006024. Primer sequences were designed using Primer3. PCR products were amplified, purified using the AMPure kit (Agencourt, Beverly, MA), and sequenced by fluorescent dye-terminator chemistry (Seqwright, Houston, TX). Known and novel single-nucleotide polymorphisms in each gene were identified from sequence analysis using Sequencher v 4.9 (Ann Arbor, MI) and confirmed in dbSNP build 130 (National Center for Biotechnology Information, Bethesda, MD).

In Vitro Functional Assessment of LIPG Mutations
All identified mutations were designated novel based on published literature if absent from dbSNP (Build 130). Functional impacts were predicted using Polyphen 2.0. All mutations except the promoter splice site Met1-18T > C mutation were generated in a LIPG cDNA clone (Origene) by site directed mutagenesis (Stratagene). Sense primers: Leu130Phe, cccacgattcagctggtgctgtaaatacc; Gly196Arg, gttgtggacagctgcagccagctgggaag; His220Gln, gcagattgttgagctcctcagcatcag; Glu388Stop, ggaattaatgctgataccggaagctag; Glu393Lys, ggataatgagccagctgcagcagtaag; Asn396Ser, gcagatgctcaggccagctgttgc; Arg476Trp, gacatcggccggtgagctctcgggtgg. Antisense primers were complementary to the sense primers. Positive clones were sequence confirmed. EL protein was generated by lipofectamine (invitrogen)-mediated transient transfection into HEK293T cells. Seventy-two hours after transfection, cells were incubated with 750 μM of heparin for 30 minutes at 37°C, and media was concentrated, followed by centrifugation at 1500g for 30 minutes (4°C). Cleared supernatant was stored at −80°C. Cells in PBS were centrifuged at 800g for 5 minutes (4°C) and lysed in 100 μL lysis buffer (0.025 mol/L ammonium chloride pH, 5 mmol/L ethyl-enediaminetetraacetic acid, 0.4 mg/mL sodium dodecyl sulfate, 8.0 mg/mL Triton-X 100 with proteinase inhibitor and heparin). After sonication and centrifugation, supernatants were stored at −80°C. Proteins resolved on NuPage 12% BisTris gels (Invitrogen, Carlsbad, CA) were transferred to polyvinylidene difluoride membranes and probed with antibodies to the N-terminus of EL (ab14797; Abcam, Cambridge, UK). Bands were visualized with supersignal chemiluminescent substrate (ThermoFisher Scientific, Waltham, MA) on X-ray film (ThermoFisher). Analyses of the lipase activity were performed using a fluorescent phospholipid substrate as described.11 A kinetic read was performed for each sample, and a time-point was chosen in the linear range of the assay to ensure that the reduced activity of LIPG mutants did not arise from insufficient substrate.

HDL Functionality Assessments
Fasting plasma and serum were collected from LIPG mutation carriers and snap frozen. For lipid efflux studies, frozen sera were analyzed by Vascular Strategies LLC using established protocols.17,18 This assay quantifies total cholesterol efflux mediated by pathways of known relevance in macrophages (ABCA1 and G1, SR-BI, and acqueous diffusion). Briefly, J774 murine macrophages were labeled with 2 μCi/mL 1H cholesterol for 24 hours in the presence of acetyl-Coenzyme A acetyltransferase inhibitor (Sandoz 58-035, Holzkirchen, Germany) and equilibrated overnight with 0.3 mmol/L 8-(4-chlorophenylthio)-3-‘5’-cycl-cys desoximono phosphate in the presence of acetyl-Coenzyme A acetyltransferase inhibitor. ApoB-depleted serum was obtained by polyethylene glycol precipitation, and 2.8% v/v ApoB-depleted serum was used as efflux acceptor for 4 hours. As controls, either 20 μg/mL APOA-I, 20 μg/mL HDL3, 2% v/v human serum, or medium alone were used. Efflux was quantified by liquid scintillation. For lipoprotein particle sizes and concentrations, proton nuclear magnetic resonance spectra of each plasma specimen (0.2 mL) were acquired in replicates using an automated 400-MHz lipoprotein analyzer at LipoScience (Raleigh, NC) following described protocols.40 For HDL subfraction analysis, 2-dimensional gel electrophoretic separation was performed by Dr Bela Asztalos using established protocols.41 HDL composition analysis was performed by isolating the HDL particles using gradient gel electrophoresis (Lipoprint, Quantimetrix, CA). In brief, 25 μL of plasma was loaded onto the gel following manufacturer’s instructions. The gels were stained with Sudan Black to locate lipoprotein bands for excision. Excised lipoproteins were used by the Bligh and Dyer method42 and analyzed by high-resolution liquid chromatography/mass spectrometry (Synapt G2 HDMS, Waters Corp, Manchester, United Kingdom) as previously described.43 For the antioxidative and anti-inflammatory functions of HDL, superoxide production and vascular cell adhesion molecule-1 expression were respectively, quantified as previously described.44

Genotyping of Cohorts
The Rotterdam, GiraFH, and PAS cohorts were genotyped using tagman probes (ABI, Foster City, CA) following manufacturer’s instructions.
Statistical Analyses
Regression modeling was used to test for association between lipid measurements and the genetic carrier status versus noncarrier status. Proc Glimmix in SAS software version 9.3 (SAS, Cary, NC) was used with a random effect variable to account for the families from which carriers were ascertained. Adjustment for age and sex was used for test of lipid parameters except for HDL percentiles, which already take into account age and sex. We assumed a dominant model, combining the heterozygous and homozygous carriers of the mutations. Fisher exact tests were used for proportional comparisons. For meta-analyses, a fixed-effect model was used, and no adjustments for multiple testing of single-nucleotide polymorphisms within each study were used or for familiarity of samples in those tests. Fisher Z score was used to capture each study’s effect and to generate a summary correlation statistic. Analyses were conducted using comprehensive meta-analysis version 2.2.050.

Results
Mutations in LIPG Have Discrete and Diverse Effects on EL Function
We sequenced a cohort of 177 unrelated individuals with extreme high HDL-C (HDL-C ≥ 90th percentile), in whom mutations in CETP, GALNT2, LCAT, lipoprotein lipase, APOAI, and ABCA1 had been excluded, and we identified 23 individuals with 8 different LIPG mutations, 6 of which are novel (Table 1; Figure 1A). To determine whether these mutations impact EL function, we used a fluorescent phospholipid substrate to quantify EL activity. All mutants showed significantly lower phospholipase activity compared with wild-type EL (Figure 1B). The LIPG mutations E391K, N396S, and R476W showed PLOF (≥50%). All the other mutations showed EL activity that was significantly different from wild type and not different from mock-transfected supernatants (CLOF) (Figure 1B). The 5′ untranslated region mutation M1-18T > C was not generated in vitro because a cDNA construct with a cytomegalovirus promoter was used. Thus, in vitro data suggest that the catalytic domain of EL (amino acids 169–274) does not tolerate missense sequence changes. However, missense mutations in the c-terminal lipase domain of EL (amino acids 347–482) still retain partial activity in vitro. Thus, mutations in LIPG have variable impact on EL function.

Table 1. LIPG Mutations Identified, Their Conservation, and Predicted Functional Effect

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Novel</th>
<th>Vertebrate Conservation</th>
<th>Predicted Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met1-18 T&gt;C</td>
<td>Yes</td>
<td>Conserved to lizard</td>
<td>Protein translation reduced</td>
</tr>
<tr>
<td>L130F</td>
<td>Yes</td>
<td>Conserved to stickleback</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>G196R</td>
<td>Yes</td>
<td>Conserved to stickleback</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>H220Q</td>
<td>Yes</td>
<td>Conserved to stickleback</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>E388X</td>
<td>Yes</td>
<td>Conserved to stickleback</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>E391K</td>
<td>Yes</td>
<td>Gin tolerated</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>N396S</td>
<td>No</td>
<td>Conserved to stickleback</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>R476W</td>
<td>No</td>
<td>Arg, Lys, Gin tolerated</td>
<td>Possibly damaging</td>
</tr>
</tbody>
</table>

Arg indicates arginine; Gin, glutamine; and Lys, lysine.

Plasma HDL-C Is Highest in CLOF Mutation Carriers
Extrapolation of the observed in vitro effects of LIPG mutations to EL activity in vivo suggests that heterozygous PLOF carriers will retain at least 75% of EL activity, whereas heterozygous CLOF carriers will retain at least 50% of EL activity. We extended the pedigrees of the probands to identify additional carriers of mutations in LIPG (Table 2) and assessed plasma HDL-C levels in mutation carriers (n=131) compared with unrelated population controls (n=756).

Carriers of LIPG mutations showed a significant 15.5 mg/dL (28.7%) increase in HDL-C compared with unrelated controls (P=0.005; Table 3). Carriers of CLOF mutations showed a 23.2 mg/dL (42.9%) increase in plasma HDL-C, whereas carriers of PLOF mutations only showed a 13 mg/dL (24.5%) increase (Table 4). No significant changes in plasma LDL-C, triglycerides, or body mass index were observed in carriers compared with controls (Table 3). No differences were observed in alcohol consumption, smoking, and history of diabetes mellitus or hypertension (data not shown).

Thus, the in vitro effects of various LIPG mutations correlated with the in vivo effects as measured by HDL-C levels. In our cohort, 1 individual was a compound heterozygote, carrying H220Q, a CLOF mutation, and N396S, a PLOF mutation. This individual would be expected to have only 25% of remaining EL activity. Consistent with this high level of EL inhibition, this individual showed a 54.6 mg/dL (100.9%) increase in plasma HDL-C compared with controls (Table 4). In addition, 2 individuals were homozygotes for the PLOF mutation N396S (Table 4; Figure 1C).

Carriers of PLOF Mutations Show Marked Variability in HDL-C Levels
We next assessed the variability of HDL-C levels in PLOF and CLOF carriers. We defined high expressivity as those with HDL-C ≥80th percentile after adjusting for age and sex, by comparing the HDL-C level of each carrier to those of age- and sex-matched individuals in the Lipid Research Clinic database. A total of 83.4% of individuals with CLOF mutations had HDL-C levels >80th percentile, compared with only 69.6% of persons with the PLOF mutation R476W and 62% of persons with the PLOF mutation N396S (N396S versus CLOF: P=0.03; R476W versus CLOF: P=ns; Fisher exact test) (Figure 1D). These data indicate that N396S and R476W represent relatively mild mutations that show variable effects on HDL-C levels in humans.

Increased Cholesterol Efflux Acceptor Capacity in LIPG Mutation Carriers
HDL-C levels are correlated with susceptibility to atherosclerosis, but measures of HDL functionality show similar or even stronger correlation with CAD. One aspect of HDL functionality, lipid efflux, may be a stronger predictor of CAD than HDL-C levels, and it was recently noted that every 1-SD increase in lipid efflux was correlated with a significant ≈25% decrease in CAD in humans. Efflux of cholesterol from macrophages to ApoB-depleted serum from CLOF mutation carriers was significantly
increased (% efflux: controls: 16.3±1.5, n=4; CLOF: 19.6±2.4, n=5; *P* =0.04) (Figure 2A). This was less apparent in PLOF mutation carriers where efflux acceptor capacity was elevated but not significantly different from controls (PLOF: 17.7±2.5, n=9; *P* =0.3) (Figure 2A).

The impact of anti-inflammatory and antioxidative effects of HDL on CAD have also been recently described. We determined whether HDL from LIPG mutation carriers displayed these properties by quantifying vascular cell adhesion molecule-1 expression and superoxide production. We found no significant differences in either of these parameters. However, a trend toward decreased superoxide production (by 53%) was observed in mutation carriers (online-only Data Supplement Figure I).

LIPG Mutation Carriers Have Larger HDL Particles and Increased Large HDL Particle Concentration

Next, we performed nuclear magnetic resonance analyses on HDL from LIPG mutation carriers. HDL from CLOF carriers was larger in size (CLOF: 10.2±0.6 nm, n=4; controls: 9.2±0.6 nm, n=6; *P* =0.04) (Figure 2B). No changes in LDL or very LDL particle sizes were observed (data not shown).

The increase in HDL size was less apparent in PLOF carriers and was not significantly different from controls (PLOF: 9.6±0.6 nm, n=9; *P*=0.3) (Figure 2A).

Table 2. Number of LIPG Mutation Carriers by Mutation

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Number of Mutation Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>N396S</td>
<td>81</td>
</tr>
<tr>
<td>R476W</td>
<td>27</td>
</tr>
<tr>
<td>L130F</td>
<td>12</td>
</tr>
<tr>
<td>H220Q</td>
<td>4</td>
</tr>
<tr>
<td>G196R</td>
<td>3</td>
</tr>
<tr>
<td>E388X</td>
<td>2</td>
</tr>
<tr>
<td>E391K</td>
<td>1</td>
</tr>
<tr>
<td>Met1-18G&gt;T</td>
<td>1</td>
</tr>
</tbody>
</table>

One individual has a H220Q and a N396S mutation.
different from controls \((P=0.1)\). Increased HDL particle size and increased large HDL particle concentration have been associated with reduced CAD.\(^{15,16}\) HDL particles were also assayed using 2-dimensional gel electrophoresis. A specific \(\approx50\%\) increase in \(\alpha1\) HDL was observed in the CLOF carriers \((CLOF: 21.7\pm4.8, n=4; \text{controls: } 14.5\pm3.8, n=5; P=0.047)\) (Figure 2D), whereas no change was observed between PLOF carriers and controls \((P=0.2)\). No significant difference was observed between PLOF carriers and controls. To further extend these findings, we assessed CAD rates in mutation carriers in the Rotterdam cohort, a prospective, population-based cohort.\(^9\) Although N396S and R476W are PLOF mutations, they are the most frequent mutations in Whites (population frequency: N396S \(\approx2.2\%\), R476W \(\approx1\%\)). Thus, these 2 mutations were genotyped in the Rotterdam cohort. As in the family-based cohort, a nonsignificant decrease in CAD was observed in mutation carriers (carriers, 17/156 \([10.9\%]\) with CAD; controls, 752/5658 \([13.3\%]\) with CAD; \(P=ns\) for all comparisons). In addition, the age at onset of CAD was significantly delayed in carriers of CLOF mutations compared with controls \((CLOF, 72.0\pm4.2 \text{ years}; \text{controls, } 51.1\pm11.7; CLOF \approx >PLOF mutations, \approx2\%\) with CAD; PLOF: 11/107 \([13.2\%]\) with CAD; controls: 111/574 \([14.7\%]\) with CAD; \(P=ns\) for all comparisons). Power calculations show we had 80\% power to observe a significant association with CAD for an effect size of \(OR \approx0.37\), owing to the low number of mutation carriers in this cohort. CLOF mutation carriers had lower CAD than either PLOF carriers or controls \((CLOF: 2/22 \approx[9.5\%]\) with CAD; PLOF: 11/107 \([13.2\%]\) with CAD; controls: 111/574 \([14.7\%]\) with CAD; \(P=ns\) for all comparisons).

### Table 4. HDL-C Elevation in Partial LOF and CLOF LIPG Mutation Carriers

<table>
<thead>
<tr>
<th>Compound</th>
<th>HDL-C (mg/dL)</th>
<th>% HDL Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>N396S</td>
<td>68.1 (28.2)</td>
<td>25.9%</td>
</tr>
<tr>
<td>R476W</td>
<td>66.9 (28.2)</td>
<td>23.2%</td>
</tr>
<tr>
<td>CLOF</td>
<td>77.3 (21.5)</td>
<td>42.9%</td>
</tr>
<tr>
<td>Compound heterozygote</td>
<td>77.3 (21.5)</td>
<td>42.9%</td>
</tr>
</tbody>
</table>

**Table 3. Plasma Lipid Levels in LIPG Mutation Carriers**

<table>
<thead>
<tr>
<th></th>
<th>Carriers</th>
<th>Unrelated Controls</th>
<th>P*: Carriers Versus Unrelated Controls</th>
<th>P*: Carriers Versus First-Degree Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N)</td>
<td>131</td>
<td>756</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Male, %</td>
<td>71/131 (54.2%)</td>
<td>367/756 (48.5%)</td>
<td>53/123 (43.1%)</td>
<td>0.97</td>
</tr>
<tr>
<td>Age, y</td>
<td>43.8 (19.7)</td>
<td>53.2 (13.5)</td>
<td>39.8 (18.8)</td>
<td>0.011</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>1.80 (0.71)</td>
<td>1.40 (0.60)</td>
<td>1.54 (0.52)</td>
<td>0.14</td>
</tr>
<tr>
<td>HDL, %ile</td>
<td>72.7 (30.5)</td>
<td>48.8 (34.9)</td>
<td>59.2 (30.6)</td>
<td>0.014</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>3.26 (1.09)</td>
<td>3.30 (0.97)</td>
<td>3.10 (1.14)</td>
<td>0.0041</td>
</tr>
<tr>
<td>TC, mmol/L</td>
<td>1.17 (0.72)</td>
<td>1.36 (0.98)</td>
<td>1.07 (0.67)</td>
<td>0.0541</td>
</tr>
<tr>
<td>TG, mmol/L</td>
<td>56.0 (1.33)</td>
<td>5.32 (1.16)</td>
<td>5.14 (1.28)</td>
<td>0.06301</td>
</tr>
<tr>
<td>BMI, kg/m(^2)</td>
<td>23.8 (3.9)</td>
<td>25.7 (4.0)</td>
<td>23.1 (5.1)</td>
<td>0.32677</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; and TG, triglyceride.

*General linear mixed model with a random variable to account for family membership and adjustment for age and sex when appropriate. Values are average (SD).

Assessment of Atherosclerosis in LIPG Mutation Carriers

We assessed the impact of LIPG mutations on risk for CAD in the 131 family-based mutation carriers compared with unrelated controls. A nonsignificant trend toward decreased CAD in mutation carriers was seen \((carriers: 14/131 [10.7\%] with CAD; controls: 111/756 [14.7\%] with CAD; OR =0.69; 95\% confidence interval [CI] =0.39–1.25; P=0.28)\) (Table 5). Power calculations show we had 80\% power to observe a significant association with CAD for an effect size of \(OR \approx0.37\), owing to the low number of mutation carriers in this cohort. CLOF mutation carriers had lower CAD than either PLOF carriers or controls \((CLOF: 2/22 [9.5\%] with CAD; PLOF: 11/107 [13.2\%] with CAD; controls: 111/574 [14.7\%] with CAD; \(P=ns\) for all comparisons). In addition, the age at onset of CAD was significantly delayed in carriers of CLOF mutations compared with controls \((CLOF: 72.0±4.2 \text{ years}; PLOF: 64.6±12.7; controls: 51.1±11.7; \text{CLOF versus controls: } P=0.01; \text{CLOF versus PLOF: } P=ns\)).
mutations on CAD in populations of small size. Again, N396S and R476W, the 2 most frequent mutations, were assessed, even though they showed only small effects on EL function. We first used the GiraFH cohort, a retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH). In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH). We first used the GiraFH cohort, a retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH). We first used the GiraFH cohort, a retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterolemia (FH).20 In contrast to the family and retrospective, multicenter cohort of 2068 individuals with familial hypercholesterol...
result in significantly increased plasma HDL-C and changes in HDL function compatible with cardioprotection. The effect of these mutations on HDL-C levels and HDL functionality is directly correlated with the impact of these mutations on EL activity. In addition, our data indicate that mutations in LIPG may confer protection against atherosclerosis.

Measures of HDL functionality show similar or even stronger correlation with CAD compared with that between HDL-C and CAD.12,13 ApoB-depleted serum from individuals with CAD displays significantly reduced efflux acceptor capacity, confirming in humans that efflux capacity is robustly and inversely correlated with atherosclerosis.13 Using identical methodology, we found that ApoB-depleted serum from CLOF mutation carriers displayed significantly improved macrophage cholesterol efflux capacity. The PC content of HDL particles is critical for efficiency of cholesterol efflux acceptors.17,18 HDL isolated from CLOF mutation carriers showed increased PC content, agreeing with the fact that EL is a phospholipase and providing the mechanism underlying the improved efflux capacity. PLOF carriers showed trends toward improved efflux capacity. We assessed the impact of HDL on antioxidative and anti-inflammatory endothelial function14 and found no significant effect of LIPG on these parameters. A nonsignificant 53% reduction in superoxide production was observed in mutation carriers, suggesting a beneficial effect of this HDL on antioxidative capacity. Overall, no significant adverse effects of LIPG reduction on HDL functionality were observed.

We next assessed the impact of LIPG mutations on CAD in humans. An important caveat is that many carriers of the 2 PLOF LIPG mutations, N396S and R476W, do not display elevated plasma HDL-C levels. Thus, if EL has an impact on atherosclerosis and this is mediated through raised HDL-C, then using only these mutations to assess the impact of EL on atherosclerosis will significantly undermine the effect. Despite this caveat, because these 2 mutations are by far the most frequent, we used them to assess the impact of LIPG on atherosclerosis.

Studies assessing the impact of genes modulating plasma HDL-C levels on atherosclerosis have been equivocal. One major factor contributing to this is the study of mild mutations with small effects on HDL-C levels as a surrogate for CLOF mutations. Selection of a few mutations with modest effects on protein function as a proxy for the inhibition of the majority of protein function often results in variable findings. However, in general, the more frequent mutations in populations tend to be mild LOF mutations, requiring the study of these to achieve adequate power.

Another factor significantly confounding such studies is the low risk for CAD in the general population. In addition, the baseline HDL-C levels from which elevation occurs significantly influence the impact of HDL-C on atherosclerosis. Increasing HDL-C from a baseline of ≥45 mg/dL does not provide much additional benefit against CAD.22,23 Other lipoproteins, such as LDL, are also important, with greater impact on CAD being observed when HDL-C is increased in the presence of high LDL-C.22 These factors led us to select for further assessment 2 cohorts with high atherosclerosis incidence: one with individuals with GiraFH, and the other with individuals with PAS. An alternate strategy was to perform association studies in a large unselected population cohort. However, because of the mild dysfunction of the 2 most common LIPG mutations and the low rate of CAD in unselected populations, we chose to study selected cohorts.

We found that mutations in LIPG result in a trend toward protection against CAD in 4 different cohorts. When a combined meta-analysis was performed, we observed a significant ≈30% reduction in CAD in LIPG mutation carriers.

The role of LIPG activity in atherosclerosis is unclear. Plasma EL concentrations are significantly increased in a cohort with

Table 5. LIPG Mutation Carriers With Atherosclerosis

<table>
<thead>
<tr>
<th>Cohort</th>
<th>LIPG Carriers With CAD</th>
<th>Controls With CAD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLOF</td>
<td>14/131 (10.7%)</td>
<td>111/756 (14.7%)</td>
<td>0.28</td>
</tr>
<tr>
<td>PLOF</td>
<td>12/108 (11.1%)</td>
<td>111/756 (14.7%)</td>
<td>0.38</td>
</tr>
<tr>
<td>Rotterdam</td>
<td>2/23 (8.7%)</td>
<td>111/756 (14.7%)</td>
<td>0.56</td>
</tr>
<tr>
<td>GiraFH</td>
<td>17/156 (10.9%)</td>
<td>752/5658 (13.3%)</td>
<td>0.37</td>
</tr>
<tr>
<td>PAS</td>
<td>11/57 (19.3%)</td>
<td>579/2011 (28.8%)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cohort Carriers</th>
<th>LIPG Carriers in CAD Cohort</th>
<th>LIPG Carriers in Control Cohort</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS sanquin</td>
<td>14/665 (2.10%)</td>
<td>34/1078 (3.15%)</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Controls indicate non-LIPG mutation carriers. Values are average (SD). CAD indicates coronary artery disease; CLOF, complete loss of function; PAS, premature atherosclerosis; and PLOF, partial loss of function.

Table 6. Meta-Analysis of LIPG Mutation Carriers With Atherosclerosis

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Carriers Without CAD</th>
<th>Controls Without CAD</th>
<th>OR</th>
<th>Lower Limit</th>
<th>Upper Limit</th>
<th>Fisher Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotterdam</td>
<td>17</td>
<td>139</td>
<td>752</td>
<td>4875</td>
<td>0.79</td>
<td>0.48</td>
<td>1.32</td>
</tr>
<tr>
<td>GiraFH</td>
<td>11</td>
<td>46</td>
<td>579</td>
<td>1432</td>
<td>0.59</td>
<td>0.30</td>
<td>1.15</td>
</tr>
<tr>
<td>PAS</td>
<td>14</td>
<td>34</td>
<td>651</td>
<td>1046</td>
<td>0.66</td>
<td>0.35</td>
<td>1.24</td>
</tr>
</tbody>
</table>

CAD indicates coronary artery disease; OR, odds ratio; and PAS, premature atherosclerosis.
metabolic syndrome and subclinical coronary heart disease,24 as well as in cultured endothelial cells in response to proinflammatory cytokines and in humans during acute inflammation,23 suggesting a correlation between EL levels and the development of atherosclerosis. However, EL enhances the selective uptake of HDL-C, although it is a scavenger receptor class B member 1-mediated pathway,26 suggesting an atheroprotective role. In contrast, overexpression of EL resulted in a significant decrease in macrophage to feces reverse cholesterol transport,27 although reverse cholesterol transport was unchanged in LIPG−/− mice.28 Several human studies have assessed the impact of variants in LIPG on atherosclerosis. However, the majority of these studies assessed the variant 584C/T (T111I), which has no functional effect on LIPG and thus is unlikely to modulate CAD risk. Two studies showed significantly reduced atherosclerosis in carriers of the T111I variant,29,30 both independent of plasma HDL-C. However, because this variant does not influence EL activity directly,31 it is possible that the association with CAD resulted from this variant being in linkage disequilibrium with other LOF LIPG mutations in these populations. A third study showed no impact of this variant on risk of coronary heart disease or plasma HDL-C levels,31 and a fourth study showed no association between this variant and CAD, although carriers of the variant showed increased plasma HDL-C.32 The intronic variants C+42T/In5 and T+2864C/In8 were associated with fewer MIs, although significance was lost after multiple testing.33 Two other SNPs in the 3′ untranslated region of LIPG also showed no altered risk for CAD.34,35 However, whether these single-nucleotide polymorphisms affect EL function is unknown.

Two studies thus far have assessed the association between a LIPG mutation known to compromise LIPG function and atherosclerosis. The first study assessed the association between the PLOF N396S mutation and a surrogate marker for CAD, intima media thickness, and found no association, although plasma HDL-C levels were mildly increased.8 However, this study was performed in only 4 N396S mutation carriers, and no conclusions can be reached from such a small study. The next study also assessed the impact of the mild N396S mutation on MI.36 Although >100,000 individuals from 20 different cohorts were assessed, overall, no impact of N396S on MI risk was observed.36 Numerous factors may have confounded this analysis. One crucial issue is HDL-C levels in the controls and not just the magnitude of increase in carriers. The impact of HDL-C on cardiovascular risk is not linear and is curtailed in people with higher HDL-C levels.22,23 Smaller increases from lower HDL levels confer a larger impact on CAD risk compared with similar increases from higher baseline HDL (>45 mg/dL).22,23 Another factor may be that the HDL-C increase was too small to detect an impact on MI. The authors used an HDL-C increase of 0.14 mmol/L to predict the impact on MI and for power calculations. However, of the 6 prospective cohorts, 3 had HDL-C increases of only 0.03, 0.04, and 0.08 mmol/L (the other 3 cohorts each had an increase of 0.14 mmol/L), thereby, perhaps, resulting in an underpowered study. Another factor is that HDL-C levels are only reported for the 6 prospective cohorts. Thus, whether HDL-C was increased in the 14 case-control cohorts is unknown. Yet another factor may be the variety of ethnicities and environments represented by the 20 cohorts and may explain the vastly varying impacts on MI for each cohort.

Recently, it was demonstrated that the mass and activity of EL isolated from individuals with cardiovascular disease was significantly higher than those without CAD,37 suggesting a direct correlation between EL activity and atherosclerosis. In addition, plasma EL activity was significantly correlated with coronary risk scores in the Framingham Study.38 Our current study using human mutations does not model the almost complete inhibition of EL achievable with drugs. However, the major impact of homozgyzous and compound heterozygous mutations on plasma HDL-C, along with the increasing efflux functionality observed with decreasing EL activity, suggests that inhibition of EL could be cardioprotective.

**Sources of Funding**

This work was supported by Xenon Pharmaceuticals Inc. (Burnaby, Canada), Merck Research Laboratories (Rahway, NJ), and the Dutch Heart Foundation (to Dr Kastelein). Dr Hayden holds a Canada Research Chair in Human Genes and Artherosclerosis de Vries, Prof Dr van Heek is an employee of Merck Research Laboratories. Drs Adelman and Collins are employees of Vascular Strategies LLC.

**Disclosures**

Dr Singaraja, Dr Tietjen, J. McEwen, and Dr Winther are employees and Dr D LEN is on the Board of Directors of Xenon Pharmaceuticals Inc. Dr Kastelein serves as a consultant to and receives research grants from Xenon Pharmaceuticals Inc. Drs Castro-Perez, Hubbard, Lin, Roddy, Wong, Mitnaul, and van Heek are employees of Merck Research Laboratories. Drs Adelman and Collins are employees of Vascular Strategies LLC.

**References**

suggests that inhibition of EL represents a promising therapeutic target for cardiovascular disease prevention. Our study implicates EL as playing a critical role in HDL metabolism and phospholipid content in HDL, thereby increasing the cholesterol efflux acceptor capacity of HDL. In line with this, HDL phospholipid content was increased in mutation carriers. Our study implicates EL as playing a critical role in HDL metabolism and phospholipid content in HDL, thereby increasing the cholesterol efflux acceptor capacity of HDL. In line with this, HDL phospholipid content was increased in mutation carriers.

Additionally, meta-analyses performed using 4 different cohorts indicate that mutations in EL are associated with a significant increase in HDL-C in both heterozygous and homozygous carriers in a gene dosage-dependent manner.

Cardiovascular disease is the leading cause of death worldwide. Despite lowering of low-density lipoprotein cholesterol with statins, significant residual risk still remains. Many epidemiological studies show that reduced plasma high-density lipoprotein cholesterol (HDL-C) is an independent risk factor for cardiovascular disease. Although heritability estimates for HDL-C are 40% to 60%, the majority of genes with roles in HDL metabolism have not been identified. In addition, few viable therapeutic targets are currently available for increasing plasma HDL-C levels. We show that mutations in endothelial lipase (LIPG) gene and high-density lipoprotein cholesterol levels. Biochim Biophys Acta. 2004;1636:40–46.


CLINICAL PERSPECTIVE

Cardiovascular disease is the leading cause of death worldwide. Despite lowering of low-density lipoprotein cholesterol with statins, significant residual risk still remains. Many epidemiological studies show that reduced plasma high-density lipoprotein cholesterol (HDL-C) is an independent risk factor for cardiovascular disease. Although heritability estimates for HDL-C are 40% to 60%, the majority of genes with roles in HDL metabolism have not been identified. In addition, few viable therapeutic targets are currently available for increasing plasma HDL-C levels. We show that mutations in endothelial lipase (LIPG; EL) result in an elevated plasma HDL-C in both heterozygous and homozygous carriers in a gene dosage-dependent manner. Additionally, meta-analyses performed using 4 different cohorts indicate that mutations in EL are associated with a significant decrease in cardiovascular disease, suggesting that inhibition of EL may be a therapeutic strategy for reducing cardiovascular disease. We show from mutation carriers was significantly better at eliciting the efflux of cholesterol from macrophages, an indication of increased cardioprotective functionality of HDL in the face of decreased EL. In addition, large α-1 HDL concentration, which shows an inverse association with cardiovascular disease, was significantly increased in mutation carriers. EL belongs to the triglyceride lipase family and acts preferentially on HDL phospholipids. Thus, mutations in EL are expected to increase phospholipid content in HDL, thereby increasing the cholesterol efflux acceptor capacity of HDL. In line with this, HDL phospholipid content was increased in mutation carriers. Our study implicates EL as playing a critical role in HDL metabolism and suggests that inhibition of EL represents a promising therapeutic target for cardiovascular disease prevention.
The Impact of Partial and Complete Loss-of-Function Mutations in Endothelial Lipase on High-Density Lipoprotein Levels and Functionality in Humans

Roshni R. Singaraja, Suthesh Sivapalaratnam, Kees Hovingh, Marie-Pierre Dubé, José Castro-Perez, Heidi L. Collins, Steven J. Adelman, Meliana Riwanto, Jasmin Manz, Brian Hubbard, Ian Tietjen, Kenny Wong, Lyndon J. Mitnaul, Margaret van Heek, Linus Lin, Thomas A. Roddy, Jason McEwen, Geesje Dallinge-Thie, Leonie van Vark-van der Zee, Germaine Verwoert, Michael Winther, Cornelia van Duijn, Albert Hofman, Mieke D. Trip, A. David Marais, Bela Asztalos, Ulf Landmesser, Eric Sijbrands, John J. Kastelein and Michael R. Hayden

Circ Cardiovasc Genet. 2013;6:54-62; originally published online December 14, 2012; doi: 10.1161/CIRCGENETICS.111.962613

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circgenetics.ahajournals.org/content/6/1/54

Data Supplement (unedited) at:
http://circgenetics.ahajournals.org/content/suppl/2012/12/14/CIRCGENETICS.111.962613.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Cardiovascular Genetics can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Cardiovascular Genetics is online at:
http://circgenetics.ahajournals.org//subscriptions/
Supplemental Material
Supplementary Figure 1: Anti-oxidative and anti-inflammatory properties of HDL. (A)

Effects of HDL isolated from LIPG mutation carriers (LOF, n=13), and wild-type controls (WT, n=9) on endothelial superoxide production were assessed, and showed a non-significant 53% reduction. (B) HDL from LIPG mutation carriers (n=13), and controls (n=9) was assessed for its effect on TNF-α-stimulated VCAM-1 expression from human aortic endothelial cells, and showed no difference, suggesting that there was no impact on anti-inflammatory properties of HDL from LIPG mutation carriers.