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Exome Sequencing and Directed Clinical Phenotyping Diagnose Cholesterol Ester Storage Disease Presenting as Autosomal Recessive Hypercholesterolemia

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for the National Heart, Lung, and Blood Institute GO Exome Sequencing Project

Objective—Autosomal recessive hypercholesterolemia is a rare inherited disorder, characterized by extremely high total and low-density lipoprotein cholesterol levels, that has been previously linked to mutations in *LDLRAP1*. We identified a family with autosomal recessive hypercholesterolemia not explained by mutations in *LDLRAP1* or other genes known to cause monogenic hypercholesterolemia. The aim of this study was to identify the molecular pathogenesis of autosomal recessive hypercholesterolemia in this family.

Approach and Results—We used exome sequencing to assess all protein-coding regions of the genome in 3 family members and identified a homozygous exon 8 splice junction mutation (c.894G>A, also known as E8SJM) in *LIPA* that segregated with the diagnosis of hypercholesterolemia. Because homozygosity for mutations in *LIPA* is known to cause cholesterol ester storage disease, we performed directed follow-up phenotyping by noninvasively measuring hepatic cholesterol content. We observed abnormal hepatic accumulation of cholesterol in the homozygote individuals, supporting the diagnosis of cholesterol ester storage disease. Given previous suggestions of cardiovascular disease risk in heterozygous *LIPA* mutation carriers, we genotyped E8SJM in >27 000 individuals and found no association with plasma lipid levels or risk of myocardial infarction, confirming a true recessive mode of inheritance.

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Conclusions—By integrating observations from Mendelian and population genetics along with directed clinical phenotyping, we diagnosed clinically unapparent cholesterol ester storage disease in the affected individuals from this kindred and addressed an outstanding question about risk of cardiovascular disease in *LIPA* E8SJM heterozygous carriers. (*Arterioscler Thromb Vasc Biol.* 2013;33:2909-2914.)

Key Words: genetics ■ hypercholesterolemia ■ myocardial infarction

Nonstandard Abbreviations and Acronyms

AGBL2	ATP/GTP binding protein-like 2
CAD	coronary artery disease
CESD	cholesterol ester storage disease
E8SJM	exon 8 splice junction mutation
LAL	lysosomal acid lipase
LDL-C	low-density lipoprotein cholesterol
MI	myocardial infarction
MRS	magnetic resonance spectroscopy
NGS	next-generation sequencing

Monogenic hypercholesterolemia is a disorder of lipid metabolism in which extremely elevated levels of total and low-density lipoprotein cholesterol (LDL-C) are caused by a single gene mutation. Mutations in *LDLR*,¹ *APOB*,² and *PCSK9*³ cause autosomal dominant hypercholesterolemia, a disease affecting ≥ 1 in 500 individuals. Autosomal recessive hypercholesterolemia occurs much less frequently, estimated to occur in 1:1 000 000 live births, and has been linked to mutations in *LDLRAP1*.⁴ In some families with apparent monogenic hypercholesterolemia, an underlying molecular defect cannot be identified in any of these known genes.

We identified a family with apparent Mendelian inheritance of high LDL-C levels that was not caused by mutations in any of the above genes known to affect LDL-C. The small size of the family pedigree precluded use of traditional linkage mapping. Next-generation sequencing (NGS), a rapid and low-cost method to perform large-scale DNA sequencing,⁵ has emerged as an important tool for uncovering the cause of inherited diseases.⁶ In this study, we used exome sequencing, a technique in which NGS is used to assess all protein-coding regions of the genome, in 3 individuals from this family to search for a rare genetic variant that cosegregated with high LDL-C levels.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Subject Recruitment

The proband (Figure 1; individual II-2) presented to the Lipid Clinic at the Academic Medical Center, University of Amsterdam, The Netherlands, at the age of 23. Her LDL-C level exceeded the 99th percentile when adjusted for age and sex. She had 2 siblings (1 of which was a monozygotic twin), both of whom shared LDL-C levels exceeding the 99th percentile. Her father and mother, a nonconsanguineous union, had LDL-C levels at the 25th and 78th percentile, respectively,

when adjusted for age and sex (Figure 1). The proband and both siblings lacked hepatosplenomegaly on abdominal examination. Based on the pedigree, an autosomal recessive mode of inheritance seemed to be the most likely explanation for the family's phenotype.

Exome Sequencing

To identify the molecular basis of hypercholesterolemia in this family, exome sequencing was performed in the proband, the proband's father, and the proband's brother (Figure 1; individuals II-2, I-1, and II-1, respectively). A total of 32 950 014 bases across the exome were targeted, and each sample was sequenced with an average of 126-fold coverage across the target. Across the exome, 82% of targeted bases were covered with >30 -fold coverage. This yielded a mean of 36 986 single nucleotide variants per individual. The average ratio of heterozygous to homozygous alleles (1.6) and ratio of transitions to transversions (2.7) per individual were expected and similar to contemporary large-scale population sequencing projects.⁷

Exome Sequencing Analysis

To exclude genetic variation unlikely to be responsible for this family's hypercholesterolemia, we relied on 3 main assumptions: (1) the causal variant(s) alters the gene's corresponding protein product; (2) the causal variant(s) is inherited in an autosomal recessive fashion; and (3) the causal variant(s) exhibits complete penetrance. For the first assumption, we only included single nucleotide substitutions and short insertions or deletions that were predicted to alter the protein sequence.

We next included either (1) compound heterozygous changes (a heterozygous variant in both affected siblings and the father located in a gene that also contained a separate heterozygous variant in both affected siblings not found in the father) or (2) variants that were homozygous in both affected siblings and heterozygous in the unaffected father. Finally, we excluded variants from further consideration if they were present in the general population at a frequency of $>1\%$, or if they were present in either heterozygous or homozygous form in the exome sequences of 235 individuals with very low LDL-C levels.

After applying this analysis, the number of variants shared among the 3 family members was reduced from 54 301 to 2 candidate single nucleotide substitutions. One was a synonymous variant predicted to alter the splice donor site of the eighth exon in the gene lipase A, lysosomal acid, cholesterol esterase (*LIPA*; c.894G>A, in the last nucleotide of exon 8), and the other was a missense change predicted to result in the substitution of Alanine for Proline at residue 384 in the gene ATP/GTP binding protein-like 2 (*AGBL2*).

Because previous reports showed a link between the c.894G>A mutation, also known as the exon 8 splice junction

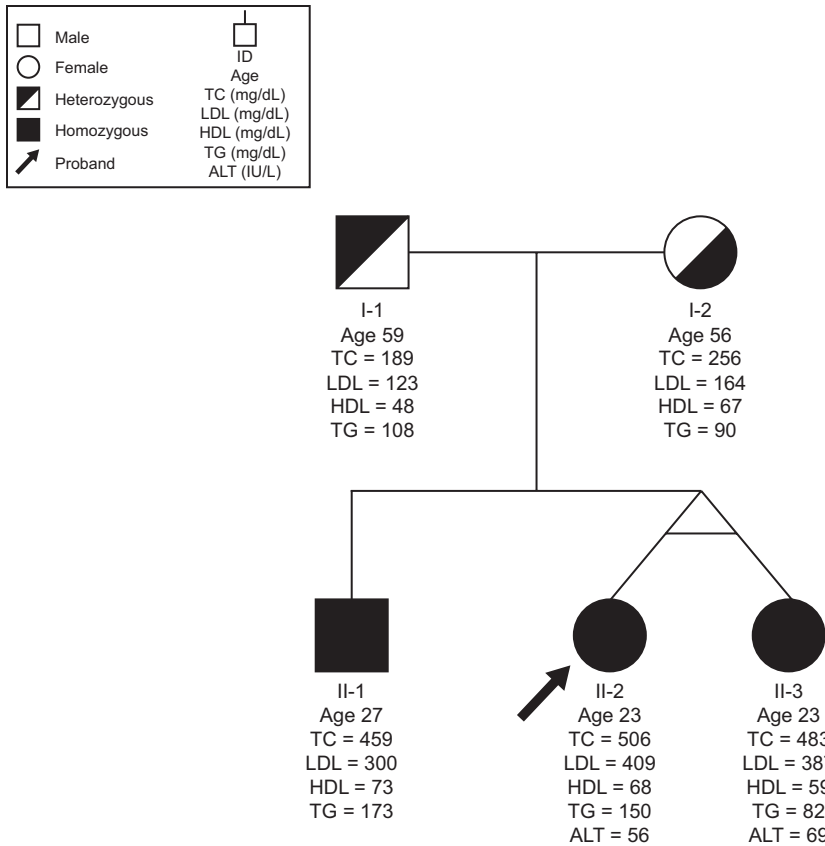


Figure 1. Pedigree of the family demonstrating autosomal recessive hypercholesterolemia. Laboratory values are shown below each individual. Individuals II-2 and II-3 are identical twins. ALT indicates alanine aminotransferase; HDL, high-density lipoprotein cholesterol; ID, individual ID from family pedigree; LDL, low-density lipoprotein cholesterol; TC, total cholesterol; and TG, triglycerides.

mutation (E8SJM), in *LIPA* and cholesterol ester storage disease (CESD),⁸ a disorder with mixed hyperlipidemia as part of the phenotypic presentation, we focused on a potential diagnosis of CESD as the most likely cause for this family’s apparent autosomal recessive hypercholesterolemia.

Functional Assessment of E8SJM

Sanger sequencing was performed and confirmed the presence of the E8SJM allele in the homozygous state in affected individuals and in the heterozygous state in both unaffected parents. Haplotype analysis revealed that both maternal and paternal E8SJM alleles were on the same haplotype as

previously reported for this mutation (haplotype 1 from Fasano et al⁹). This does not appear to be a result of consanguinity because the proband was found to share 53% and 49% of her exome identical-by-descent with her brother and father, respectively, eliminating cryptic consanguinity. The skipping of exon 8 was confirmed in all individuals carrying the mutated allele (Figure 2).

Although the affected individuals did not present with clinically apparent hepatic disease, given the previous reports linking mutations in *LIPA* with CESD, we reassessed the affected individuals for the level of hepatic cholesterol ester using magnetic resonance spectroscopy (MRS), a technique

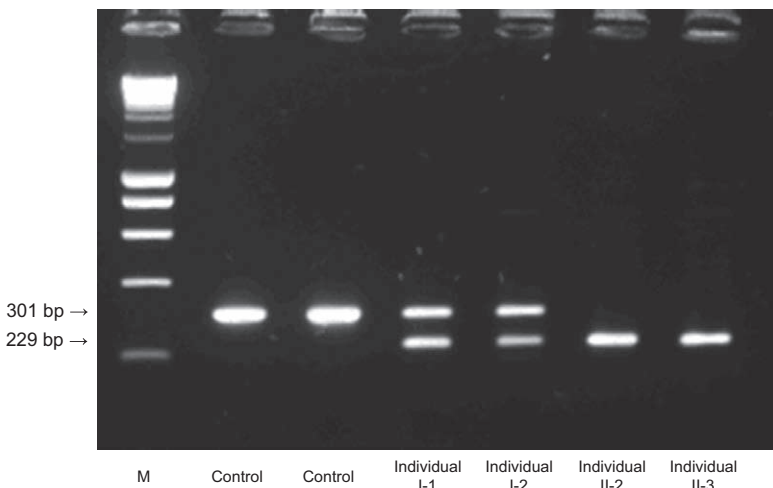


Figure 2. Reverse transcription polymerase chain reaction of *LIPA* demonstrating skipping of exon 8 as a result of exon 8 splice junction mutation (E8SJM). The upper and lower bands correspond to the expected products either containing (301 bp) or lacking (229 bp) exon 8, respectively. Control cDNA from individuals not carrying E8SJM demonstrates the expected product containing exon 8. Heterozygous carriers of E8SJM (individuals I-1 and I-2) demonstrate the presence of 1 wild-type transcript and 1 transcript lacking exon 8, whereas homozygous E8SJM carriers (individuals II-2 and II-3) demonstrate complete skipping of exon 8. M indicates molecular weight marker.

shown to correlate well with histological lipid distribution.¹⁰ In individuals II-1, II-2, and II-3, MRS demonstrated a distinct cholesterol peak separate from the larger and expected triglyceride peak at 1.25 ppm. The ratios between triglyceride at 1.25 ppm and cholesterol at 0.9 ppm were 0.57, 0.34, and 0.40 for individuals II-1, II-2, and II-3, respectively, indicating the presence of an excess of hepatic cholesterol deposition (Figure 3). The elevated cholesterol peak at 0.9 ppm was not identified in individual I-1. Individuals II-1, II-2, and II-3 had normal hepatic size as measured on the MRI portion of the study.

Population Impact of E8SJM

Given previous reports suggesting that serum lipids levels are increased in heterozygous E8SJM carriers,¹¹ we genotyped the E8SJM variant in 13 194 individuals of European ancestry. Triglyceride, high-density lipoprotein cholesterol, and LDL-C levels were available in 13 194, 13 144, and 12 805 individuals, respectively. In these individuals, the E8SJM was present with an allele frequency of 0.16% and no association was observed with any of these 3 lipid fractions (Table 1).

Furthermore, to also assess the impact of partial loss of *LIPA* function on risk for myocardial infarction (MI) or coronary artery disease (CAD) in the population, we genotyped the E8SJM variant in 27 472 individuals of European ancestry (12 747 cases with MI/CAD, 14 725 controls free of MI and CAD). In these individuals, the E8SJM was present with an allele frequency of 0.11% and there was no association of E8SJM with risk for MI or CAD (odds ratio for MI or CAD in carriers=0.85; $P=0.6$).

Discussion

Traditional Mendelian genetic analyses have relied on positional cloning and sequencing the genetic regions under linked

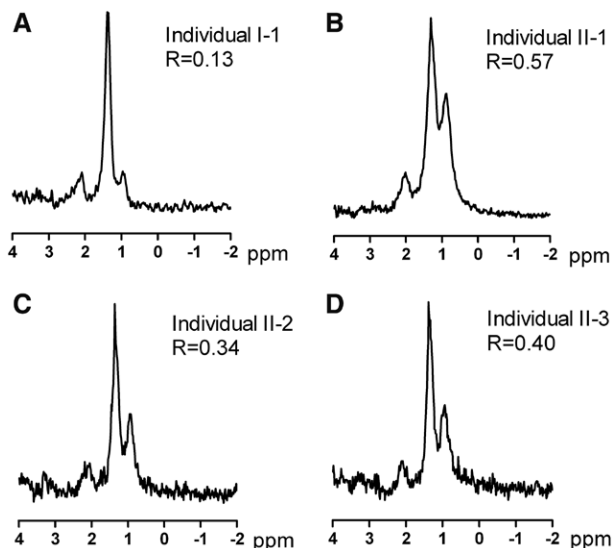


Figure 3. Water suppressed magnetic resonance spectroscopy spectra demonstrating hepatic cholesterol deposition in homozygous carriers of *LIPA* exon 8 splice junction mutation (E8SJM). R indicates ratio between peaks at 1.25 and 0.9 ppm. **A**, Individual I-1, the unaffected father of the proband, demonstrates a normal ratio. **B** to **D**, Individuals II-2, II-3, and II-1, respectively, demonstrate elevated ratios.

Table 1. Association of E8SJM and Plasma Lipid Levels in the Population

Trait	n	MAF	Effect, mg/dL*	95% CI	P Value
LDL-C	12 581	0.16%	-0.059	-13.3 to 13.2	0.9
HDL-C	12 839	0.16%	4.46	-0.5 to 9.5	0.08
TG	13 443	0.16%	-0.16	-0.3 to 0.02	0.08

CI indicates confidence interval; E8SJM, exon 8 splice junction mutation; MAF, minor allele frequency; and n, number of individuals contributing to the analysis.

*Effect: change in mg/dL for each copy of the minor allele.

peaks to identify causal defects responsible for monogenic disorders. These techniques are unfortunately of limited use in small families such as the one presented in the current study. NGS, however, now allows for the potential identification of candidate genes underlying Mendelian disorders in families regardless of the pedigree size. In this study, we performed NGS across the exome in 3 individuals from a family with suspected autosomal recessive hypercholesterolemia and identified homozygous E8SJM alleles in *LIPA* that cosegregated with the clinical diagnosis of hypercholesterolemia.

Lysosomal acid lipase (LAL), encoded by the gene *LIPA*, is responsible for hydrolyzing cholesterol esters and triglycerides that are delivered to lysosomes. Mutations in *LIPA* that completely inactivate LAL have previously been identified as the molecular cause of Wolman disease, a rapidly lethal disease of infancy, characterized by hepatosplenomegaly, abdominal distension, adrenal calcification, and steatorrhea with extensive storage of cholesterol esters and triglycerides in the liver, spleen, and other organs in the first weeks of life.^{12,13}

A related disorder, CESD, is associated with a less severe phenotype.^{14,15} Characterized by massive hepatic accumulation of cholesterol esters, hepatomegaly, steatosis, and mixed hyperlipidemia, CESD is caused by mutations in *LIPA* that result in near complete loss of LAL activity with enough residual enzymatic activity to hydrolyze triglycerides but not cholesterol esters.

The identification of homozygous E8SJM alleles in *LIPA* was surprising in this family because it has been previously identified as a cause of CESD.¹⁶ E8SJM has been shown to cause subtotal loss of gene function resulting in only 2% to 4% normally spliced *LIPA* mRNA transcripts and LAL activity.¹⁶ Homozygosity for E8SJM has previously been reported in individuals with hepatic disease and mixed hyperlipidemia, characterized by elevated levels of LDL-C and triglyceride with decreased high-density lipoprotein cholesterol levels (Table 2).

The homozygous individuals in the current study presented with a very different phenotype and would not have been clinically diagnosed with CESD. Their lipid profile is characterized by extremely elevated LDL-C with normal to high high-density lipoprotein cholesterol and normal triglyceride levels, whereas previously described E8SJM homozygotes have been noted to have increased LDL-C with low high-density lipoprotein cholesterol and elevated triglyceride levels (Table 2). In addition, the hepatic phenotype in the homozygous individuals from the current study appears to consist of only a subtle elevation in alanine aminotransferase (Table 2)

Table 2. Phenotypic Consequences of Homozygosity for *LIPA* E8SJM

Study	ID	TC, mg/dL	LDL-C, mg/dL	HDL-C, mg/dL	TG, mg/dL	AST, IU/L	ALT, IU/L	Associated Signs
Fasano et al ⁹		298	221	35	216	58	110	HSM
		323	N/A	N/A	259	2–3×ULN	2–3×ULN	HSM
		189	130	40	93	56	110	HM
		263	197	31	178	75	102	HM
Muntoni et al ¹⁶		337	264	16	216	N/A	N/A	HSM
Current study	II-2							
	At presentation	506	409	68	150	N/A	56	None
	On statin therapy	187	104	66	38	30	32	
	II-3							
	At presentation	483	387	59	82	N/A	69	None
	On statin therapy	127	47	66	28	33	37	
	II-1							
	At presentation	459	300	73	173	N/A	N/A	None
On statin therapy	140	81	47	64	45	61		

ALT indicates alanine aminotransferase (upper limit of normal in current study, 34 IU/L); AST, aspartate aminotransferase (upper limit of normal in current study, 40 IU/L); E8SJM, exon 8 splice junction mutation; HDL-C, high-density lipoprotein cholesterol; HM, hepatomegaly; HSM, hepatosplenomegaly; ID, individual ID from family pedigree (Figure 1); LDL-C, low-density lipoprotein cholesterol; N/A, not available; TC, total cholesterol; TG, triglycerides; and ULN, upper limit of normal.

without the typical hepatosplenomegaly (hepatomegaly and splenomegaly are present in >99% and 74% of patients with CESD, respectively).¹⁷

Given the previous associations between *LIPA* E8SJM and CESD (for homozygous carriers) and polygenic hypercholesterolemia and potentially increased risk of MI/CAD (for heterozygous carriers),¹¹ we performed directed phenotypic and genetic follow-up analyses to address 2 questions: (1) Do the homozygous carriers within this pedigree have hepatic hallmarks of CESD?; and (2) Are the heterozygous parents at increased risk for MI/CAD? Using noninvasive hepatic MRS, we demonstrated the presence of abnormal quantities of hepatic cholesterol in the homozygous E8SJM carriers of this family. This finding is entirely consistent with previously reported hepatic MRS findings in patients with LAL deficiency (previously reported MRS ratios, 0.24–0.5)¹⁸ and confirms the diagnosis of CESD in the 3 offspring. A liver biopsy was not thought to be clinically indicated given the absence of increased transaminase levels combined with previous reports surrounding the causal role of *LIPA* E8SJM in CESD and the confirmatory MRS findings. Although a seemingly subtle distinction, this diagnosis is clinically important because the offspring should be followed for the progression of hepatic disease and may be potential candidates in the future for enzyme replacement therapy that is currently in development.¹⁹ In addition, this finding illustrates that CESD may have a more variable phenotypic presentation than previously appreciated.

To assess the potential of increased cardiovascular disease risk in the heterozygous parents, we genotyped *LIPA* E8SJM in the population. The population frequency of *LIPA* E8SJM has previously been estimated to be between 0.21% and 0.25% in individuals of European descent^{20,21} and has been associated with a polygenic hypercholesterolemia phenotype,¹¹ prompting the hypothesis that it may be associated with increased risk of MI/CAD. We now firmly establish

that this variant is rarer than previously estimated (allele frequency=0.11%). We consider our estimate of the carrier frequency for European individuals to be more accurate than previous reports given the larger numbers of individuals assessed (27 472 in the current study compared with 4112 in a previous report).²¹

In this large genetic study, we observed no association of heterozygosity with plasma lipid levels or risk for MI/CAD. Although we cannot definitively exclude a weak association with MI/CAD or serum lipid levels, we had 93% power to detect a 2-fold increased risk of MI/CAD at an α of 0.05 and 94% power to detect a variant explaining 0.1% of the phenotypic variance in LDL-C at an α of 0.05. These findings suggest that the E8SJM acts in a truly recessive fashion and that heterozygous loss of function does not result in a distinct lipid or MI phenotype.

It is uncertain why the presentation of CESD in this family differed from those described in previous reports. The E8SJM in this family occurs on the same haplotype as previously reported for this mutation, supporting a common founder, ancestor for this mutation and suggesting that the milder-than-expected phenotype is not explained by a simple difference of local genetic background in *LIPA*. In addition to the E8SJM in *LIPA*, we identified rare homozygous alleles in *AGBL2* carried by all 3 affected offspring. At this time it is unclear what, if any, phenotypic effect this confers. There may be a genetic factor (in *AGBL2* or elsewhere) conferring a protective hepatic effect; however, given the lack of family members with hepatic disease as a comparator, we are underpowered to discover such a variant.

In summary, we report homozygosity for E8SJM in *LIPA* as a cause of clinically unapparent CESD presenting as autosomal recessive hypercholesterolemia. The discovery of E8SJM in *LIPA* in this family highlights both the blessing and the curse of using NGS in genetic discovery studies; along with the potential unbiased discovery of the causal variant

comes tens of thousands of additional variants unrelated to the phenotype of interest and the possibility of unexpected findings. We suggest integrating Mendelian and population genetics with directed clinical testing as a powerful way to discern signal from noise in the next generation of genetic discovery studies.

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Disclosures

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Significance

Autosomal recessive hypercholesterolemia is a rare inherited disorder previously linked to mutations in *LDLRAP1*. In this report, we use exome sequencing and clinical phenotyping to diagnose cholesterol ester storage disease in a small family with apparent autosomal recessive hypercholesterolemia. Cholesterol ester storage disease is caused by mutations in *LIPA* and typically presents with hepatic disease and mixed hyperlipidemia. This study reveals a broader phenotypic presentation for loss of function mutations in *LIPA* than previously appreciated and suggests that *LIPA* mutations may be considered in the clinical evaluation of autosomal recessive hypercholesterolemia.

Materials and Methods

Case history, sample collection and diagnostic procedures

The family was recruited from the outpatient clinic of the Department of Vascular Medicine at the Academic Medical Center, Amsterdam, the Netherlands. The proband (Figure 1; individual II-2) was referred to our clinic after being diagnosed with extremely elevated low density lipoprotein cholesterol (LDL-C) levels (409 mg/dL; >99th percentile when adjusted for age and sex) diagnosed during routine laboratory testing at work. We noted that her siblings had similar profiles and the following molecular diagnostic procedures were performed after all participants gave written informed consent.

Genomic DNA was prepared from 5 ml whole blood on an AutopureLS apparatus according to the manufacturer's protocol (Gentra Systems, Minneapolis, USA). The coding regions of *LDLR*, *APOB* (amino acids 3441 to 3615) and *PCSK9* were sequenced as previously described¹. *LDLRAP1* was also sequenced given the family's apparent autosomal recessive pattern of inheritance. Sequence analysis was performed by direct sequencing using the Big Dye Terminator ABI Prism Kit, version 1.1 (Applied Biosystems, Foster City, CA, USA). The products of the sequencing reactions were run on a Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA, USA) and sequence data were analyzed by the use of the Sequencer package (GeneCodes Co, Ann Arbor, MI, USA).

Plasma concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured by commercially available kits (Boehringer Mannheim, Mannheim, Germany) in EDTA samples derived from the participants after an overnight fast. LDL-C concentrations were calculated by the Friedewald formula².

Exome Sequencing

Whole exome sequencing was performed at the Broad Institute. Briefly, genomic DNA underwent library construction and in-solution hybrid selection as previously described³ using an Agilent (Agilent Technologies, Santa Clara, CA, USA) whole exome kit targeting 33Mb of genomic sequence. The target includes 188,260 exons from 18,560 genes. The resulting exome-enriched DNA was sequenced using 75-base pair paired-end reads on an Illumina GA-II sequencer⁴.

Exome Sequencing Analysis

Raw sequence reads from the Illumina sequencers were aligned to the human genome reference (HG19) using the Burroughs-Wheeler Alignment tool⁵ in paired-end mode. The aligned reads underwent base quality recalibration using the Genome Analysis Toolkit (GATK)^{6, 7}. Duplicated reads from sequencing the

same DNA molecule and sequencing reads not corresponding to the exome target were discarded.

Single nucleotide substitutions and short insertion and deletion events were identified and genotyped using the GATK UnifiedGenotyper tool in multisample mode. The quality scores of the resulting variants were recalibrated using the GATK Variant Score Recalibration and the functional consequence of each variant was predicted using SnpEff⁸. Plink⁹ was used to estimate the proportion of the exome that is shared identical-by-descent between the family members.

Each variant was also annotated with its corresponding allele frequency in the National Heart Lung and Blood Institute's Exome Sequencing Project (ESP) (<http://evs.gs.washington.edu/EVS>). As controls, we used 201 samples with extremely low levels of LDL-C that underwent exome sequencing as part of the ESP and the exomes from 34 individuals with suspected monogenic hypobetalipoproteinemia that were sequenced locally.

The haplotype containing the *LIPA* c.894G>A mutation was computationally reconstructed from the sequencing data using a set of common markers in *LIPA* as previously described¹⁰. Intronic variants used in the haplotype reconstruction were individually reviewed to ensure both read depth and sequencing quality were sufficient for accurate genotyping.

LIPA exon 8 splicing assay

Blood was drawn in a BD Vacutainer® CPT Mononuclear Cell Preparation Tube (BD, MJ, USA) and RNA was isolated within 2 hours using Trizol reagent (Ambion, Life technologies, CA, USA) according to the manufacturer's protocol. cDNA was prepared from 1.0 µg of RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, CA, USA). A sequence fragment spanning the eighth exon of *LIPA* was amplified by polymerase chain reaction using 1 µl of the cDNA preparation with the following primers: forward primer in exon 7: 5'- GAA GTG GCT GGG TAC CCA CG-3' and reverse primer in exon 10: 5'- CCA GAC TGC AGT CGG CAC AAG-3'. The resulting DNA products were visualized on an agarose gel to confirm the splicing defect. *LIPA* RNA was also isolated from individuals not carrying the c.894G>A mutation.

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS) was performed on a 3T MR scanner (Philips Healthcare, Best, The Netherlands) using body coil transmission and a 16ch receive torso coil. A 20x20x20-mm voxel was positioned in the right liver lobe. The voxel was placed distant from blood vessels in the liver. Spectra were acquired during free breathing by using first-order iterative shimming and a water suppressed STEAM sequence (TR/TE 3000/12 ms, 32 acquisitions). Prior to averaging, individual spectra were zero order phase corrected and the central TG

peaks (triglyceride, at 1.25 ppm) were aligned to the same location. Ratios between TG and cholesterol peaks were calculated by fitting the peaks at 1.25 and 0.9 ppm using a spectroscopic analysis package (jMRUI)¹¹.

Population genetics of LIPA E8SJM

We genotyped *LIPA* c.894G>A (rs116928232) in a total of eleven previously described cohorts, to evaluate the phenotype of heterozygous carriers. To assess the effect of *LIPA* E8SJM on serum lipid levels, we genotyped rs116928232 in 1) the Atherosclerosis, Thrombosis, and Vascular Biology Italian Study¹²; 2) the Malmo Diet and Cancer Cohort¹³; 3) the PROCARDIS study¹⁴; and 4) the Ottawa Heart Genomics Study (OHGS)¹⁵. In addition to these cohorts, to assess the effect of *LIPA* E8SJM on risk of myocardial infarction/coronary artery disease (MI/CAD), we genotyped rs116928232 in 5) the AMC-Premature Atherosclerosis Study (AMC-PAS)¹⁶; 6) the PennCath study¹⁷; 7) the Verona Heart Study¹⁸; 8) the Women's Health Initiative¹⁹; 9) the Welcome Case Control Consortium Coronary Heart Disease Study²⁰; 10) the German MI Family Study²⁰; and 11) the Stockholm Heart Epidemiology Programme. Cohorts 1-8 were genotyped using the Illumina HumanExome v1.0 SNP array (Illumina, CA, USA) using GenomeStudio v2010.3 module version 1.8.4 along with the custom cluster file StanCtrExChp_CEPH.egt Cohorts 9-10 were genotyped using Taqman technology (ABI, CA, USA) and cohort 11 was genotyped using the Sequenom platform (Sequenom, CA, USA). All genotyping was performed at the Broad Institute (Cambridge, MA).

Samples were excluded if one or more of the following criteria were met: missing greater than 5% of genotypes on the Illumina HumanExome array; discordant self-reported and genotype-inferred gender; an inbreeding coefficient less than -0.2 or greater than 0.2 across all markers on the array; duplicated samples; or proportion of genotypes IBD > 0.2 with another sample. Principal components were calculated using Eigenstrat 4.2²¹ and population outliers were removed. Quality control procedures were performed using PLINK⁹ and R (The R Project for Statistical Computing, Vienna, Austria).

The association between MI/CAD and rs116928232 was assessed using a fixed-effects Mantel-Haenszel meta-analysis. The association of rs116928232 with LDL-C, HDL-C, and log-transformed triglyceride levels was assessed for each cohort with linear regression using ten principal components of ancestry, age, and gender as covariates. A meta-analysis using inverse standard-error weighting was performed to combine the individual association results. The association testing was performed using PLINK⁹ and the meta-analysis was performed using METAL²² and R (The R Project for Statistical Computing, Vienna, Austria).

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