

Targeted next-generation sequencing to diagnose disorders of HDL cholesterol^S

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Abstract A low level of HDL cholesterol (HDL-C) is a common clinical scenario and an important marker for increased cardiovascular risk. Many patients with very low or very high HDL-C have a rare mutation in one of several genes, but identification of the molecular abnormality in patients with extreme HDL-C is rarely performed in clinical practice. We investigated the accuracy and diagnostic yield of a targeted next-generation sequencing (NGS) assay for extreme levels of HDL-C. We developed a targeted NGS panel to capture the exons, intron/exon boundaries, and untranslated regions of 26 genes with highly penetrant effects on plasma lipid levels. We sequenced 141 patients with extreme HDL-C levels and prioritized variants in accordance with medical genetics guidelines. We identified 35 pathogenic and probably pathogenic variants in HDL genes, including 21 novel variants, and performed functional validation on a subset of these. Overall, a molecular diagnosis was established in 35.9% of patients with low HDL-C and 5.2% with high HDL-C, and all prioritized variants identified by NGS were confirmed by Sanger sequencing. Our results suggest that a molecular diagnosis can be identified in a substantial proportion of patients with low HDL-C using targeted NGS.—Sadananda, S. N., J. N. Foo, M. T. Toh, L. Cermakova, L. Trigueros-Motos, T. Chan, H. Liany, J. Collins, S. Gerami, R. R. Singaraja, M. R. Hayden, G. A. Francis, J. Frohlich, C. C. Khor, and L. R. Brunham. Targeted next-generation sequencing to diagnose disorders of HDL cholesterol. *J. Lipid Res.* 2015. 56: 1993–2001.

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A low concentration of plasma HDL cholesterol (HDL-C) is one of the most common lipid abnormalities and an important risk factor for CVD. Low HDL-C predicts increased CVD risk, even among patients with aggressively treated LDL cholesterol (LDL-C) (1–3). HDL-C levels are highly heritable (4) and display both locus and allelic heterogeneity, with multiple variants in several genes leading to very high or very low HDL-C. The genetic architecture of HDL-C is notable among polygenic traits in that rare variants with presumed large effect sizes are present in a substantial proportion of patients with very low or very high HDL-C, usually in the same genes that cause extremely rare Mendelian disorders of HDL-C (5–12). This suggests that it may be possible to establish a molecular diagnosis in many patients with extreme HDL-C levels. However, detection of the specific molecular abnormality in patients with extreme HDL-C is rarely performed outside of specialized research laboratories because of the cost, complexity, and time required to do so, and uncertainty regarding the clinical utility of establishing a molecular diagnosis.

The development of next-generation sequencing (NGS) technologies has created new opportunities for the routine use of sequencing in clinical medicine. Targeted NGS panels have been effectively used for newborn carrier screening and for the diagnosis of inherited cardiomyopathy, hereditary cancers, and other conditions (13), but to date have not been established for disorders of HDL-C. The objective of this study was to evaluate the accuracy

Abbreviations: HDL-C, HDL cholesterol; HGMD, Human Gene Mutation Database; ID, identification number; LDL-C, LDL cholesterol; MAF, minor allele frequency; NGS, next-generation sequencing; UTR, untranslated region.

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and diagnostic yield of a targeted NGS panel to establish the molecular diagnosis of abnormal HDL-C in a range of patients with extreme HDL phenotypes visiting a specialty lipid clinic. Our results indicate that this approach can reliably and accurately identify pathogenic variants in a substantial proportion of patients with low levels of HDL-C.

MATERIALS AND METHODS

Patients

We recruited consecutive patients with HDL-C levels below the 10th percentile or greater than the 90th percentile using age- and gender-adjusted population data from the Lipid Research Clinics (14), hereafter referred to as “extreme HDL-C,” regardless of other lipid parameters, from the Healthy Heart Program Prevention Clinic at St. Paul’s Hospital, Vancouver, Canada, a large specialty lipid clinic serving a multi-ethnic patient population. We did not attempt to exclude patients with potential secondary causes of low HDL-C (e.g., hypertriglyceridemia) or high HDL-C (e.g., alcohol use). Related individuals were not specifically recruited. Patients were excluded if they could not speak English or were unable or unwilling to provide written informed consent. As a positive control, we included patients in whom a molecular diagnosis of a Mendelian disorder of HDL had previously been established by Sanger sequencing (15). Clinical data were abstracted from the patients’ medical records. In cases where multiple sets of laboratory values were available, we used the most recent values. All subjects provided written informed consent. This study was approved by the Clinical Research Ethics Board of the University of British Columbia.

DNA sequencing

DNA was isolated from venous blood samples using DNeasy kits (Qiagen) or saliva using Oragene kits (DNA Genotek) and quantified using the Quant-iT PicoGreen assay (Life Technologies). Sequencing libraries were prepared according to the manufacturer’s instructions (Illumina, San Diego, CA). In-solution hybridization capture was performed with SeqCap EZ library reagents (Roche-Nimblegen). Captured DNA was quantified by the KAPA system (Kapa Biosystems) and diluted to a final concentration of 5 nM. Sequencing was performed on an Illumina MiSeq instrument in 2×151 bp mode. Reads were mapped to the reference human genome (hg19) using Burrows-Wheeler alignment tool v0.5.9 in paired read mode (maximal exact match algorithm) and processed following the recommended guidelines by the Genome Analysis Toolkit “Best Practices for Variant Calling” (available at <http://www.broadinstitute.org/genome-analysis-toolkit>). Variants were called using the Genome Analysis Toolkit v3 Unified Genotyper. We applied the following variant-level and genotype-level quality filters for variant calling: quality by depth (QD) < 2.0 ; root mean square of mapping quality (MQ) < 40.0 ; rank sum test of mapping quality (MQRankSum) < -12.5 ; rank sum test of read position (ReadPosRankSum) < -8.0 , < 20 times base coverage; $< Q20$ genotype quality; and allele balance < 0.15 for heterozygous calls and < 0.85 for homozygous calls. All single nucleotide variants and indels that passed these quality control filters were annotated with the SIFT, Polyphen 2 [HumDiv], Polyphen 2 [HumVar], PROVEAN, and Condel tools, and matched against public databases of variants (1000 Genomes, HapMap, and National Heart, Lung, and Blood Institute exome variant server). We then prioritized variants in HDL genes according to the following criteria: 1) variants that are reported to be disease-causing in the Human Gene Mutation Database

(HGMD) (16); 2) disruptive variants [nonsense, splice-site (two nucleotides on either side of the intron/exon boundary) and frameshift] that are novel or rare [minor allele frequency (MAF) $< 1\%$ in public databases and MAF $< 4\%$ in study samples]; and 3) novel or rare (MAF $< 1\%$) missense variants that were predicted to be deleterious by all of SIFT, Polyphen 2 [HumDiv], Polyphen 2 [HumVar], PROVEAN, and Condel. Variants that met these criteria were validated by bidirectional Sanger sequencing of PCR amplicons (primer sequences available on request).

Biochemical assays

ABCA1 cDNA sequences were cloned into the pCDNA3.1 vector and the W590L and R2200* mutations generated by site-directed mutagenesis. Immunoblotting and immunofluorescence in transiently transfected HEK293 cells were carried out as previously described (15). Cholesterol efflux assays were performed, as previously described (15), in transiently transfected HEK293 cells. Data represent the mean \pm SD of at least three independent experiments, each performed in triplicate.

LCAT assays were performed as recommended by the manufacturer (Calbiochem) using 5 μ l of human plasma. The fluorescent intensities at 390 nm and 470 nm were measured at time = 0, 2, 4, and 8 h. LCAT activity was calculated as the rate of change in the ratio of fluorescent intensities at 470 nm and 390 nm. Data represent the mean \pm SD of three independent experiments, each performed in triplicate.

Statistical analyses

Results are presented as mean \pm SD. Differences between groups were compared with two-tailed Student’s *t*-test, or one-way ANOVA for three or more groups, or for comparison between proportions, chi-squared test. Calculations were performed in GraphPad Prism software. $P < 0.05$ was considered statistically significant.

RESULTS

Sequencing extreme HDL-C patients

We generated a customized NGS panel to capture the exons, intron/exon boundaries, and flanking untranslated regions (UTRs) of 26 genes with known roles in plasma lipid metabolism based on data from the HGMD and manual curation of the literature (Table 1). We included genes in which rare variants are known to cause highly penetrant effects on plasma levels of HDL-C, LDL-C, TG, and lipoprotein (a), as well as on the response to lipid-lowering therapy. We generated a probe library using Nimblegen SeqCap technology to capture these targets.

To assess the accuracy and diagnostic yield of this panel, we sequenced 141 patients with extreme levels of HDL-C, including 64 patients with extremely low HDL-C and 77 patients with extremely high HDL-C. This patient population was hypothesized to harbor a substantial percentage of damaging variants in HDL-related genes (5–12). The clinical characteristics of these patients are shown in Table 2. Patients with low HDL-C also had lower total plasma cholesterol and LDL-C, as well as higher TGs, higher BMI, and a higher frequency of diabetes mellitus, consistent with previous observations (17). Most patients in both groups were of self-reported European ancestry.

We performed high-throughput sequencing of this cohort to a mean depth of $1,559 \pm 290$ reads per base after quality

TABLE 1. List of genes sequenced

Phenotype	Gene	MIM Number	Disease	Locus	Number of Exons
Low HDL-C	<i>ABCA1</i>	600046	Tangier disease	9q31.1	50
Low HDL-C	<i>APOA1</i>	107680	ApoA-I deficiency	11q23-q24	5
Low HDL-C	<i>APOA2</i>	107670	ApoA-II deficiency	1q23.3	4
Low HDL-C	<i>LCAT</i>	606967	Familial LCAT deficiency/ fish eye disease	16q22.1	6
Low HDL-C	<i>NPC1</i>	607623	Niemann-Pick disease	18q11.2	27
Low HDL-C	<i>PLTP</i>	172425	—	20q13.12	17
High HDL-C	<i>CETP</i>	118470	—	16q21	17
High HDL-C	<i>GALNT2</i>	602274	—	1q41-q42	16
High HDL-C	<i>LIPG</i>	603684	—	18q21.1	11
High HDL-C	<i>SCARB1</i>	601040	—	12q24.31	13
High LDL-C	<i>ABCG5</i>	605459	Sitosterolemia	2p21	16
High LDL-C	<i>ABCG8</i>	605460	Sitosterolemia	2p21	13
High LDL-C/low LDL-C	<i>APOB</i>	107730	FH	2p24-p23	29
High LDL-C	<i>LDLR</i>	606945	FH	19p13.2	18
High LDL-C	<i>LDLRAP1</i>	605747	FH	1p36-p35	13
High LDL-C/low LDL-C	<i>PCSK9</i>	607786	FH	1p32.3	12
Low LDL-C	<i>MTTP</i>	157147	Abetalipoproteinemia	4q24	19
High TG	<i>APOA5</i>	606368	—	11q23	4
High TG	<i>APOC2</i>	608083	Chylomicronemia	19q13.2	4
High TG	<i>APOE</i>	107741	Dysbetalipoproteinemia	19q13.2	4
High TG	<i>GPIHBP1</i>	612757	—	8q24.3	4
High TG	<i>LMF1</i>	611761	—	16p13.3	20
High TG	<i>LPL</i>	609708	Chylomicronemia	8p22	10
Low TG/high HDL-C	<i>APOC3</i>	107720	—	11q23.3	4
High lipoprotein (a)	<i>LPA</i>	152200	—	6q26	40
Statin myopathy	<i>SLCO1B1</i>	604843	—	12p	15

The MIM number is from the Online Mendelian Inheritance in Man database. FH, familial hypercholesterolemia.

filtering. Ninety-three percent of target bases were covered by 20 or more reads. Ninety-four percent of targeted exons had $\geq 50\%$ of bases covered with an average of 15 or more reads. The mean GC (guanine-cytosine) content of exons with $< 50\%$ of bases covered with 15 or more reads was $70.8 \pm 4\%$ compared with $52.0 \pm 8\%$ for exons with $\geq 50\%$ of bases covered with 15 or more reads, suggesting that local genomic features interfered with the capture of these loci.

We identified 668 variants that met quality filtering criteria, including 357 exonic variants, 37 intronic variants, 212 3'UTR variants, 37 5'UTR variants, 18 variants downstream of the 3'UTR, and 7 variants upstream of 5'UTR. We then prioritized these variants (supplementary Fig. 1) based on established medical genetics recommendations (18), as follows. Variants known to be disease-causing for abnormalities of HDL-C in HGMD were designated as “pathogenic.” Novel or rare variants (MAF $< 1\%$ in public databases and MAF $< 4\%$ in study samples) in HDL-related genes were designated as “probably pathogenic” if they resulted in a premature stop, indel, or splice site alteration, or for missense variants, if they were predicted to be deleterious by five different variant prediction algorithms (SIFT, Polyphen 2 [HumDiv], Polyphen 2 [HumVar], PROVEAN, and Condel).

Thirty-five variants in 38 individuals met these prioritization criteria, including 29 variants in low HDL-C genes and 6 variants in high HDL-C genes (Table 3). Among the 35 patients with prioritized variants, 10 patients (28.5%) had > 1 variant. Variants in the *ABCA1* gene were most frequent, followed by variants in *LCAT*. No variants that met our prioritization criteria were identified in *APOA2*, *GALNT2*, *APOC3*, or *PLTP*. Twenty-one of the prioritized variants we identified were novel, including ten novel variants in *ABCA1*,

three novel variants in *LCAT*, two novel variants in each of *APOA1*, *NPC1*, *LIPG*, and one novel variant in each of *CETP* and *SCARB1* (Table 3).

Of note, three patients with extremely low HDL-C were found to harbor homozygous or compound heterozygous mutations in the *APOA1*, *ABCA1*, and *LCAT* genes (Table 4). This included a 43-year-old female subject [patient identification number (ID) 114] with extremely low HDL-C (0.1 mmol/l) and ApoA-I (0.05 g/l), found to be homozygous for a novel premature truncation in the *APOA1* gene (p.Arg34*); a 21-year-old male subject (patient ID 18301) with near undetectable HDL-C (0.1 mmol/l) found to be

TABLE 2. Characteristics of patients with extreme HDL-C

	High HDL-C Patients	Low HDL-C Patients	P
Number	77	64	—
Age (mean \pm SD)	58.4 \pm 10	53.4 \pm 13	0.01
Gender (% male)	26.0	79.7	< 0.0001
TC (mean \pm SD, mmol/l)	5.87 \pm 1.3	4.15 \pm 1.2	< 0.0001
HDL-C (mean \pm SD, mmol/l)	2.47 \pm 0.4	0.66 \pm 0.2	< 0.0001
LDL-C (mean \pm SD, mmol/l)	3.06 \pm 1.1	2.13 \pm 0.9	< 0.0001
TG (mean \pm SD, mmol/l)	0.88 \pm 0.4	3.88 \pm 3.4	< 0.0001
ApoA-I (mean \pm SD, g/l)	2.14 \pm 0.3	1.07 \pm 0.2	< 0.0001
BMI (mean \pm SD, kg/m ²)	25.0 \pm 16	29.7 \pm 6	< 0.0001
CAD (%)	6.5	12.5	0.2
Diabetes mellitus (%)	0.0	27.0	< 0.0001
Current smoker (%)	4.8	14.1	0.07
Lipid lowering medication (%)	59.5	62.9	0.6
Self-reported ancestry			
European (%)	81.8	71.8	0.3
Asian (%)	11.7	14.1	0.3
Other/not reported (%)	6.5	14.1	0.3

TC, total cholesterol; CAD, coronary artery disease. P values represent two-tailed t-test for comparison of means and chi-squared test for comparison of proportions.

TABLE 3. Pathogenic and probably pathogenic variants identified in patients with extreme HDL-C

	Ref. Seq.	Nuc. Change	Amino Acid Change	rsID/Novel	HGMD	Functional Prediction	MAF	Phenotype
Low HDL genes								
<i>ABCA1</i>	NM_005502	c.1196T>C	p.Val399Ala	rs9282543:G	DM	Tolerated	0.002	Low
<i>ABCA1</i>	NM_005502	c.1755C>A	p.Asp585Glu	Novel	—	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.1769G>T	p.Trp590Leu	Novel	DM	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.1913G>A	p.Arg638Gln	Novel	DM	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.2328G>C	p.Lys776Asn	rs138880920	DP	Damaging	0.003	High
<i>ABCA1</i>	NM_005502	c.2540C>T	p.Pro847Leu	Novel	—	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.3338delT	p.Gln980Serfs*9	Novel	—	—	—	Low
<i>ABCA1</i>	NM_005502	c.3121C>G	p.Leu1041Val	rs192935024	—	Damaging	0.001	High
<i>ABCA1</i>	NM_005502	c.3541C>A	p.Ala1046Asp	rs141021096	DM	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.3946C>T	p.Ser1181Phe	rs76881554	DM	Damaging	0.0006	High
<i>ABCA1</i>	NM_005502	c.4449delC	p.Leu1484Cysfs*17	Novel	—	—	—	Low
<i>ABCA1</i>	NM_005502	c.4939C>T	p.Thr1512M	Novel	DM	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.5039G>A	p.Arg1680Gln	rs150125857	DM	Damaging	0.0002	Low
<i>ABCA1</i>	NM_005502	c.5550G>T	p.Tryp1699Cys	rs146934490	DM	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.5449C>T	p.Arg1817*	Novel	—	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.5702_5703dupGA	p.Ile1902Glufs*12	Novel	—	—	—	Low
<i>ABCA1</i>	NM_005502	c.7002C>T	p.Arg2200*	Novel	—	Damaging	—	Low
<i>ABCA1</i>	NM_005502	c.6730G>A	p.Val2244Ile	rs144588452	DM	Tolerated	0.0004	Low
<i>APOA1</i>	NM_000039	c.138C>T	p.Arg34*	Novel	—	Damaging	—	Low
<i>APOA1</i>	NM_000039	c.382T>A	p.Lys131Met	rs4882	—	Damaging	—	Low
<i>APOA1</i>	NM_000039	c.391_393del	p.Lys131del	Novel	—	—	—	Low
<i>LCAT</i>	NM_000229	c.451C>T	p.Thr147Ile	rs121908050	DM	Damaging	—	Low
<i>LCAT</i>	NM_000229	c.487G>A	p.Arg159Gln	Novel	DM	Damaging	Low	—
<i>LCAT</i>	NM_000229	c.694T>A	p.Ser232Thr	rs4986970	DM	Tolerated	0.0084	Both
<i>LCAT</i>	NM_000229	c.1043C>A	p.Thr348Ile	Novel	—	Damaging	—	Low
<i>LCAT</i>	NM_000229	c.1103G>T	p.Gly368Val	Novel	DM	Damaging	—	Low
<i>NPCI</i>	NM_000271	c.665A>G	p.Asn222Ser	rs55680026	DM	Tolerated	0.003	Both
<i>NPCI</i>	NM_000271	c.3308G>T	p.Gly1012Cys	Novel	—	Damaging	—	Low
<i>NPCI</i>	NM_000271	c.3689T>C	p.Leu1230Ser	Novel	—	Damaging	—	High
High HDL genes								
<i>CETP</i>	NM_000078	c.118+1-118+4delGTAA	splice site	Novel	DM	—	—	High
<i>CETP</i>	NM_000078	c.1376A>G	p.Asp459Gly	rs2303790	DM	Damaging	0.0062	High
<i>LIPG</i>	NM_006033	c.716T>C	p.Ile239Thr	Novel	DM	Damaging	—	High
<i>LIPG</i>	NM_006033	c.1069G>T	p.Glu273*	Novel	—	Damaging	—	High
<i>LIPG</i>	NM_006033	c.1426C>T	p.Arg476Trp	rs117623631	DM	Damaging	0.003	High
<i>SCARB1</i>	NM_001082959	c.715G>A	p.Gly239Arg	Novel	—	Damaging	—	Low

MAF is based on 1000 Genomes CEU (Utah residents with ancestry from northern and western Europe) data. Functional prediction based on SIFT. DM, disease-causing mutation in HGMD; DP, disease-associated polymorphism in HGMD; Ref. Seq., reference sequence; Nuc., nucleotide; rsID, reference SNP identification. Phenotype refers to whether the variant was detected in a patient (or patients) with low HDL-C, high HDL-C, or in both groups of patients.

compound heterozygous for one known disease-causing mutation in *ABCA1* (p.Trp590Leu) and one novel premature truncation in *ABCA1* (p.Arg2200*); and a 21-year-old male subject (patient ID 34) with extremely low HDL-C (0.1 mmol/l) and less severely reduced ApoA-I levels (0.9 g/l), found to be homozygous for a novel predicted deleterious missense variant in *LCAT* (p.Thr348Ile), as well as heterozygous for a previously identified variant in *ABCA1* (p.Val2244Ile) (19). These results established molecular diagnoses of the rare Mendelian disorders ApoA-I deficiency, Tangier disease, and familial *LCAT* deficiency, respectively, in these three individuals. Another individual (patient ID 35) who is the sibling of patient ID 34 and had a less dramatic reduction in HDL-C (0.5 mmol/l) was found to be heterozygous for the same *LCAT* variant (p.Thr348Ile), establishing a gene-dosage relationship for this variant with HDL-C levels.

One individual with extremely high HDL-C (3.2 mmol/l) was compound heterozygous for two known disease-causing variants in *LIPG*, p.Arg476Trp, and p.Ile239Thr (20). One individual of self-reported Japanese ancestry with high HDL-C (1.9 mmol/l) was heterozygous for p.Asp459Gly in

CETP, a known high HDL-C-causing variant that is present in 2–3% of individuals of Asian ancestry, but absent in individuals of European ancestry (<http://www.1000genomes.org>). One patient with low HDL-C (0.52 mmol/l) was heterozygous for both a novel 2 bp insertion in *ABCA1* leading to premature protein truncation (p.Glu1902Ilefs*1912) and a novel predicted damaging mutation in *SCARB1* (p.Gly239Arg). This observation is consistent with previous data that mutations in *ABCA1* are dominant to the effects of mutations in *SCARB1* (21).

The distribution of prioritized alleles in genes known to cause low HDL-C was highly skewed toward patients with low HDL-C, with 44/50 (88%) of such alleles occurring in patients with low HDL-C and only 6/50 (12%) in patients with high HDL-C. Similarly, 83% of prioritized alleles in genes known to cause high HDL-C were observed in patients with high HDL-C. Among patients with low HDL-C, 34/35 (97.1%) of the prioritized alleles observed were in genes known to cause low HDL-C, and only 2.9% were in genes known to cause high HDL-C. These observations suggest that our prioritization criteria were effective, in general, at identifying variants with penetrant effects on

TABLE 4. Individuals with a new molecular diagnosis of HDL

Patient ID	Gender	Age	TC	HDL-C	LDL-C	TG	ApoA-I	Gene	Type	Variant
Low HDL-C patients										
114	F	43	3.48	0.10	2.30	2.49	0.05	<i>APOA1</i>	Homo.	p.Arg34*
18301	M	21	1.80	0.10	1.36	0.85	0.27	<i>ABCA1</i>	Cmpd. het.	p.Arg2200* p.Trp590Leu
34	F	21	2.30	0.10	1.70	1.10	0.90	<i>LCAT</i> <i>ABCA1</i>	Homo. Het.	p.Val2244Ile p.Val2244Ile
13	M	63	5.18	0.33	3.73	2.44	1.04	<i>ABCA1</i>	Het.	p.Arg638Gln
96	M	59	3.70	0.36	–	4.06	0.89	<i>LCAT</i>	Het.	p.Arg159Gln
41	M	62	3.87	0.39	2.28	2.65	1.15	<i>LCAT</i> <i>ABCA1</i>	Het. Het.	p.Ala165Thr p.Arg1680Gln
28	M	41	3.10	0.40	1.30	3.00	0.60	<i>APOA1</i> <i>LCAT</i>	Het. Het.	p.Lys131del p.Ser232Thr
4	F	67	3.15	0.44	1.71	2.21	0.62	<i>ABCA1</i>	Het.	p.Arg1817*
21	M	61	4.70	0.50	2.50	3.70	1.00	<i>ABCA1</i>	Het.	p.Asp585Glu
91	M	62	3.40	0.50	1.80	2.40	0.76	<i>ABCA1</i>	Het.	p.Pro847Leu
35	M	26	4.00	0.50	2.70	1.70	1.14	<i>LCAT</i> <i>ABCA1</i>	Het. Het.	p.Thr348Ile p.Val2244Ile
87	M	49	3.20	0.52	1.60	2.20	0.98	<i>ABCA1</i> <i>SCARB1</i>	Het. Het.	p.Glu1902Ilefs*12 p.Gly239Arg
117	F	58	6.63	0.59	5.30	1.61	1.12	<i>ABCA1</i>	Cmpd. het.	p.Thr1512Met p.Gln980Serfs*9
9	F	40	4.90	0.60	–	8.70	1.13	<i>LCAT</i>	Het.	p.Ser232Thr
90	M	48	3.60	0.60	2.30	1.60	1.11	<i>LCAT</i>	Het.	p.Gly368Val
20	M	56	1.83	0.65	0.79	0.85	1.14	<i>ABCA1</i>	Het.	p.Val399Ala
115	F	54	4.20	0.66	2.40	2.50	1.30	<i>ABCA1</i> <i>LCAT</i>	Cmpd. het. Het.	p.Trp1699Cys p.Ala1046Asp
139	M	60	3.43	0.66	1.16	3.55	1.16	<i>ABCA1</i>	Het.	p.Ser232Thr
134	M	71	3.40	0.70	2.00	1.50	0.99	<i>LCAT</i>	Het.	p.Val399Ala
14	M	31	6.60	0.70	4.40	3.20	1.01	<i>LCAT</i>	Het.	p.Thr147Ile
57	M	35	4.10	0.70	2.90	1.00	0.96	<i>APOA1</i>	Het.	p.Ser232Thr
53	M	47	4.16	0.71	2.49	2.12	0.93	<i>ABCA1</i>	Het.	p.Lys131Met
66	F	53	3.80	1.00	2.40	0.80	1.11	<i>ABCA1</i>	Het.	p.Cys1484Leufs*17 p.Trp590Leu
High HDL-C patients										
36	F	77	6.80	3.15	3.40	0.69	–	<i>LIPG</i>	Cmpd. het.	p.Ile239Thr p.Arg476Trp
48	F	61	5.10	2.20	2.20	1.47	2.38	<i>CETP</i> <i>ABCA1</i>	Het. Het.	p.Asp459Gly p.Leu1041Val
44	M	71	5.10	2.20	2.20	2.00	1.97	<i>CETP</i>	Het.	Splice site
100	M	89	5.46	2.13	2.87	1.01	1.69	<i>LIPG</i>	Het.	p.Glu273*

TC, total cholesterol; Het., heterozygous; Cmpd. het., compound heterozygous; Homo., homozygous.

the lipid phenotypes in question. A notable exception is the *NPC1* gene, in which we detected pathogenic or probably pathogenic variants in two individuals with low HDL-C (3.1% of low HDL-C patients) and two individuals with high HDL-C (2.6% of high HDL-C patients), all in the heterozygous state, suggesting that these variants may not contribute to the differences in HDL-C in these patients.

We performed Sanger sequencing of PCR amplicons to confirm the 35 prioritized variants, present as 50 genotypes. All 50 of these genotypes were bi-directionally confirmed by Sanger sequencing, indicating that the NGS assay had 100% concordance with the gold standard (95% confidence interval 92.9–100.0%). We also assessed the reproducibility of the NGS assay by performing technical replication of one sample in two independent NGS runs. After quality filtering was performed, we observed 100% concordance in quality filtered variant calls between runs.

Diagnostic yield for extreme HDL-C

We considered a molecular diagnosis to have been made if a patient had a pathogenic or probably pathogenic variant detected in a gene known to cause the phenotype present in that patient (i.e., in a low HDL-C gene in a patient

with low HDL-C and vice versa). We excluded variants in *NPC1* from this analysis because of the observation that a similar proportion of patients with high versus low HDL-C had variants in this gene. Overall, a molecular diagnosis was established in 23 out of 64 patients with low HDL-C (35.9%) and 4 out of 77 patients with high HDL-C (5.2%) (Table 4). The percentage of patients in whom a molecular diagnosis was made did not appreciably change when we excluded patients of non-European ancestry [patients with a molecular diagnosis 15/46 (32.6%) with low HDL-C and 3/63 (4.8%) with high HDL-C], suggesting that these results were not substantially affected by population stratification.

Within the low HDL-C group of patients, HDL-C levels were significantly lower among those in whom a molecular diagnosis was made compared with those in whom no molecular diagnosis was made (0.51 ± 0.2 mmol/l vs. 0.74 ± 0.1 mmol/l, $P < 0.0001$) (Fig. 1). Similarly, within the low HDL-C group, patients in whom a molecular diagnosis was made had lower TG levels (2.44 ± 1.6 mmol/l vs. 4.52 ± 3.9 mmol/l, $P = 0.02$) and a trend toward a lower prevalence of type 2 diabetes (13.0% vs. 36.6%, $P = 0.08$), compared with those in whom no molecular diagnosis was made. In contrast, among patients with high HDL-C, the HDL-C level

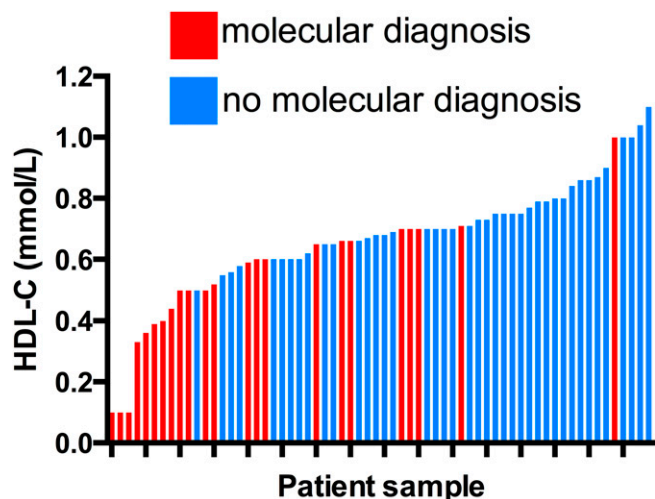


Fig. 1. HDL-C levels in patients with or without a molecular diagnosis of low HDL. Each patient is represented by one column in order of increasing HDL-C level from left to right. Red columns indicate patients in whom a molecular diagnosis of low HDL-C was made by NGS, and blue columns indicate those in whom no molecular diagnosis of low HDL-C was made.

did not differ between those with or without a molecular diagnosis (2.42 ± 0.5 mmol/l vs. 2.47 ± 0.4 mmol/l, $P = 0.8$).

Identification of known mutations

To assess the sensitivity of our assay to detect previously identified mutations, we sequenced six patients with

known disease-causing rare mutations in the *ABCA1* gene previously identified by Sanger sequencing (15). This included five individuals heterozygous for missense mutations in *ABCA1* and one individual compound heterozygous for mutations in *ABCA1*. All seven genotypes were correctly identified by NGS. However, it is noteworthy that one of these mutations, c.4176-11T>G, previously shown to be pathogenic (15), would not have passed conservative prioritization criteria based on its distance from the intron/exon junction (11 nucleotides upstream of the exon start site).

Functional significance of pathogenic variants

To investigate the functional significance of variants identified by our prioritization approach, we performed functional validation of a subset of these variants. We generated the *ABCA1* p.Trp590Leu and p.Arg2200* variants, identified in patient ID 18301, and transiently expressed them in HEK293 cells. Both variants displayed similar levels of protein expression by Western blot (Fig. 2A) and similar patterns of subcellular localization by immunofluorescence (Fig. 2B), as compared with wild-type *ABCA1*. We then tested the ability of cells transfected with these variants to efflux radiolabeled cholesterol in response to ApoA-I. The p.Arg2200* variant displayed complete abrogation of ApoA-I-dependent cholesterol efflux activity, whereas the p.Trp590Leu variant displayed an $\sim 50\%$ reduction in efflux compared with wild-type *ABCA1* (Fig. 2C), indicating that these two variants represent complete and

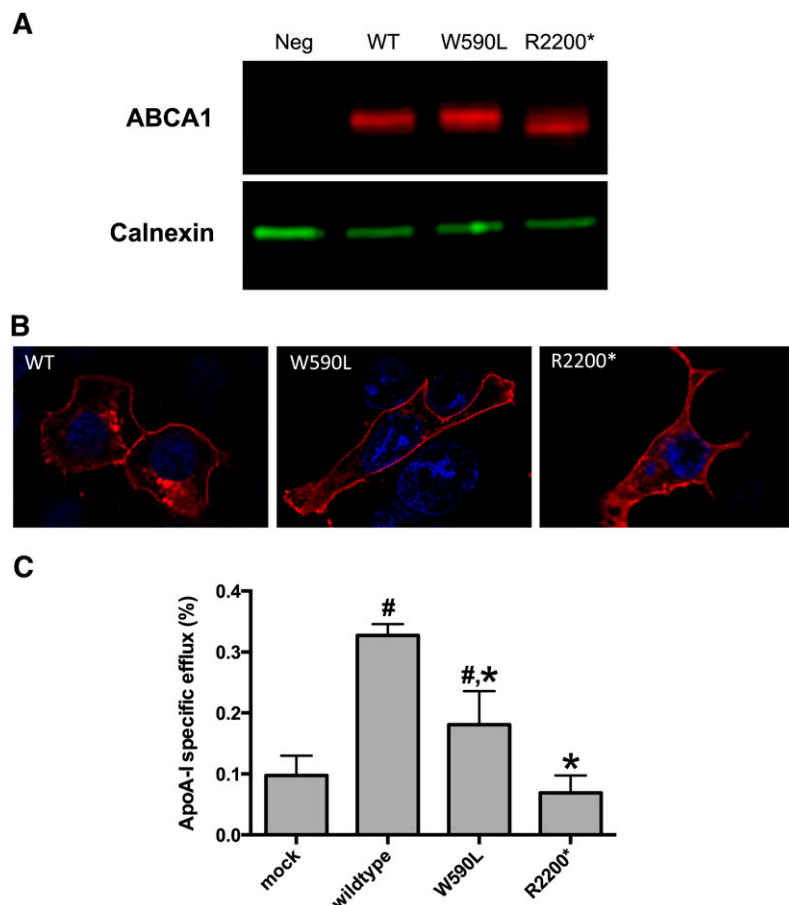


Fig. 2. Functional characterization of variants in *ABCA1*. A: Western blot analysis showing normal protein expression of wild-type and mutant *ABCA1*. The two *ABCA1* variants, as well as wild-type *ABCA1*, were generated in vitro and expressed in HEK293 cells. Cell lysates were probed with antibodies to *ABCA1* and calnexin (as loading control). Neg, negative control. B: Immunofluorescence results showing normal subcellular localization of wild-type, W590L, and R2200* *ABCA1* proteins. C: HEK293 cells were transiently transfected with wild-type, W590L, and R2200* *ABCA1*, and cholesterol efflux measured in the presence or absence of ApoA-I. # Indicates a statistically significant difference compared with mock transfected and * indicates a statistically significant difference compared with wild-type *ABCA1* at $P < 0.05$ using a one-way ANOVA.

partial loss-of-function alleles, respectively. Similar results have been reported previously for another mutation in *ABCA1* (p.Trp590Ser) that affects the same amino acid position (22).

We measured *LCAT* activity in plasma from six individuals with variants in the *LCAT* gene compared with four patients with wild-type *LCAT* and similar HDL-C levels. This included the novel variant p.Thr348Ile, observed in one patient in the homozygous state and in one patient in the heterozygous state. We also included the p.Ser232Thr and p.Gly368Val variants, both of which have been previously reported to cause low HDL-C (5, 23), but have not been functionally characterized. We observed p.Ser232Thr in three patients and p.Gly368Val in one patient, all in the heterozygous state. Relative to patients with wild-type *LCAT* and similar HDL-C levels, heterozygotes for either p.Ser232Thr or p.Thr348Ile variants had significant reductions in plasma *LCAT* activity, with some residual activity (Fig. 3). The patient homozygous for p.Thr348Ile, as well as the patient heterozygous for p.Gly368Val, had complete abrogation of *LCAT* activity with residual activity that was not significantly different than the negative control sample. These data indicate that patients with these variants in *LCAT* have impaired plasma *LCAT* activity, consistent with the concept that these variants represent loss-of-function alleles.

DISCUSSION

We have described a targeted NGS panel for molecular diagnosis of disorders of lipid metabolism. We assessed the performance of this panel in a population of patients with extreme HDL-C levels without previously identified mutations, as well as a collection of patients with known rare mutations that impact HDL-C. The major findings of this study are: 1) that this panel is highly accurate and has high analytic sensitivity to identify pathogenic and probably

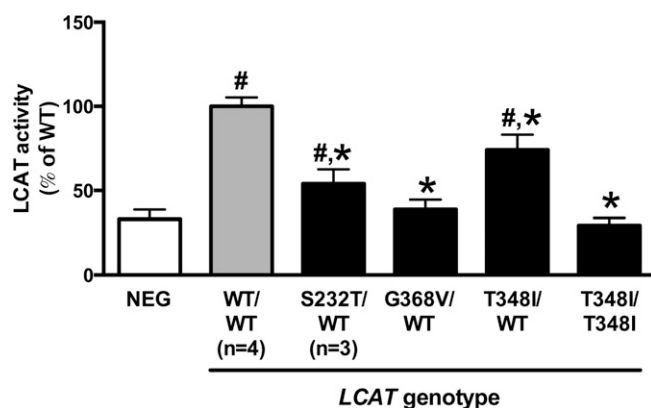


Fig. 3. *LCAT* activity in plasma from patients with wild-type or variant *LCAT* genes. *LCAT* activity was determined in plasma using a fluorescent assay. Values represent the mean \pm SD of triplicate samples from individual patients unless otherwise indicated. # Indicates a statistically significant difference compared with negative control and * indicates a statistically significant difference compared with wild-type, both at $P < 0.05$ using a one-way ANOVA.

pathogenic variants that impact HDL-C; 2) the report of several novel variants in HDL-related genes with associated clinical phenotypes and functional validation of a subset of these; and 3) that a molecular diagnosis can be established in a substantial percentage of patients with extremely low HDL-C visiting a specialty lipid clinic.

Our results showed high analytic accuracy of this assay to detect pathogenic variants. It is noteworthy that while our assay accurately detected a known pathogenic mutation in the *ABCA1* gene (c.4176-11T>G) (15) with high levels of confidence ($Q > 30$, 3,188 depth of coverage), the location of this mutation, 11 nucleotides 5' of the start of an exon, means that it would have been excluded by most conservative prioritization approaches that generally identify splice site variants two nucleotides up- or downstream of the intron/exon junction. This example highlights the ongoing challenge of determining pathogenicity by computational approaches.

We showed that the NGS assay could successfully identify the molecular causes of rare Mendelian disorders of HDL-C, namely, ApoA-I deficiency, Tangier disease, and familial *LCAT* deficiency. Notably, none of these three patients displayed the classical physical examination findings associated with these conditions (i.e., enlarged yellow tonsils in Tangier disease or corneal opacities in familial *LCAT* deficiency). This suggests that the sequencing-based approach described here may be more sensitive to identify patients with rare Mendelian disorders of HDL-C compared with an approach based on classical stigmata of these conditions. Previously, we have employed Sanger sequencing to identify pathogenic mutations in patients with Mendelian disorders of HDL-C (15). For large genes such as *ABCA1* (50 exons), this approach is costly, labor-intensive, time-consuming, and generally requires sequential testing of candidate genes if no pathogenic mutation is identified in the initial screening. In contrast, the targeted resequencing strategy we describe here allows processing of multiple samples and multiple genes in parallel, thus substantially reducing both the cost and time on a per-patient and per-gene basis.

While our study was designed primarily to detect known pathogenic variants, it is noteworthy that 21 of the prioritized variants we detected were novel. Importantly, we confirmed functionality of some of these variants by demonstrating either reduced activity in vitro or reduced enzyme activity ex vivo. These results highlight the research and discovery potential of targeted gene panels for both known disease genes and important candidate genes. In this regard, we included *NPC1* as a candidate gene based on the observation that patients with the autosomal recessive disorder, Niemann-Pick disease, have reduced levels of plasma HDL-C (24–26). Our results showing a similar proportion of patients with low versus high HDL-C carrying predicted damaging variants in *NPC1* (3.1% vs. 2.6%) do not support a role for heterozygous variants in this gene causing low HDL-C in the general population. For this reason, we did not consider the finding of a pathogenic or probably pathogenic variant in *NPC1* to constitute a molecular diagnosis of low HDL-C. Larger sample sizes and

functional testing may help to clarify the impact of rare heterozygous variants in *NPC1* on HDL-C levels.

One previous study evaluated the use of a targeted NGS panel for lipid disorders in patients with abnormal TG or LDL-C levels and in patients with lipodystrophy and maturity-onset diabetes of the young (27). That study also reported a high level of analytic accuracy in detecting known mutations (95.2%). The mean depth of coverage achieved by that assay (345.1×) was substantially less than was achieved by our assay (1,559×), which relates to the smaller genomic capture region of our assay. Together with these previous results on disorders of TG and LDL-C, the current study, demonstrating high accuracy to identify pathogenic variants causing abnormalities of HDL-C, suggest that targeted NGS panels can accurately identify pathogenic variants across the entire range of clinical dyslipidemias.

We identified a pathogenic or probably pathogenic variant in 35.9% of patients with low HDL-C, suggesting a high diagnostic yield in this patient population. Notably, among patients with low HDL-C, the HDL-C level was significantly lower among those in whom a pathogenic variant was identified compared with those in whom no such variant was identified (Fig. 1). This suggests that identification of a molecular defect in patients with low HDL-C may identify a more extreme subpopulation of patients, and likewise, that the diagnostic yield will be highest among patients with the lowest levels of HDL-C. Indeed, had we included only patients with an HDL-C of less than or equal to 0.7, 0.6, or 0.5 mmol/l, the molecular diagnosis rate would have increased to 51.2, 65.2, or 91.7%, respectively. This further indicates that these established HDL genes account for the vast majority of cases of extremely low HDL-C, and that there are likely to be few additional major genes causing this phenotype that remain to be discovered. The observation that patients with low HDL-C in whom no pathogenic variant was identified had higher TG levels and a trend toward a higher prevalence of diabetes mellitus is consistent with the concept that the low HDL-C phenotype in these patients may be secondary to other metabolic factors, rather than being primarily due to a major genetic cause.

In contrast to the low HDL-C group, the diagnostic yield in patients with extremely high HDL-C was lower in our cohort (5.2%), suggesting that there may be additional unknown genetic or nongenetic factors that impact this phenotype, or alternatively, that a greater proportion of such patients have multiple variants in genes with more modest effect sizes that may not have been detected by our approach (12).

There are several limitations of our study. Our assay was primarily designed to detect single nucleotide variants and small indels. As such, larger indels or complex rearrangements may not have been detected. As bioinformatics approaches to identifying structural and copy number variants improve, it is expected that detection of this category of variant will increase. Importantly, out of the 200 curated mutations in the *ABCA1* gene, only 6 (3%) are large indels or complex rearrangements, suggesting that the majority of pathogenic variants will be detected by our assay. Second,

despite applying stringent prioritization criteria, it is not possible to conclusively determine the pathogenicity of novel variants by computational methods alone. This is particularly the case for missense variants, and it is likely that some of the novel missense variants detected in our study that were predicted to be damaging are in fact benign. We used a relatively stringent bioinformatics approach, by requiring that five different computational algorithms all agreed in their predictions that a novel missense variant was deleterious, for us to classify it as probably pathogenic. Notwithstanding the limitations of bioinformatics approaches to predict functionality, our functional validation of a subset of novel variants provides confidence that many of the variants detected in this study are functional. In addition, the prioritized variants showed strong enrichment with the associated phenotype: 88% of prioritized alleles in low HDL genes were in patients with low HDL-C. In contrast, analysis of all rare coding variants in low HDL-C genes without prioritization displayed a more equal distribution: 60% of rare nonprioritized coding alleles in low HDL genes were in patients with low HDL-C. This indicates that our prioritization approach selected variants with penetrant effects on these lipid phenotypes. Lastly, we biochemically characterized only a subset of the variants detected. While our functional data is consistent with the concept that many of the variants we detected do impair the function of the encoded proteins, we did not perform exhaustive functional characterization of all novel variants detected. This also reflects the reality of clinical practice, in which novel variants are frequently discovered but functional characterization may not be possible.

Identifying the molecular cause of HDL-C or other lipid or lipoprotein parameters provides diagnostic closure and allows rapid cascade screening in the family members of affected individuals. In patients with mutations causing elevated LDL-C, introduction of a molecular diagnostic program has been shown to significantly increase the appropriate use of lipid-lowering therapy (28, 29). Similarly, identification of molecular abnormalities in patients with low HDL-C could guide personalized cardiovascular prevention and risk-reduction strategies, and may improve patient compliance with therapeutic lifestyle modification or pharmacological interventions. Moreover, targeted therapies for specific disorders of HDL metabolism are in development (30, 31), and the identification of specific molecular defects in patients with abnormal HDL-C levels may identify candidates for these future therapies.

In summary, we have described a novel targeted NGS panel for disorders of lipid metabolism. Our validation study in patients with extreme levels of HDL-C indicates that this assay is highly accurate for the identification of pathogenic variants and that the diagnostic yield of such an approach is high, particularly among patients with very low HDL-C. Targeted sequencing approaches hold the promise to refine diagnostic categories and may lead to new opportunities for personalized treatment and prevention strategies in patients with abnormalities of plasma lipid levels. **FF**

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