In search of a genetic explanation for LDLc variability in an FH family: common SNPs and a rare mutation in *MTTP* explain only part of LDL variability in an FH family

Michael Winther,* Shoshi Shpitzen,† Or Yaacov,†,§ Jakob Landau,§ Limor Oren,† Linda Foroozan-Rosenberg,† Naama Lev Cohain, Daniel Schurr,† Vardiela Meiner,†† Auryan Szalat,†,§§ Shai Carmi,§ Michael R. Hayden,* Eran Leitersdorf,† and Ronen Durst[1],†,*****

Centre for Molecular Medicine and Therapeutics,* University of British Columbia, Vancouver, Canada; Center for Research, Prevention, and Treatment of Atherosclerosis,† Radiology Department,** Department of Genetics and Metabolic Diseases,†† Internal Medicine Ward,§§ and Cardiology Division,*** Hadassah-Hebrew University Medical Center, Jerusalem, Israel; and Hebrew University-Hadassah Braun School of Public Health and Community Medicine,§ Jerusalem, Israel

ORCID ID: 0000-0001-5159-1419 (M.R.H.)

Abstract **We previously identified a highly consanguineous familial hypercholesterolemia (FH) family demonstrating segregation of the JD Bari mutation in the LDL receptor as well as a putative cholesterol-lowering trait. We aimed to identify genes related to the latter effect. LDL cholesterol (LDLc) values were normalized for FH affectation status, age, and gender. Using genome-wide SNP data, we examined whether known SNPs gleaned from a genome-wide association study could explain the variation observed in LDLc. Four individuals with markedly reduced LDL levels underwent whole exome sequencing. After prioritizing all potential mutations, we identified the most promising candidate genes and tested them for segregation with the lowering trait. We transfected a plasmid carrying the top candidate mutation, microsomal triglyceride transfer protein (*MTTP*) R634C, into COS-7 cells to test enzymatic activity. The SNP score explained 3% of the observed variability. *MTTP* R634C showed reduced activity (49.1 nmol/ml) compared with the WT allele (185.8 nmol/ml) ($P = 0.0012$) and was marginally associated with reduced LDLc in FH patients ($P = 0.05$). Phenotypic variability in a FH pedigree can only partially be explained by a combination of common SNPs and a rare mutation and a rare variant in the *MTTP* gene. LDLc variability in FH patients may have nongenetic causes.**—Winther, M., S. Shpitzen, O. Yaacov, J. Landau, L. Oren, L. Foroozan-Rosenberg, N. Lev Cohain, D. Schurr, V. Meiner, A. Szalat, S. Carmi, M. R. Hayden, E. Leitersdorf, and R. Durst. **In search for a genetic explanation for LDLc variability in an FH family: common SNPs and a rare mutation in *MTTP* explain only part of LDL variability in an FH family.** ***J. Lipid Res.*** **2019.** 60: **1733–1740.**

Supplementary key words low density lipoprotein • genetics • cholesterol metabolism • diseases/dyslipidemia • gene expression/polymorphisms • genes in lipid dysfunction • low density lipoprotein/assembly • low density lipoprotein cholesterol • familial hypercholesterolemia • single nucleotide polymorphism • microsomal triglyceride transfer protein

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by a high LDL cholesterol (LDLc) content in the serum. FH occurs in one in 250−500 in heterozygous (HTZ) form and one in a million in homozygous (HMZ) form (1). In most cases, the disorder results from mutations in the LDL receptor (*LDLR*) gene. Mutations in the *APOB-100* gene and the recently identified proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene result in phenocopies of the disease (2). Over a thousand different mutations in *LDLR* have been recorded to date. Owing to the high content of LDL in the blood, this disorder predisposes patients to early atherosclerosis, premature coronary heart disease, cerebrovascular accidents, calcification of cardiac valves leading to their dysfunction, and even early death. It is thought that at least 5% of all early myocardial infarctions are in FH individuals.

Although FH is caused by mutations in single genes, individuals with FH show striking phenotypic variability.

This work was supported by Israel Science Foundation Grant 1053/12 awarded to R.D. S.C. was supported by the Abisch-Frenkel Foundation for the Promotion of Life Sciences.

Manuscript received 28 December 2018 and in revised form 21 June 2019.

Published, JLR Papers in Press, August 6, 2019
DOI https://doi.org/10.1194/jlr.M092049

Abbreviations: FH, familial hypercholesterolemia; GCTA, Genome-wide Complex Trait Analysis; GWAS, genome-wide association study; HMZ, homozygous; HTZ, heterozygous; LDLc, LDL cholesterol; LDLR, LDL receptor; MTTP, microsomal triglyceride transfer protein.

[1] To whom correspondence should be addressed.
e-mail: durst@hadassah.org.il

The online version of this article (available at http://www.jlr.org) contains a supplement.

LDLc levels and age of onset of cardiovascular disease are highly variable (3). The mutational heterogeneity of FH explains some of the phenotypic variation found among FH homozygotes in whom a strong correlation is found between residual receptor activity and severity of the disease (4, 5). However, the correlation between mutation type and LDL levels is complex. Significant variations in LDLc levels that cannot be explained by gender and age differences are seen even in individuals carrying exactly the same mutation. Studies have shown that while the mutation type and residual activity of the mutated *LDLR* can explain some of the variability in cholesterol and LDL levels, factors such as age, gender, and apoE genotype also have significant effects (6, 7). In a study of 177 untreated FH patients in the United Kingdom, Humphries et al. (8) demonstrated a large range of total cholesterol levels ranging from just above 5 mmol/l to over 15 mmol/l. Mutations in genes that are known to reduce LDLc, particularly *APOB* mutations known to cause hypobetalipoproteinemia but also mutations in *PCSK9*, *APOE*, and *ANGPTL3* were found to reduce LDLc in some FH patients (9–12) These and other studies (13) demonstrate that the large variability in phenotypic FH is influenced by both genetic and environmental modifiers.

Several large pedigrees segregating *LDLR* mutations have been described in which some carriers of an *LDLR* mutation have untreated normal cholesterol levels, suggesting the existence of a gene with a cholesterol-lowering effect. Hobbs et al. (14), the first to suggest the existence of such a gene, described a 9-year-old male who was HMZ for a point mutation changing Ser156 to Leu, and whose plasma cholesterol level was higher than 500 mg/dl. The proband's mother, although HTZ for this mutation, had an LDLc level in the 28th percentile for the population. Further genotyping of the family identified the mutant gene in HTZ form in 17 of the mother's relatives, five of whom had normal LDLc values. The inheritance pattern of the cholesterol-suppressing trait was consistent with single gene-dominant transmission (14). Through linkage analysis, the *LDLR* locus, *apoB-100*, and *apoE* were excluded as potential cholesterol-lowering genes (14). In a subsequent study, the same group tested the kinetics of *apoB*-containing particles. They demonstrated higher than normal catabolism of LDL particles, suggesting that the cholesterol-lowering effect could be due to either decreased secretion of *apoB*-containing lipoproteins or enhanced clearance of LDL precursor lipoproteins (15). Sass et al. (16) described a French Canadian FH family with a 5 kb deletion in the *LDLR*, resulting in complete loss of function of the allele (17). Part of the cholesterol-lowering effect could be attributed to an *apoE2* allele, but could not explain it in full, leading the authors to conclude that the existence of other cholesterol-lowering genes is highly likely. The above two examples indicate that in some FH pedigrees, a strong effect of cholesterol lowering can segregate within the family.

In a previous report, we described a large and highly inbred FH family (labeled family 408) with segregation of the JD Bari mutation c.2483A>G; p. p.Tyr828Cys (herein termed Y828C) (supplemental Fig. S2) (18). The *LDLR* mutation in this family resides in the receptor's cytoplasmic tail and causes defective receptor internalization with virtual absence of *LDLR* activity (19, 20). Because several FH mutation carriers in family 408 had normal or even low LDL concentrations, it was assumed that this particular family segregates for one or more genes that lower LDLc, thus compensating for the high cholesterol expected in carriers of the FH mutation (18). The mutation causing the cholesterol-lowering effect in this family has not yet been discovered but was putatively linked, using tandem repeat analysis, to the long arm of chromosome 13. Subsequently we failed, however, to find a cholesterol-lowering gene within this locus.

The aim of the present study was to identify the putative LDLc-reducing mutation in family 408. To exclude common variants causing cholesterol reduction, we used SNP arrays to genotype the entire pedigree and then calculated a score for each individual based on prior genome-wide association studies. After this analysis failed to explain most of the observed variability in LDLc, we carried out exome sequencing to search for rare variants that might explain the observed cholesterol-lowering effect.

MATERIALS AND METHODS

Participants

All participants were members of family 408, of whom 143 individuals were identified through the MedPed project, and for 119 of whom high-quality DNA was available (19). The study was approved by the institutional review board of the Hadassah-Hebrew University Medical Center in accord with the Helsinki Declaration. All participants signed their informed consent to take part in the study. Patients who underwent MRI signed an additional consent form for the procedure.

SNP array analysis

All members of the pedigree were genotyped using the PsychArray-24 kit (v1.1; Illumina iScan). Quality control was performed using PLINK (21, 22) to assure a genotyping rate greater than 95% per person, a missingness rate per variant lower than 2%, and minor allele frequency above 1%. We also removed variants with suspected Mendelian errors and variants deviating from Hardy-Weinberg equilibrium ($P < 10^{-5}$). LDLc values were adjusted to account for the known Y828C *LDLR* mutation by dividing the individuals into three subgroups based on the genotype (9.5% HMZ carriers, 46.7% HTZ carriers, and 43.8% WT), and subtracting from each individual the mean LDLc of the respective subgroup.

Association was analyzed by the software, Genome-wide Complex Trait Analysis (GCTA) (23), which employs a linear mixed model that can account for any degree of relatedness between study subjects. We used default parameters, except that to increase power we opted to use the LOCO "leave one chromosome out" method. Association summary figures were prepared in R using the qqman package (https://CRAN.R-project.org/web/packages/qqman/index.html). We also ran an association analysis using PLINK's qfam command, with similar results to those obtained by GCTA.

We calculated a polygenic score using SNPs found to affect LDLc in a large recent genome-wide association study (GWAS)

($P < 10^{-4}$) (24). To select SNPs for the score, we performed linkage disequilibrium clumping in PLINK. The weight of each SNP was its reported effect size, and strand flipping was performed where necessary. The score for each individual was calculated in PLINK as the sum of the SNP effects for all effect alleles carried by that individual. The score was correlated to the adjusted LDLc level using regression analysis in R.

Whole exome sequencing

Using whole exome sequencing, we analyzed DNA samples from four of our FH family members. All four were HTZ for the JD Bari mutation, but three had normal LDLc levels, suggesting that they had a cholesterol-lowering trait (herein: "affecteds"), and one ("control") had a high cholesterol level as anticipated for an FH family member. Genomic DNA was randomly fragmented, and the fragments were amplified, purified, and hybridized to Agilent's SureSelect Biotinylated RNA Library (BAITS) for enrichment. Each captured library was then loaded onto a HiSeq2000 platform (Illumina), and we performed high-throughput sequencing to ensure that each sample met the desired average sequencing depth. SOAPaligner (soap2.21) was used to align the reads to the human reference genome with a maximum of three mismatches. The mean depth of target region, the percent coverage of target region, and the fraction of target covered greater than or equal to four times were on average 79.26, 98.68, and 96.22, respectively. Candidate SNPs were filtered with the following criteria: sequencing depth between 4 and 200, estimated copy number no more than 2, and distance between two SNPs larger than 5 bp. Common SNPs with minor allele frequencies >5% in dbSNP were excluded. Noncoding SNPs were also excluded. LDLc was compared between carriers and noncarriers of candidate SNPs.

Cell culture

Naïve COS-7 cells demonstrate negligible microsomal triglyceride transfer protein (*MTTP*) mRNA levels and were therefore chosen for the transfection assays. Untransfected COS-7 cells served as a reference. HepG2 cells (which do possess endogenous *MTTP* activity) and COS-7 cells were maintained in DMEM medium containing 50 ml of certified fetal bovine serum, 6 ml of PEN-STREP solution, and 6 ml of 200 mM L-glutamine solution at 37°C and 5% CO_2. To test for the dominant negative effect of the R634C mutation, we transfected HepG2 cells with WT and mutated plasmid and measured *MTTP* activity.

RNA extraction and RT-qPCR analysis

RNA was extracted using TRI-Reagent (Sigma-Aldrich) according to the manufacturer's instructions and resuspended in 100 μl of diethylpyrocarbonate water. From each sample we extracted 1 μg of RNA for reverse transcription with the qScript cDNA kit (Quanta), as described by the manufacturer. RT-quantitative (q) PCR reactions using software 2.3 of the StepOnePlus™ real-time PCR (Applied Biosystems) were run in triplicate using PerfeCTa primers and PerfeCTa SYBR Green FastMix (Quanta). Relative quantification of the large *MTTP* subunit transcript was performed in relation to a standard curve. Quantified values for *MTTP* were normalized against the input determined by a housekeeping gene (*HPRT1*) (Quanta).

Plasmid transfection

COS-7 and HepG2 cells were transfected using the jetPRIME® kit (Polyplus-transfection®) as described by the manufacturer. Three types of constructs were used for the transfection: *i*) pcDNA3.1 (+) plasmid containing WT (N) *MTTP*; *ii*) pcDNA3.1 (+) plasmid containing mutated *MTTP* (Keyclone Technologies, San Diego, CA); and *iii*) GFP plasmid to evaluate transfection success. The coding sequence of *MTTP* cDNA constructs was verified by Sanger sequencing.

Aliquots of transfected COS-7 and HepG2 cells were taken for RNA extraction, protein extraction, and *MTTP* activity assay. Activity assays were replicated six times. Transfected cells were maintained for 48 h after transfection to allow *MTTP* and GFP expression. GFP expression was analyzed on a NIKON-Ti inverted fluorescence microscope.

MTTP activity assay

MTTP activity was evaluated using an *MTP* activity assay kit (Sigma-Aldrich) according to the manufacturer's protocol. This kit provides a quantitative homogeneous fluorometric method for assessing transfer activity by measuring the increase in fluorescence that occurs as a labeled neutral lipid substrate is transferred from donor to acceptor. Student's *t*-test was used to compare *MTTP* activity between preparations.

MRI analysis

All patients underwent MRI of the liver, scanned on a 1.5T (Avanto, Siemens Healthcare, Gemany) fitted with an eight-channel body array surface coil. Chemical shift-encoded spoiled gradient-echo acquisitions provided the T1-weighted in-phase and out-of-phase sequences. Acquisition parameters were 400 cm FOV, 6 mm slice thickness, 25 slices, 70 flip angle (to minimize T1-related bias), 390 kHz receiver bandwidth; TR = 113 ms, TE/ΔTE = 2.38/1.98 ms (total of four echos). Separate in-phase and out-of-phase images were used for the evaluation. A region of interest on the liver was marked on each image for signal intensity evaluation. The results were then inserted into the formula to calculate the fat-signal fraction (FF = liver SIIP − liver SIOP)/2 × liver SIIP (SI, signal intensity; IP, in-phase; OP, out-of-phase).

RESULTS

SNP array analysis

After adjusting the LDLc values according to age and gender, we performed a genome-wide association analysis using the linear mixed model of GCTA (23). The results showed many associations with LDLc at genome-wide significant *P* values (supplemental Fig. S1A). On further inspection, the associated loci were found to be concentrated on chromosome 19 linked to the known *LDLR* mutation. When we adjusted the LDLc phenotype to the known *LDLR* genotype, no genome-wide associated SNPs could be detected (qq-plot in supplemental Fig. S1B).

To determine whether the variance in LDLc values could be explained, at least in part, by the combined effect of multiple genes (a polygenic model), we computed the polygenic score for each individual in the pedigree according to the effect sizes reported in a recent large-scale GWAS (24). Regression of the *LDLR* mutation-corrected LDLc values on the polygenic score revealed minimal correlation between SNP scores and corrected LDLc values (adjusted $r^2 = 0.002$, $P = 0.26$). However, when we repeated the analysis after omitting two outlier individuals with an apparently strong cholesterol-lowering effect, the model explained approximately 3% of the variance (**Fig. 1**) (adjusted $r^2 = 0.028$, $P = 0.045$). This may suggest that whereas the model

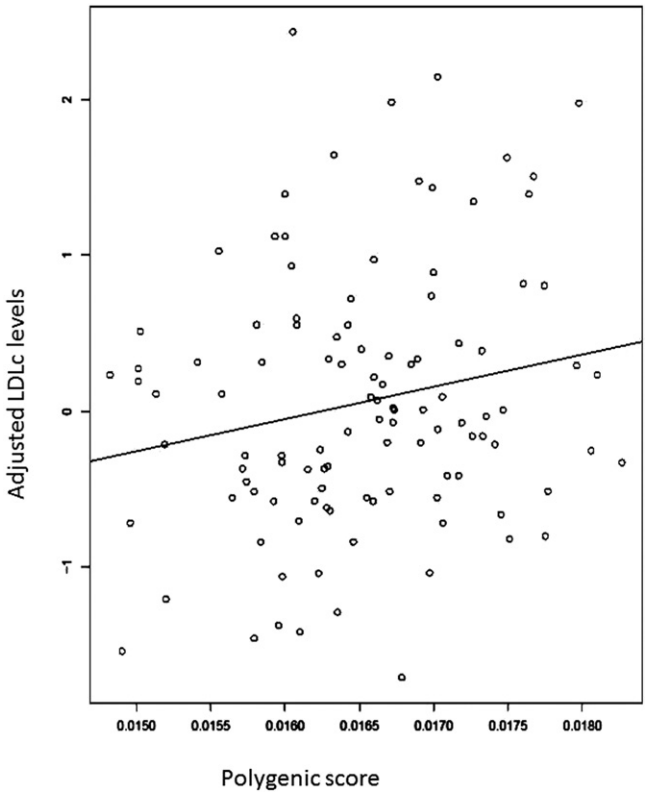

Fig. 1. Correlation between LDLc values and polygenic score. Polygenic scores for LDLc levels were generated using SNPs found to affect LDLc in a large recent GWAS ($P < 10^{-4}$) (24). The score of each individual in the pedigree was calculated in PLINK. LDLc levels were adjusted as follows: First, the expected LDLc based on age and sex was subtracted. Then, a Z-score was calculated separately for each genotype group of the Y828C mutation. The figure shows the adjusted LDLc versus the polygenic score, demonstrating that ~3% of the variability in LDLc can be explained by the polygenic score (adjusted $r^2 = 0.028$, $P = 0.045$).

can explain part of the variability in LDLc, those results are offset by the strong LDLc-lowering effects of some individuals within the pedigree. This effect may be coded by a rare variant in a gene causing a robust cholesterol-lowering effect. We therefore performed whole exome sequencing of those individuals with extreme phenotypes in an attempt to find a rare variant that could explain the cholesterol-lowering effect.

Whole exome sequencing analysis

We performed whole exome analysis on four FH family members. SNPs passing filtering criteria as described in the Materials and Methods (Whole exome sequencing section) were further filtered based on the following assumptions to account for both Mendelian and non-Mendelian inheritance patterns: *i*) Homozygote assumption: The three affected individuals carry HMZ variation at the same position, whereas the control does not. Under this assumption, six tolerated SNPs [based on SIFT and PolyPhen scores (25, 26)] and no damaging SNPs were obtained. *ii*) Heterozygote assumption: The three affected individuals carry HTZ variation at the same position, whereas the control carries no variation at that position. Under this assumption, we obtained a total of 168 SNPS of which 78 were predicted to be damaging. *iii*) Additive model assumption: The three affected individuals have either type of variation (HTZ or HMZ) at the same position, while the control has no variation. Under this assumption, we obtained 186 SNPS of which 84 were predicted to be damaging. *iv*) Polygenic model assumption: Two of the three affected individuals have any type of variation at the same position, while neither the third affected individual nor the control carries the variation. A total of 951 SNPS were obtained. Of these, it was predicted by SIFT or PolyPhen software that 480 would be damaging.

The next step required manual curation of each result to obtain candidate genes that would fit the predetermined model of inheritance and would likely play a role in cholesterol homeostasis. A total of 271 LDLc homeostasis-related genes were listed from PubMed and OMIM and were cross-referenced with the list of genes from the sequencing analysis. Of the 180 variations of interest that appeared in both lists, 86 were predicted to be damaging. Among these variants, 16 were shared between at least two affected individuals but not with the control. All variations were nonsynonymous. Finally, five variants met the criteria of manual Integrative Genomics Viewer quality control (27) (**Table 1**). Among these variants, a variant in the *MTTP* was the likeliest to be cholesterol lowering. *MTTP* codes for a key protein in the assembly and secretion of apoB-containing lipoproteins in the liver and intestine. Mutations in *MTTP* are the molecular basis of abetalipoproteinemia, a rare autosomal recessive disorder characterized by the absence of circulating apoB-containing lipoproteins (such as VLDL) of both intestinal and hepatic origin (28). A recent reanalysis of a LDLc GWAS with >100,000 individuals also demonstrated strong association with *MTTP* (29). It should be noted that this signal was not seen in smaller GWAS studies, suggesting that the SNP effect is modest.

The p.Arg634Cys mutation (Table 1) (herein termed R634C) was reported only once in gnomAD with an allele frequency of 0.000008955 and 0.000004062 and the entire gnomAD population respectively. Multiple lines of computational evidence support a deleterious effect on the gene or gene product (https://varsome.com/variant/hg19/MTTP%20R634C). We further tested 127 FH individuals from other pedigrees sharing similar ancestry, representing 254 alleles. None carried R634C.

Linkage analysis of the *MTTP* mutation

R634C in *MTTP* was genotyped in 119 family members. LDLc concentrations for all family members were normalized relative to the 50th percentile in the same FH affectation status, age, and gender. To account for FH affectation status, the family members were divided into two groups: HTZ for the p.Tyr828Cys (Y828C) *LDLR* mutation and WT for the *LDLR*. Each of these groups was further divided into two subgroups (**Fig. 2**): R634C-mutated *MTTP* (both HTZ and HMZ) and nonmutated (N) *MTTP*. Statistical analysis for the effect of *MTTP* mutation on LDLc levels in the various groups was carried out twice: once for all family

TABLE 1. A list of the candidate mutations that met the criteria of manual Integrative Genomics Viewer quality control

Coordinates	12	35	60	30	Substitution	dbSNP Identification	Gene Name
4,100532349,1,C/T	1/1	0/1	0/1	0	R634C	Novel	*MTTP*
4,110638824,1,C/T	0/1	0/1	0	0	D111N	rs114816312:T	*PLA2G12A*
9,4663252,1,A/C	0/1	0	0/1	0	S202R	rs72695803:C	*PPAPDC2*
17,41063162,1,C/A	0	0/1	0/1	0	L265M	Novel	*G6PC*
19,11240282,1,A/G	0/1	0/1	0/1	0/1	Y828C	rs28942085:G	*LDLR*

Individuals with IDs 12, 35, and 60 are affected; 30 is the control. Analysis of candidate mutations suggested five potential LDLc-lowering mutations. Of these, two (in *MTTP* and *G6PC*) are novel. *MTTP* is a likely candidate because it is known to cause marked lowering of LDLc. *LDLR* is the JD Bari mutation known to cause FH. *PLA2G12A*, phospholipase A2 group XIIA; *PPAPDC2*, phospholipid phosphatase 6; *G6PC*, glucose-6-phosphatase catalytic subunit.

members (Fig. 2) and once excluding children under the age of 8 years (for whom the correlation between age and cholesterol is nonlinear). The *P* values for a difference in LDLc levels between carriers and noncarriers of the R634C *MTTP* mutation (for *LDLR* mutation carriers) were $P = 0.05$ and $P = 0.04$ for the entire cohort and when excluding children under 8, respectively. While these *P* values do not survive Bonferroni correction ($0.05/4 = 0.0125$), possibly due to the small sample size, they are suggestive of an association. Thus, an assay to show the pathogenicity of the *MTTP* R634C mutation was carried out.

MTTP activity assay

As shown in **Fig. 3**, *MTTP* activity in transfected COS cell plasmids was significantly reduced by the R634C mutation (49.1 nmol/ml vs. 185.8 nmol/ml for mutated and

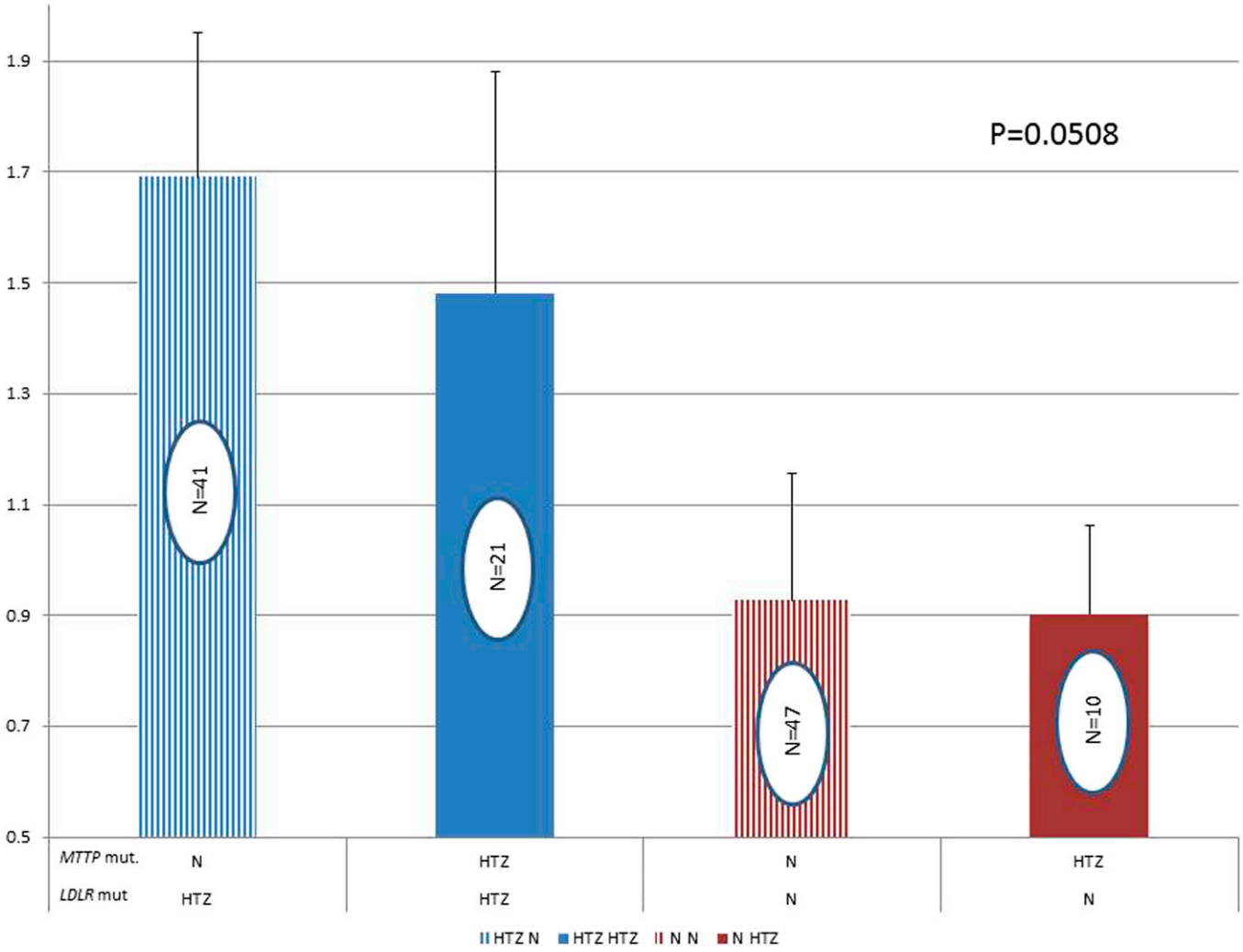

Fig. 2. LCLc levels in carriers and noncarriers of the R634C *MTTP* mutation stratified by *LDLR* mutation status in all 119 family members. The results suggest generally higher levels of LDLc in the HTZ *LDLR* group, but which can be lowered in carriers of the *MTTP* mutation. Similar results were obtained when children under the age of 8 are excluded. N, noncarriers.

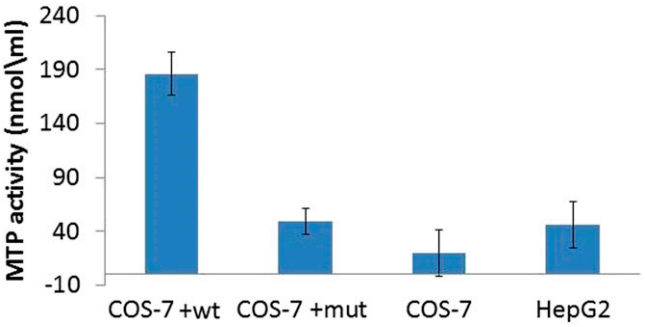

Fig. 3. MTTP protein activity assay in COS and HepG2 cells. *MTTP* activity in COS cells transfected with the mutant (mut) *MTTP* plasmid is significantly lower than in COS cells transfected with wild-type (wt) *MTTP*. *MTTP* activity is similar between COS cells transfected with the mutant MTTP and nontransfected cells. *MTTP* transcripts in COS cells transfected with mutant or wild-type *MTTP* plasmids were similar. HepG2 cells with intrinsic *MTTP* activity were used as a reference for the assay's performance.

nonmutated *MTTP*, respectively; $P = 0.0012$). Mutated *MTTP* activity did not differ significantly from that in nontransfected COS cells. In nontransfected COS cells, the level of activity was lower than in HepG2 cells, which possess endogenous *MTTP* activity (Fig. 3). Transcript mRNA levels were similar between the mutated and nonmutated cells, suggesting that there was no difference in transfection efficacy between the cultures. *MTTP* mutations in humans may cause significant phenotype, only reported in the homozygote state. To test the hypothesis that our mutation might have a dominant negative effect, we transfected COS cells with WT and mutated alleles. The activity of the mutated allele-transfected cells was similar to the nontransfected Hep2G cell line. Thus, we could not demonstrate a dominant negative effect with the mutation (supplementary Fig. S3).

MRI analysis

Drugs that inhibit *MTTP* are known to cause hepatosteatosis. To test for the effect or the R634C *MTTP* mutation on liver fat content, liver fat was analyzed by MRI spectroscopy (**Table 2**) (30) in eight HTZ *LDLR* mutation carriers. Half were HTZ for the R634C *MTTP* mutation and half were WT. ANOVA revealed no significant difference between the two groups, pointing to a lack of a significant difference in hepatosteatosis, and indicating that the R634C *MTTP* mutation is not associated with significant fat infiltration of the liver. Thus, it may be possible to find *MTTP* inhibitors that do not cause hepatic fatty changes.

DISCUSSION

FH is a genetic condition in which the cellular function of *LDLR* is damaged, preventing evacuation of LDLc particles from the blood into the cells. LDLc concentrations in some members of the FH family described in this study were lower than expected. In attempting to explain this observed variability, we first looked at the SNP-based polygenic score for LDLc in the pedigree. After individuals with very strong cholesterol-lowering effects were omitted from the analysis, the score could explain ∼3% of the observed variability in LDLc. To determine whether a rare variant was causing a major change in LDLc, we employed exome sequencing in a search for a rare variant with a strong LDLc-reducing effect. A novel mutation (R634C) in the *MTTP* gene, not previously described in dbSNP, was identified. FH patients carrying this *MTTP* mutation demonstrated lower LDLc values than noncarriers. Together, both SNP score and the rare variant search could explain, at most, only part of the variability in LDLc observed in the pedigree. LDLc variability in our pedigree may only be partly explained by SNP score or rare variants. It is therefore assumed that LDLc variability in FH patients may be more complex and also involve epigenetic and environmental influences.

MTTP is located in the endoplasmic reticulum and plays a central role in lipoprotein assembly, catalyzing the transport of triglyceride, cholesteryl ester, and phospholipid between vesicles (31). *MTTP* is predicted to have three major structural domains: an N-terminal β-barrel (amino acid residues 22−297); a middle α-helical domain (residues 298−603); and a C-terminal β-sheet (residues 604−894) (32, 33). The N-terminal β-barrel domain mediates interaction with the N-terminus of apoB; the middle α-helical domain associates with both protein disulfide isomerase and apoB; and the C-terminal β-sheet domain contains both the lipid-binding and the lipid-transfer activities of *MTTP* (32, 34). The R634C mutation is located in the lipid-binding and lipid-transfer activity domains. Thus, we speculate that the replacement of a positively charged amino acid (R) by a neutral/negatively charged amino

TABLE 2. Liver fat content by MRI and fibro tests in four *MTTP* mutation carriers and four noncarriers

Sample Number	Age at MRI	*LDLR* Mutation	*MTTP* Mutation	BMI	MRI Results (%)[a]
408-15	55	HTZ	N	33.2	40.4
408-23	49	HTZ	N	23.5	24.2
408-50	50	HTZ	N	34.3	24.3
408-37	41	HTZ	N	24.9	0.8
408-14	43	HTZ	HTZ	24.7	12.6
408-74	60	HTZ	HTZ	28.5	18
408-95	50	HTZ	HTZ	28.9	24.4
408-9	47	HMZ	HTZ	19.9	0
P				0.35	0.4

N, noncarrier.
[a]MRI results in percentage of liver fat infiltration.

acid (C), combined with the location of the mutation, damages the protein's activity. An in vitro assay in transfected cells demonstrated a significant reduction in the mutated allele compared with the WT gene. The similar mRNA levels indicate that the difference in activity was not due to lower levels of mRNA in the transfected cells but was rather the result of either lower enzyme activity or reduced protein level. Human heterozygotes for *MTTP* mutation were not reported to have a significant phenotype. We tested the option that the R634C mutation may have a dominant negative effect but could not demonstrate such an effect. Our assumption is that in FH mono- and heterozygotes given the markedly elevated LDLc at baseline, even a small haploinsufficiency effect will have a statistically significant effect on LDL.

Drugs that inhibit *MTTP* activity are used to treat FH. These drugs, however, have problematic side effects, particularly fatty liver changes (35). An analysis of liver fat contents in four FH patients carrying the *MTTP* mutation and four controls revealed no significant differences in either MRI or plasma biomarkers. It is possible that the inhibitory effect of the mutation is not associated with changes in liver fat content, suggesting that such changes do not necessarily result from inhibition of *MTTP* activity.

In conclusion, in this study of a large consanguineous FH family exhibiting a cholesterol-lowering trait, we sought to better understand genetic modifiers of the FH trait. SNP score could explain only 3% of the observed variability in LDLc. Exome sequencing performed in the search for rare LDLc-reducing variants identified an R634C *MTTP* mutation that reduces LDLc when present in HTZ FH-mutation carriers. We showed that this mutation reduces *MTTP* activity in transfected COS-7 cells, and that this reduction is not associated with a tendency toward an increase in liver fat. Our data thus support the concept that an FH phenotype can be modified by *MTTP* mutations as well as by common SNPs. However, these at best explain only part of the observed variability in LDLc among FH patients. Other causes may be relevant for the observed LDLc variability. These may include epigenetic as well as environmental factors.

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