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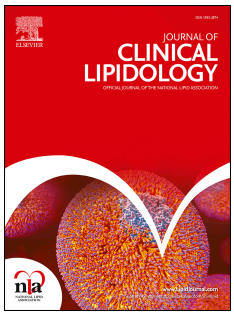

ABCG5/G8 gene is associated with hypercholesterolemias without mutation in candidate genes and non-cholesterol sterols

Itziar Lamiquiz-Moneo, Lucía Baila-Rueda, Ana M. Bea, Rocío Mateo-Gallego, Sofía Pérez-Calahorra, Victoria Marco-Benedí, Antonio Martín-Navarro, Emilio Ros, Montserrat Cofán, José Carlos Rodríguez-Rey, Miguel Pocovi, Ana Cenarro, Fernando Civeira

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Full title

ABCG5/G8 gene is associated with hypercholesterolemias without mutation in candidate genes and non-cholesterol sterols

Short title

ABCG5/G8 mutations in genetic hypercholesterolemia

Authors

Itziar Lamiquiz-Moneo[a], Lucía Baila-Rueda[a] , Ana M. Bea[a], Rocío Mateo-Gallego[a], Sofía Pérez-Calahorra[a], Victoria Marco-Benedí[a], Antonio Martín-Navarro[a], Emilio Ros[b], Montserrat Cofán[b], José Carlos Rodríguez-Rey[c], Miguel Pocovi[d], Ana Cenarro[a], Fernando Civeira[a].

Affiliation

[a] Unidad Clínica y de Investigación en Lípidos y Arteriosclerosis, Hospital Universitario Miguel Servet, Instituto de Investigación Sanitaria Aragón (IIS Aragón), CIBERCV, Universidad de Zaragoza, Zaragoza, Spain.

[b] Unitat de Lipids, Servei d'Endocrinologia i Nutrició, Institut d'Investigacions Biomediques August Pi i Sunyer, Hospital Clinic and CIBER Fisiopatologia de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), Barcelona, Spain

[c] Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria, and Instituto de Investigación Valdecilla (IDIVAL), Santander, Cantabria, Spain.

[d] Universidad de Zaragoza, Departamento de Bioquímica, Biología Molecular y Celular, 50009, Zaragoza, Spain

***Corresponding author**

Name: Itziar Lamiquiz-Moneo

Address: Unidad Clínica y de Investigación en Lípidos y Arteriosclerosis, Hospital Universitario Miguel Servet, Avenida Isabel La Católica 1-3, 50009, Zaragoza, Spain

Telephone number: (34) 976765500 (EXT 2895)

E-mail address: itziarlamiquiz@gmail.com

Keywords

Genetic hypercholesterolemia, *ABCG5/G8*, non-cholesterol sterols, cholesterol absorption

Abstract

Context

Approximately 20-40% of clinically-defined familial hypercholesterolemia cases do not show a causative mutation in candidate genes (mutation-negative FH), and some of them may have a polygenic origin.

Objective

The aim of this work was to study the prevalence of *ABCG5/G8* genetic variants in mutation-negative FH, as defects in these genes relate to intestinal hyperabsorption of cholesterol and thus *ABCG5/G8* variants could explain in part the mechanism of hypercholesterolemia.

Design, Setting and patients:

We sequenced the *ABCG5/G8* genes in 214 mutation-negative FH and 97 controls. Surrogate markers of cholesterol absorption (5α-cholestanol, β-sitosterol, campesterol, stigmasterol and sitostanol) were quantified by high performance liquid chromatography tandem mass spectrometry in both studied groups.

Results

We found 8 mutation-negative FH patients (3.73%) with a pathogenic mutation in *ABCG5/G8* genes. We observed significantly higher concentration of surrogate markers of cholesterol absorption in mutation-negative FH than in controls. In addition, we found significantly higher concentrations of cholesterol absorption markers in mutation-negative FH with *ABCG5/G8* defects than in mutation-negative, *ABCG5/G8* negative FH. A gene score reflecting the number of common single nucleotide variants associated with hypercholesterolemia was significantly higher in cases than in controls ($p=.032$). Subjects with a gene score above the mean had significantly higher 5α-cholestanol and stigmasterol than those with a lower gene score.

Conclusions

Mutation-negative FH subjects accumulate an excess of rare and common gene variations in *ABCG5/G8* genes. This variation is associated with increased intestinal absorption of

cholesterol, as determined by surrogate makers, suggesting that these *loci* contribute to hypercholesterolemia by enhancing intestinal cholesterol absorption.

Abbreviations

GH: genetic hypercholesterolemias

FH: Familial hypercholesterolemia

TC: Total cholesterol

TG: triglyceride

LDLc: Low density lipoprotein cholesterol

SNVs**:** single nucleotide variations

CHD: cardiovascular disease

Mutation-negative FH: Familial hypercholesterolemia without mutation in candidate genes

HDLc: High density lipoprotein cholesterol

HPLC-MS/MS: high performance liquid chromatography tandem mass spectrometry

BMI: Body mass index

Apo: Apolipoprotein

miRNAs: microRNAs

Introduction

Genetic hypercholesterolemias (GH) are a heterogeneous group of lipid disorders caused by monogenic and polygenic defects and characterized by very high plasma concentrations of total cholesterol (TC) due to increased low-density lipoprotein cholesterol (LDL-C) and high risk of premature coronary heart disease (CHD). Familial hypercholesterolemia (FH) is the most common monogenic GH [1], with autosomal codominant transmission and with a current estimated prevalence of about 1:200-250 in the general population [2, 3]. FH is caused by mutations in *LDLR*, the gene coding for the LDL receptor; *APOB*, coding for apolipoprotein (apo) B; *PCSK9* [4, 5], which codes for the enzyme proprotein convertase subtilisin/kexin type 9, or *APOE* genes [6]. In addition, a rare recessive form of FH is also caused by mutations in the *LDLRAP1* gene [7]. However, a causative mutation in candidate genes is not found in approximately 20-40% of clinically defined FH cases [8], suggesting that there are either other as yet unidentified genetic causative *loci* or these cases represent severe polygenic hypercholesterolemia. Actually, affected subjects with clinical FH but without mutations in candidate genes, accumulate some common single nucleotide variations (SNVs) with a small LDL-C raising effect [9] that do not fully explain the high LDL-C phenotype of these subjects.

Different GWAS have shown that at least one hundred *loci* are associated with LDL-C concentration in the population [10, 11]. One of these *loci* is *ABCG5/G8*; this gene complex encodes the proteins ABCG5 and ABCG8, which form a heterodimer conveyor located in the membrane of enterocytes and hepatocytes. It has been shown that ABCG5/G8 limits the intestinal absorption of cholesterol and phytosterols and promotes their hepatobiliary secretion. Severe functional mutations in *ABCG5/G8* cause sitosterolemia, a rare autosomal disorder characterized by an increase of phytosterols in blood, up to 30 times greater than normal [12]. In addition, studies have shown that *ABCG5/G8* variation is associated with cholesterol and non-cholesterol sterol plasma levels [13-17]. However, the contribution of *ABCG5/G8 loci* variation to mutation-negative FH has not been previously examined.

Considering that mutation-negative FH subjects usually have higher non-cholesterol sterol concentrations than other types of GH [18], probably due to intestinal sterol hyperabsorption, we hypothesized that genetic variations in *ABCG5/G8* are involved in some forms of mutation-negative FH. Hence, the aim of this study was to analyze common and rare mutations in *ABCG5/G8* in a large cohort of mutation-negative FH and assess their association with non-cholesterol sterols and LDL-C, to establish the genetic contribution of these *loci* in this type of GH.

Material and Methods

Subjects

Unrelated subjects (n = 214) 18–79 years of age with a clinical diagnosis of non-FH GH: LDL-C above the 95th percentile of the Spanish population [19], triglycerides (TG) below 200 mg/dL, and familial presentation (at least one first-degree relative with the same phenotype) from the Lipid Clinics at Hospital Universitario Miguel Servet, Zaragoza and Hospital Clinic, Barcelona were selected for this study. In all subjects, the absence of a pathogenic mutation in *LDLR, APOB* and *PCSK9* genes was confirmed by the Lipochip ® platform [20]. Exclusion criteria were the presence of an ε2/ε2 genotype or the p.(Leu167del) mutation in the *APOE* gene. Exclusion criteria were secondary causes of hypercholesterolemia including obesity (body mass index >30 kg/m2), poorly controlled type-2 diabetes (HbA1c >8%), renal disease with glomerular filtration rate <30 ml/min and/or macroalbuminuria, liver diseases (ALT> 3 times upper normal limit), hypothyroidism (TSH >6 mIU/L), pregnancy, autoimmune diseases and treatment with protease inhibitors. Assessment of cardiovascular risk factors, personal and family history of cardiovascular disease, intake of drugs affecting intestinal or lipid metabolism, and anthropometric measurements were performed in all participants. The normolipemic group (n= 97) consisted of healthy, unrelated men and women volunteers aged 18–79 years, who underwent a medical examination at the Hospital Miguel Servet of Zaragoza. Exclusion criteria for control subjects were personal or parental history of premature cardiovascular disease or dyslipidemia, current acute illness, or use of drugs that might

influence glucose or lipid metabolism. All subjects signed an informed consent to a protocol previously approved by our local ethics committee (Comité Ético de Investigación Clínica of Aragón, Zaragoza, and of Hospital Clínic, Barcelona, Spain).

Clinical and laboratory determinations

Cases and controls were assessed for personal and familial history of cardiovascular disease, medication use and cardiovascular risk factors. EDTA plasma and serum samples were collected after 10-12 hours of fasting in all participants after at least 6 weeks without lipid-lowering drugs in subjects without prior cardiovascular disease. TG levels were determined by standard enzymatic methods. High-density lipoprotein cholesterol (HDL-C) was measured directly by an enzymatic reaction using cholesterol oxidase (UniCel DxC 800; Beckman Coulter, Inc., Brea, CA, USA). Lipoprotein(a), apo A1, apo B and C-reactive protein were determined by IMMAGE kinetic nephelometry (Beckman-Coulter, Inc.). In patients with prior cardiovascular disease, baseline lipid values were obtained from medical records.

Genetic analysis

Whole blood genomic DNA was isolated by using standard methods. Promoters, coding regions and intron-exon boundaries of *ABCG5* (NM_022436) and *ABCG8* (NM_022437) were amplified by PCR and purified by ExoSap-IT (USB). Amplified fragments were sequenced by the Sanger method using the BigDye 3.1 sequencing kit (Applied Biosystems) in an automated ABI 3500xL sequencer (Applied Biosystems). DNA sequences were analyzed using VariantReporter™ software (Applied Biosystems). *APOE* genotypes were determined by DNA sequencing of exon 4 as previously described [21].

To evaluate the pathogenicity of new identified genetic variants, we used PolyPhen-2 [22] and Mutation Taster [23]. The effect of variants in potential splicing sites was predicted with NetGene2 [24] and NNSplice [25]. We refer to non-synonymous variants as sequence variations causing amino acid substitutions (missense variants) or introducing a premature stop codon (nonsense variants). Intronic variants were considered when they were located in intron-exon boundaries.

In order to compare the frequency of identified variants in the general population we have compiled the allele frequencies of the identified variants from the 1000 Genomes Project [26] and ExAc Browser Data [27].

Mutations were defined as genetic variants with a frequency lower than 1% in the general population. A mutation was defined as pathogenic when it was not present in controls and bioinformatic analysis prediction defined it as "damaging" (Polyphen-2) or "disease causal" (MutationTaster). Single Nucleotide Polymorphism (SNP), was defined as genetic variants with a frequency higher than 1% in the general population.

Gene score

A gene score using the sum of the risk alleles of 27 genetic variants with allelic frequencies statistically different between cases and controls was calculated for each subject.

Determination of serum sterols

Serum concentrations of cholesterol, 5α-cholestanol, β-sitosterol, campesterol, stigmasterol, sitostanol, desmosterol and lanosterol were quantify by high performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in 206 non-FH GH subjects without lipid-lowering drugs and 97 controls according to the method previously described [28]. In 8 subjects non-cholesterol sterols were not quantified because 7 had prior CHD and one had not provided a serum sample. Data were expressed as mg/dL, as well as normalized to total cholesterol. Briefly, 100 µL of serum were transferred to screw-capped vials, and 6 µl of deuterium-labeled internal standard, [2H6] cholesterol-26,26,26,27,27,27 (7.9 mM) for non-cholesterol sterols, and [2H7] cholesterol-25,26,26,26,27,27,27 for cholesterol, were added. Alkaline hydrolysis was performed for 20 min at 60ºC in an ultrasound bath, followed by a double extraction with 3 µl of hexane. The extract was loaded onto the solid-phase extraction cartridge (1 mg, Discovery DSC-18, Supelco, Spain), which was preconditioned with 400 µl of methanol and gravity eluted. The non-cholesterol sterols and cholesterol were desorbed with 1.4 µL

of 2-propanol by gravity, and 40 µL of the final mixture was injected into the HPLC-MS/MS system.

Statistical Analyses

Analyses were performed using SPSS version 20.0 (Chicago, Ilinois, USA). The nominal level for significance was $p< 0.05$. Normal distribution of variables was analyzed by the Kolmogorov–Smirnov test. Quantitative variables with normal distribution were expressed as mean ± standard deviation and were analyzed with Student t-test. Variables with a skewed distribution were expressed as medians and interquartile ranges and were analyzed with the Mann-Whitney U test. Qualitative variables were expressed as a percentage and were analyzed by Chi-square test.

Results

The main clinical and biochemical characteristics of the two groups: 214 mutation-negative FH patients and 97 normolipemic controls are presented in Table 1. By study design, compared to normolipidemic controls, mutation-negative FH participants had significantly higher values of total cholesterol, triglycerides, HDL-C, LDL-C, lipoprotein(a), and apo B, as well as a higher prevalence of hypertension and cardiovascular disease. No differences in age, body mass index (BMI) or *APOE* genotype variability were found between patients and controls.

Non-cholesterol sterol-to-TC ratios are presented in Table 2. 5α-cholestanol, stigmasterol and sitostanol ratios were significantly higher in cases than in controls (*p=.023, p<.001 and p=.003*, respectively). The sum of all cholesterol absorption surrogate markers and phytosterols was also significantly higher in mutation-negative FH than in controls (*p= .028*).

The frequency of SNVs in *ABCG5/G8* genes found in mutation-negative FH was compared to the frequency in controls, in the 1000 Genomes Project and ExAc Browser Data. Appendix table 1 shows the 29 SNVs identified which allelic frequencies were significantly different from those identified in controls or described in the 1000 Genomes Project or in the ExAc Browser Data. They include 27 SNPs and two mutations: p.(Gly269Arg) in *ABCG5* and p.(Gly512Arg) in *ABCG8*.

Table 3 shows the clinical characterization of patients carrying pathogenic mutations identified by sequencing in *ABCG5* and *ABCG8.* A total of eight patients out of 214 (3.73%) were carriers of 6 pathogenic mutations: p.(Asn578Ser), p.(Gly288Arg), p.(Arg198Gln), p.(Gly269Arg), and p.(Asn285Ser) in *ABCG5* and p.(Gly512Arg) in *ABCG8.* One of them is described for the first time in this work: p.(Asn578Ser) in *ABCG5.* None of these pathogenic mutations were present in controls. Appendix table 2 shows bioinformatic analysis of six pathogenic mutations.

Subjects with mutation-negative FH with pathogenic mutations in *ABCG5/G8* genes had significantly higher non-cholesterol sterol-to-TC ratios (5α-cholestanol, β-sitosterol, campesterol, and stigmasterol) and cholesterol absorption than subjects without mutation in *ABCG5/G8* genes (*p=.042, p=.045, p=.034, p=.047* and *p=.030*, respectively). TC

concentrations did not show significant differences between the groups (Table 4). There were no differences in desmosterol and lanosterol, cholesterol synthesis markers, between both groups (Supplemental Table 3). In one out of eight patients with mutation-negative FH with pathogenic mutation in *ABCG5/G8* genes, cholesterol absorption markers were not determined because serum without lipid lowering drugs was not available.

Among the 27 common genetic variants with significantly different frequencies between cases, 1000 Genomes Project and ExAc Browser Data, only c.*380T>G, located in the 3´ UTR of *ABCG5* gene was associated with statistically significant differences in non-cholesterol sterol to TC ratios (5α-cholestanol, β-sitosterol, stigmasterol and campesterol) and cholesterol absorption. T allele carriers had higher intestinal cholesterol absorption than subjects carrying the G allele (*p=.011, p=.00, p=.002, p=.022* and *p=.002*, respectively), but TC concentrations were not significantly different between genotypes (Table 5).

A gene score reflecting the total number of risk alleles at the 27 SNVs associated with hypercholesterolemia was significantly higher in cases than controls (*p=.032*). Subjects with scores above the mean (>33 points) had significantly higher non-cholesterol sterol to TC ratios (5α-cholestanol and stigmasterol) than subjects with the lowest score (*p=.034* and *p=.029*, respectively). Cholesterol absorption was non-significantly higher in subjects with the highest score compared to those with the lowest score (*p=.077*) (Table 6).

Discussion

This study shows that *ABCG5/G8* gene variation would play a role in the pathogenesis of genetic hypercholesterolemia unrelated to *LDLR, APOB, PCSK9* and *APOE* genes. As previously reported [29], subjects with GH unrelated to common genetic defects disclose higher surrogate markers of intestinal cholesterol absorption than controls. There are, however, three novel findings that can be highlighted from this work.

First, *ABCG5/G8* genetic variation contributes to GH through rare pathogenic mutations with large effects and with common variants with small effects. Patients with rare *ABCG5/G8* pathogenic mutations disclose approximately twice the serum concentrations of surrogate markers of intestinal cholesterol absorption than those not carrying mutations. However, the percentage of common mutation-negative FH subjects carrying these rare pathogenic *ABCG5/G8* mutations is low, approximately 4%. We did not find any pathogenic mutation in the control group; hence we could consider that these major defects are involved in the high LDL-C phenotype in these patients. However, we must take into account that there could be other SNVs in other genes, which could explain part of the phenotype of these subjects, and the number of normolipemic controls in not high enough to exclude completely their presence in the normolipemic population. We found 8 patients carrying six pathogenic mutations. Five mutations were in *ABCG5* and one mutation was in *ABCG8.* The mutations p.(Arg198Gln), p.(Gly269Arg), p.(Asn296Ser) and p.(Gly288Arg) in ABCG5 are located in the cytosolic N-terminal region, prior to the first transmembrane domain. In this N-terminal region of ABCG5, one mutation causing sitosterolaemia has been previously described [30]. The mutation p.(Asn578Ser) in ABCG5 is located in the extracellular domain, and this position seems to interact with the amino acid Arg419. The mutations p.(Arg419Pro) and p.(Arg419His) at the same residue have been shown to cause sitosterolaemia. The mutation p.(Gly512Arg) in *ABCG8* is located in the transmembrane α-helix domain; a close mutation in this domain, p.(Leu501Pro), also causes sitosterolaemia [31].

Second, our results show that mutation-negative FH subjects accumulate risk alleles of *ABCG5/G8* with small effects on non-cholesterol sterols, suggesting that, in most of these GH

cases, the contribution of *ABCG5/G8* genetic variation has a limited effect within a polygenic background, as occurs in other genetic dyslipidemias [32]. Among common SNVs frequently found in mutation-negative FH, only one SNVs in 3´UTR showed significant variations in non-cholesterol sterols by itself. Variants in 3´UTR have been previously involved in the pathogenesis of some mutation-negative FH. An important mechanism of post-transcriptional regulation linked to the 3´UTR implicates the binding of microRNAs (miRNAs), small non-protein coding RNAs that regulate gene expression at the post-transcriptional level. [33]. The frequency of the c.*380T>G variant in *ABCG5*, located in 3´UTR, is significantly lower in mutation-negative FH than in controls, and T allele carriers had higher intestinal cholesterol absorption than subjects carrying the G allele. The c.*380T>G variant has been previously associated with sitosterolaemia [34]. The mir-494 is a miRNA, which has been previously associated with this variant [35]. Bioinformatic analysis of this variant showed that T allele carriers disclose mir-494, which is not present in G allele carries. For this reason, we propose that mir-494 could regulate the absorption of non-cholesterol sterols and could explain in part the pathogenesis of the hypercholesterolemia in these subjects.

Finally, our study supports the multifactorial origin of most mutation-negative FH, in whom intestinal hyperabsorption of sterols plays a minor role that does not fully explain the etiology of these hypercholesterolemias, and with different contributions among subjects depending on the number of risk alleles they carry. Those subjects that accumulate many risk alleles of *ABCG5/G8* show a clearer hyperabsorption mechanism that could explain some of the LDL-C variation. We have recently analyzed a group of mutation-negative FH families with hyperabsorption in the proband. The cosegregation analysis showed a substantial contribution of hyperabsorption on the LDL-C concentration, although with high variation among the families [29].

An interesting issue is the potential relationship between *ABCG5/G8* gene variation and CHD risk. The C allele of the c.1199C>A, p.(Thr400Lys) variant in *ABCG8* has been previously associated with CHD [36]. In our study, the frequency of this risk allele variant was significantly higher in mutation-negative FH subjects than in the 1000 Genomes Project,

suggesting that the association with CHD previously reported could be mediated by its effect on LDL-C. However, the association of plasma phytosterols with CHD has not been clearly established [37]. Actually, none of 8 subjects with severe pathogenic mutations in *ABCG5/G8* in our study had CHD. Further studies are required to establish the potential association of *ABCG5/G8* variation and atherosclerosis.

Our study has limitations: We have not performed functional studies of the ABCG5/ABCG8 variants detected, but due to their large number their analysis would require a different approach. We have analyzed surrogate markers for intestinal cholesterol absorption; although very well validated; they have some limitations, and may be influenced by diet, sex, age, BMI, and *APOE* genotype. However, the differences in non-cholesterol sterols between mutation-negative FH subjects and controls and between patients with and without pathogenic *ABCG5/G8* mutations were not modified by any of these confounding factors. Subjects with hyperabsorption-associated *ABCG5/G8* mutations had no higher LDL cholesterol concentrations than the other hypercholesterolemic subjects of genetic cause. Our interpretation is that hypercholesterolemias with hyperabsorption do not present a more severe phenotype than those other genetic forms not associated with this mechanism and by the moment of unknown origin.

In conclusion, the sequencing analysis of a large group of subjects with genetic hypercholesterolemia and no pathogenic mutation in *LDLR*, *APOB*, *PCSK9* or *APOE* genes, shows that they accumulate an excess of rare and common gene variations in *ABCG5/G8* genes. This variation is associated with surrogate markers of increased intestinal cholesterol absorption, suggesting that these *loci* contribute to their hypercholesterolemia by raising intestinal cholesterol absorption.

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Disclosure summary

I certify that neither I nor my co-authors have a conflict of interest as described above that is relevant to the subject matter or materials included in this work.

Author contributions

ILM performed the experiments and contributed to the writing of the manuscript. FC contributed substantially to the conception and design and to the writing of the manuscript; all authors participated in acquisition, analysis and interpretation of the data, in the drafting of the article or critical revision and in final approval of the version to be published.

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Table 1. Clinical and biochemical characteristics of controls and mutation-negative FH.

		Controls (n = 97)	Mutation-negative FH (n = 214)	*p*
Age, years		44.5 ± 17.3	47.0 ±12.4	0.135
Men, n (%)		37 (38.1)	112 (47.6)	0.020
Body mass index, kg/m^2		25.3 ± 4.59	24.5 ± 2.69	0.149
Total cholesterol by HPLC MS/MS, mg/dL		201 ± 45.3	302 ± 59.8	<0.001
Triglycerides, mg/dL		75.0 (49.0-103)	112 (86.0-157)	<0.001
LDL cholesterol, mg/dL		122 (114-146)	224 (205-244)	<0.001
HDL cholesterol, mg/dL		53.0 (45.0-64.3)	55.0 (47.0-68.0)	0.042
Lipoprotein(a), mg/dL		15.7 (7.26-30.6)	44.3 (16.1-98.4)	<0.001
Apolipoprotein B, mg/dL		98.8 ± 20.0	154 ± 32.3	<0.001
Apolipoprotein A1, mg/dL		157 (138-177)	157 (137-180)	0.503
Glucose, mg/dL		85.5 (79.0-92.0)	87.0 (82.0-96.0)	0.169
Hypertension, n (%)		6 (6.38)	32 (15.0)	0.035
Diabetes, n (%)		0 (0)	1 (0.47)	0.509
Cardiovascular disease, n (%)		0 (0)	7 (3.27)	0.073
Tobacco, n (%)	Non smoker	53 (54.6)	100 (46.7)	0.330
	Current smoker	22 (22.7)	61 (28.5)	
	Former smoker	22 (22.7)	53 (24.8)	
APOE genotype, n (%)	E2/E3	10 (10.3)	14 (6.54)	0.107
	E3/E3	69 (71.1)	136 (63.5)	
	E4/E3	15 (15.5)	57 (26.6)	
	E4/E4	1 (1.03)	6 (2.8)	
	E4/E2	2 (2.06)	1 (0.46)	

Quantitative variables are expressed as means ± standard deviations, except for variables not following normal distribution that are expressed as medians (interquartile ranges). Qualitative variables were expressed as %. The *p* value was calculated by Student's t test or Mann-Whitney U and Chi-square, as appropriate.

HPLC-MS/MS: high performance liquid chromatography tandem mass spectrometry.

Table 2. Non-cholesterol sterols in controls and mutation-negative FH.

Non-cholesterol sterol ratios to cholesterol x10^3	Controls (n = 97)	Mutation-negative FH (n = 206)	*p*
5α-cholestanol	2.06 (1.57-2.47)	2.11 (1.66-2.89)	0.023
β-sitosterol	1.60 (0.985-2.05)	1.84 (1.33-2.74)	<0.001
Campesterol	0.935 (0.703-1.22)	0.939 (0.699-1.33)	0.435
Stigmasterol	0.102 (0.064-0.148)	0.171 (0.097-0.291)	<0.001
Sitostanol	0.076 (0.040-0.154)	0.046 (0.027-0.107)	0.008
Cholesterol absorption*	4.88 (3.70-6.10)	5.24 (4.14-7.34)	0.028

*Cholesterol absorption denotes the sum of sterol ratios to cholesterol x10^3 of 5α-cholestanol, β-sitosterol, campesterol, stigmasterol and sitostanol

Quantitative variables with not following normal distribution were expressed as median (interquartile range). The *p* value was calculated by Mann-Whitney U.

Table 3. Clinical characteristics of patients carrying pathogenic mutations in *ABCG5/G8* genes.

Gene	Nucleotide change	Predicted aminoacid change	Age (years)	Sex	BMI (kg/m^2)	TC mg/dL	TG* mg/dL	Apo B, mg/dL	*APOE* Genotype	Non q
ABCG5	rs146534033 c.1733A>G	p.(Asn578Ser)	49	W	19.8	308	149	157	ε3/ε3	
			49	W	25.5	312	180	118	ε3/ε4	
	rs139264483 c.862G>T	p.(Gly288Cys)	56	M	25.4	263	166	123	ε3/ε4	
	rs141828689 c.593G>A	p.(Arg198Gln)	58	W	25.0	316	156	136	ε2/ε3	
			48	M	26.7	315	211	153	ε3/ε3	
	rs552803459 c.805G>A	p.(Gly269Arg)	46	W	26.4	362	104	130	ε3/ε3	
	c. 887A>G	p.(Asn296Ser)	48	M	22.9	309	102	159	ε3/ε3	
ABCG8	rs376069170 c.1534G>A	p.(Gly512Arg)	51	M	25.0	272	189	109	ε3/ε3	

BMI: Body mass index. TC: Total cholesterol. TG: triglyceride. ApoB: Apolipoprotein B. M: Men. W: Woman

Quantitative variables are expressed as means, except for variables not following normal distribution that are expressed as medians*.

Table 4. Lipids and non-cholesterol sterols in mutation-negative FH subjects with mutation in *ABCG5/G8* genes and in mutation-negative FH without mutations in *ABCG5/G8* genes.

	Mutation-negative FH with *ABCG5/G8* mutation* (n = 7)	Mutation-negative FH without *ABCG5/G8* mutation (n = 199)	*p*
Age (years)	49.9 ± 3.89	46.9 ±12.5	0.111
Sex, men, n (%)	4 (50%)	108 (52.4%)	0.893
Body Mass Index, Kg/m^2	24.5 ± 2.39	24.5 ± 2.72	0.946
Cholesterol by HPLC MS/MS, mg/dL	296 ± 83.2	290 ± 61.1	0.820
Triglycerides, mg/dL	157 ± 38.6	120 ± 49.8	0.039
LDL cholesterol, mg/dL	229 (206-243)	223 (205-244)	0.760
HDL cholesterol, mg/dL	47 (43.3-50.8)	56.0 (48.0-68.0)	0.015
Apolipoprotein B, mg/dL	158 ± 25.9	154 ± 32.6	0.775
5α-cholestanol-to-TC x 10^3	3.13 (2.05-8.59)	2.11 (1.66-2.86)	0.042
β-sitosterol-to-TC x 10^3	3.03 (1.86-11.5)	1.84 (1.33-2.72)	0.045
Campesterol-to-TC x 10^3	1.64 (0.907-4.94)	0.938 (0.691-1.32)	0.034
Stigmasterol-to-TC x 10^3	0.447 (0.167-0.819)	0.167 (0.097-2.89)	0.047
Sitostanol-to-TC x 10^3	0.084 (0.057-0.314)	0.046 (0.027-0.107)	0.088
Cholesterol absorption†	7.97 (5.48-21.3)	5.14 (4.13-7.15)	0.030

*Referred to patients described in detail in Table 3.

†Cholesterol absorption denotes the sum ratios to cholesterol x10^3 of 5α-cholestanol, β-sitosterol, campesterol, stigmasterol and sitostanol

Quantitative variables are expressed as mean ± standard deviation, except for variables not following normal distribution that are expressed as median (interquartile range). The *p* value was calculated by Student's t test or Mann-Whitney U, as appropriate.

Table 5. Non-cholesterol sterols in mutation-negative FH subjects with the rs2278356 variant and in mutation-negative FH without the rs2278356 variant. .

rs2278356 c.*380T>G	Mutation-negative FH with variant c.*380T>G (n=97)	Mutation-negative FH without variant c.*380T>G (n=109)	*p*
Cholesterol by HPLC MS/MS, mg/dL	292 ± 62.8	290 ± 61.1	0.838
Triglycerides, mg/dL	118 ± 46.8	124 ± 52.8	0.317
LDL cholesterol, mg/dL	218 (205-239)	230 (206-250)	0.021
HDL cholesterol, mg/dL	56.0 (48.5-67.0)	55.0 (46.0-68.0)	0.459
Apolipoprotein B, mg/dL	151 ± 33.6	157 ± 31.3	0.176
5α-cholestanol to TC x 10^3	2.07 (1.69-2.82)	2.37 (1.68-3.41)	0.011
β-sitosterol to TC x 10^3	1.68 (1.26-2.40)	2.28 (1.42-3.40)	0.001
Campesterol to TC x 10^3	0.831 (0.642-1.31)	1.16 (0.757-1.64)	0.002
Stigmasterol to TC x 10^3	0.142 (0.082-0.278)	0.179 (0.097- 0.329)	0.022
Sitostanol to TC x 10^3	0.043 (0.025-0.088)	0.052 (0.027-0.116)	0.420
Cholesterol absorption†	4.66 (4.03-6.44)	6.12 (4.34-8.32)	0.002

†Cholesterol absorption denotes the sum ratios to cholesterol x10^3 of 5α-cholestanol, β-sitosterol, campesterol, stigmasterol and sitostanol

Quantitative variables are expressed as mean ± standard deviation, except for variables not following normal distribution that were expressed as median (interquartile range). ln(5α-cholestanol to TC x 10^3), ln(β-sitosterol to TC x 10^3), ln(campesterol to TC x 10^3), ln(stigmasterol to TC x 10^3), ln(sitostanol to TC x 10^3) and ln(cholesterol absorption) were used to calculate *p* for trend adjusted by confusing factors: body mass index, sex and age.

Table 6. Non-cholesterol sterols in mutation-negative FH according to SNV gene score.

	Mutation-negative FH with lower gene score (n = 100)	Mutation-negative FH with higher gene score (n =106)	*p*
Cholesterol by HPLC MS/MS, mg/dL	282 ± 62.5	298 ± 60.3	0.074
Triglycerides, mg/dL	121 ± 48.6	121 ± 51.3	0.967
LDL cholesterol, mg/dL	225 (204-245)	224 (205-241)	0.586
HDL cholesterol, mg/dL	58.5 (48.0-69.0)	55.0 (46.0-67.0)	0.420
Apolipoprotein B, mg/dL	153 ± 34.7	155 ± 30.5	0.659
5α-cholestanol to TC x 10^3	2.036 (1.65-2.82)	2.33 (1.78- 3.38)	0.034
β-sitosterol to TC x 10^3	1.86 (1.31- 2.67)	1.89 (1.40-3.01)	0.149
Campesterol to TC x 10^3	0.938 (0.643-1.38)	0.972 (0.761-1.56)	0.068
Stigmasterol to TC x 10^3	0.129 (0.078-0.271)	0.192 (0.101-0.321)	0.029
Sitostanol to TC x 10^3	0.050 (0.032- 0.097)	0.042 (0.027-1.04)	0.953
Cholesterol absorption*	4.99 (3.97-7.06)	5.58 (4.15-8.37)	0.077

*Cholesterol absorption denotes the sum ratios to cholesterol x10^3 of 5α-cholestanol, β-sitosterol, campesterol, stigmasterol and sitostanol.

Quantitative variables not following normal distribution were expressed as median (interquartile range). ln(5α-cholestanol to TC x 10^3), ln(β-sitosterol to TC x 10^3), ln(campesterol to TC x 10^3), ln(stigmasterol to TC x 10^3), ln(sitostanol to TC x 10^3) and ln(cholesterol absorption) were used to calculate *p* for trend adjusted by confusing factors: body mass index, sex and age.

- Phytosterols as markers of intestinal cholesterol absorption are elevated in FH-
- The present study demonstrates an increase in *ABCG5/G8* mutations in FH-
- Subjects with mutations in *ABCG5/G8* have increased plasma phytosterols
- We show for the first time that *ABCG5/G8* is involved in the etiopathogenesis of FH-

Supplemental Table 1**.** Allele frequencies of genetic variants with allelic frequencies significantly different in mutation-negative FH from those identified in controls or described in the 1000 Genomes Project.

Gene	Nucleotide change	Predicted aminoacid change	Bioinformatic Analysis	Frequency in mutation-negative FH	Frequency in controls	*p*[1]	Frequency in the 1000 Genomes population	*P*[2]	Frequency in ExAc	*P*[3]
ABCG5	rs6756629 c.148C>T	p.(Arg50Cys)	Damaging	0.047	0.067	0.243	0.076	0.0457	0.066	0.1094
	rs9789463 c.502-395T>G	-	Benign	0.210	0.242	0.378	0.347	<0.001	0.224	0.665
	rs4549146 c.339A>G	p.(Val113Val)	Benign	0.276	0.351	0.062	0.422	<0.001	0.4319	<0.001
	rs4557032 c.502-192C>T	-	Benign	0.259	0.273	0.717	0.344	0.0017	-	-
	rs114938914 c.502-256G>A	-	Benign	0.040	0.062	0.225	0.077	0.0099	0.1416	0.007
	rs552803459 c.805G>A	p.(Gly269Arg)	Damaging	0.005	0.000	0.339	0.000	0.0296	<0.001	<0.001
	rs4148187 c.1324+164C>T	-	Benign	0.264	0.253	0.761	0.334	0.0093	-	-
	rs200839584 c.1570G>A	p.(Val524Ile)	Benign	0.005	0.000	0.339	0.000	0.0296	<0.001	<0.001
	rs150002815 c.1320T>A	p.(Asn440Lys)	Benign	0.012	0.000	0.129	0.000	0.0006	<0.001	<0.001
	rs2278356 c.*380 T>G	-	Benign	0.270	0.340	0.1183	0.320	0.0339	-	-
	rs2278357 c.*416G>A	-	Benign	0.092	0.124	0.226	0.157	0.0011	-	-

ABCG8	rs3806471 c.-19T>A	-	Benign	0.237	0.227	0.7791	0.297	0.0204	0.417	<0.001
	rs72647315 c.-15A>C	-	Benign	0.050	0.050	0.9390	0.000	0.0296	0.000	<0.001
	rs11887534 c.55G>A	p.(Asp19Asn)	Benign	0.042	0.096	0.0082	0.080	0.0107	0.077	0.007
	rs4148209 c.64-21C>A	-	Benign	0.291	0.273	0.6476	0.371	0.0038	0.388	<0.001
	rs4148210 c.64-7C>T	-	Benign	0.275	0.278	0.9243	0.372	<0.001	0.386	<0.001
	rs4148211 c.161A>G	p.(Tyr54Cys)	Benign	0.279	0.273	0.8741	0.371	<0.001	0.387	<0.001
	rs4148213 c.165+105G>C	-	Benign	0.296	0.278	0.6576	0.372	0.0058	-	-
	rs4148214 c.561+43T>C	-	Benign	0.322	0.412	0.0280	0.461	<0.001	0.561	<0.001
	rs114197606 c.964+178G>A	-	Benign	0.002	0.000	0.4494	0.032	0.0057	-	-
	rs376069170 c.1534G>A	p.(Gly512Arg)	Probably damaging	0.002	0.000	0.4494	0.000	0.0025	0.000	<0.001
	rs4148217 c.1199C>A	p.(Thr400Lys)	Benign	0.033	0.000	0.0105	0.201	<0.001	0.2146	<0.001
	rs4245794 c.1212-77T>C	-	Benign	0.068	0.093	0.2811	0.107	0.0209	-	-
	rs34198326 c.1411+104A>G	-	Benign	0.052	0.124	0.0015	0.090	0.0128	-	-
	rs371711306 c.1845G>C	p.(Met615Ile)	Benign	0.050	0.000	0.3403	0.000	0.030	0.000	<0.001
	rs55741639 c.965-58A>G	-	Benign	0.023	0.000	0.0314	0.016	0.2072	-	-
	rs4953027 c.1412-152A>G	-	Benign	0.289	0.117	<0.001	0.247	0.0956	-	-

	rs10709506 c.1411+90delA	-	Benign	0.132	0.240	0.0009	0.153	0.3054	-	-
	rs28517482 c.1412-8C>T	-	Benign	0.343	0.196	0.0002	0.397	0.0548	0.599	<0.001

The *p* value was calculated by Chi-square test, by comparing mutant versus wild-type allelic frequencies between mutation-negative FH and controls (p^1), between mutation-negative FH and 1000 Genomes Project (p^2) and between mutation-negative FH and ExAc Browser Data (p^3).

Supplemental Table 2. Bioinformatic Analysis of pathogenic mutations

Gene	Nucleotide change	Predicted aminoacid change	Bioinformatic Analysis				Frequency in the 1000 Genomes population	Frequency in the ExAc
			SIFT (score)	PredictSNP2* (probability)	Polyphen-2 (score)	Mutation taster (probability)		
ABCG5	rs146534033 c.1733A>G	p.(Asn578Ser)	Tolerated (0.10)	Damaging (87%)	Probably Damaging (0.984)	Disease causing (0.984)	0.000	<0.001
	rs139264483 c.862G>T	p.(Gly288Cys)	Damaging (0.00)	Damaging (87%)	Probably Damaging (0.999)	Disease causing (0.999)	0.000	<0.001
	rs141828689 c.593G>A	p.(Arg198Gln)	Damaging (0.02)	Damaging (87%)	Probably Damaging (1.000)	Disease causing (0.999)	0.00399	0.0023
	rs552803459 c.805G>A	p.(Gly269Arg)	Damaging (0.00)	Damaging (87%)	Probably Damaging (1.000)	Disease causing (0.999)	0.000	<0.001
	c. 887A>G	p.(Asn296Ser)	Damaging (0.02)		Possibly damaging (0.635)	Disease causing (0.999)	-	-
ABCG8	rs376069170 c.1534G>A	p.(Gly512Arg)	Tolerated (0.37)	Damaging (87%)	Possibly damaging (0.682)	Disease causing (0.997)	0.000	0.000

*PredictSNP2 used CADD, DANN, FATHMM and FunSeq2 as bioinformatic predictors.

Supplemental Table 3. Cholesterol synthesis markers in mutation-negative FH subjects with mutation in *ABCG5/G8* genes and in mutation-negative FH without mutations in *ABCG5/G8* genes.

	Mutation-negative FH with *ABCG5/G8* mutation* (n = 7)	Mutation-negative FH without *ABCG5/G8* mutation (n = 199)	*p*
Lanosterol-to-TC x 10^3	0.11 (0.05-0.31)	0.11 (0.07-0.16)	0.961
Desmosterol -to-TC x 10^3	2.18 (0.89-6.12)	2.24 (1.73-2.91)	0.940

*Referred to patients described in detail in Table 3.

Lanosterol and demosterol are expressed as ratios to cholesterol x 10^3

Variables are expressed as median (interquartile range). The *p* value was calculated by Mann-Whitney U.