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ARTICLE

Genetic determinants of plasma HDL-cholesterol levels in familial hypercholesterolemia

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The objective of this study was to determine the extent to which common genetic variants can explain the variation of high-density lipoprotein cholesterol (HDL-C) plasma levels in familial hypercholesterolemia (FH). FH is characterized by elevated low-density lipoprotein cholesterol levels and premature cardiovascular disease (CVD). Although low HDL-C levels have been shown to affect the severity of the clinical phenotype, little is known about the factors that determine HDL-C levels in these patients. A cohort of 1002 heterozygous FH patients was genotyped for polymorphisms in the genes encoding for ATP-binding cassette transporter A1, apolipoprotein (apo) AIV, apoCIII, apoE, cholesteryl transfer ester protein, hepatic lipase, lipoprotein lipase, and two paraoxonases. Multiple linear regression showed that, together, these polymorphisms explain only 3.9% of the variation of HDL-C plasma levels. When significant two-way interactions between the polymorphisms were also taken into account, the explained variation rose to 12.5%. In a regression model that also incorporated sex, smoking, alcohol use, body mass index, and concomitant beta-blocker use as covariates, the explained variation of HDL-C plasma levels even increased to 32.5%. This study provides direct evidence that multiple, modestly penetrant, but highly prevalent, polymorphisms can explain a substantial part of the variation of HDL-C plasma levels in a representative large cohort of heterozygous FH patients.
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Introduction

Familial hypercholesterolemia (FH) is a common, hereditary disorder, characterized by elevated levels of plasma low-density lipoprotein cholesterol (LDL-C) and premature cardiovascular disease (CVD). FH is caused by mutations in the low-density-lipoprotein receptor (*LDL-R*) gene, leading to an insufficient uptake of LDL-C from the circulation.[1] Characteristically, the mean age of onset of CVD is between 40 and 45 years in male FH patients and 10 years later in female FH patients.[1,2] Although the cause of FH is monogenic, there is wide variation in the onset and severity of atherosclerotic disease in these patients.[3] This is often suggested to be related to environmental and additional genetic risk factors. In this respect, low high-density-lipoprotein cholesterol (HDL-C) has been shown to be an independent risk factor for the development of CVD in FH patients.[4–8] Therefore, early identification of FH

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patients with low HDL-C, in addition to the development of therapeutic strategies to specifically raise HDL-C, may be warranted.[9] HDL-C levels, however, are affected by sex, obesity, smoking, diet, alcohol consumption, exercise, and medication use, in addition to numerous genetic factors,[10,11] but the relative contribution of these factors to HDL-C levels in FH patients is not known.[12] It is assumed that the combined effects of multiple common genetic variants might explain a large part of HDL-C variation.[13] We therefore determined the contribution of polymorphisms in a number of HDL-C-related gene loci to the variation of HDL-C plasma levels in FH.

Methods

Study design and study population

The present investigation was a retrospective, multicenter, cohort study. The study design and study population have been described elsewhere.[8] Briefly, lipid clinics in the Netherlands submit DNA samples from clinically suspected FH patients to a central laboratory for LDL-receptor mutation analysis.[14] We randomly selected hypercholesterolemic patients from this DNA-bank database with the aid of a computer program (Microsoft Excel). These patients had been referred from 27 lipid clinics throughout the Netherlands. A total of 2400 FH patients were included in this study. The FH diagnostic criteria were based on internationally established criteria.[15–17]

Phenotypic data (including detailed information on factors known to influence plasma HDL-C levels) were acquired by reviewing patient's medical records by a trained team of data collectors.[18] Guidelines for data collection from medical records were constructed for the purpose of the study and have been published.[18] Written informed consent was obtained from all living patients. The Ethics Institutional Review Board of each participating hospital approved the protocol.

Factors known to influence plasma HDL-C levels

Smoking was defined as ever having smoked (yes/no). Body mass index (BMI) was calculated from height and length (kg/m^2). Alcohol use was determined at the time of the first visit to the lipid clinic (yes/no). Hypertension was defined when the diagnosis had been made and when antihypertensive medication was prescribed, or if three consecutive measurements of blood pressure were >140 mmHg systolic or >90 mmHg diastolic. Concomitant beta-blocker and/or diuretic use were assessed at the time of determination of plasma HDL-C concentration (yes/no). Diabetes mellitus was defined when the diagnosis had been made and medication (insulin or oral antidiabetics) was prescribed, or by a fasting plasma glucose of >6.9 mmol/l.

Laboratory analysis

All laboratory parameters were measured in fasting blood samples after at least 6 weeks of withdrawal of any lipid-lowering medication. The presented values are those from as close to the first lipid clinic visit as possible, with a maximum time-span of two years. Plasma total cholesterol, HDL-C, and triglycerides (TG) were measured by standard enzymatic methods. LDL-C concentrations were calculated by means of the Friedewald formula.[19] Mutations in the *LDL-R* gene were assessed as described previously.[20]

Selection of polymorphisms

Using the current literature, we selected 25 DNA polymorphisms in nine genes that are known to affect HDL-C levels or that could potentially affect HDL-C levels. We selected the following 23 biallelic polymorphisms in eight HDL-C-related genes for the present analysis: $ABCA1_{C69T}$, $ABCA1_{ins319}$, $ABCA1_{Arg219Lys}$, $ApoAIV_{Thr347Ser}$, $ApoAIV_{Gln360His}$, $ApoCIII_{C(-641)A}$, $ApoCIII_{C(-482)T}$, $ApoCIII_{Thr(-455)Cys}$, $ApoCIII_{C1100T}$, $ApoCIII_{C3175G}$, $ApoCIII_{T3206G}$, $CETP_{C(-629)A}$, $CETP_{Taq1B}$, $CETP_{C(-631)A}$, $CETP_{Ile405Val}$, $HL_{C(-514)T}$, $LPL_{T(-93)G}$, $LPL_{Asp9Asn}$, $LPL_{Asn291Ser}$, $LPL_{Ser447Stop}$, $PON1_{Leu55Met}$, $PON1_{Gln192Arg}$, and $PON2_{Ser311Cys}$, and we included the triallelic *ApoE* polymorphism, determined by arginine/cysteine variations at codons 112 and 158, in our analyses.

Genetic analyses

Genomic DNA was extracted from peripheral blood leukocytes according to a standard protocol. Genotyping was performed by Roche Molecular Systems, CA, USA. For all biallelic sites, genotypes were generated using a PCR and immobilized probe assay, as described previously by Cheng *et al*.[21] Laboratory personnel had no access to either identifiable or clinical information.

Statistical analysis

All polymorphism genotypes were treated as categorical variables. The effect of individual polymorphism genotypes on HDL-C concentration was estimated by linear regression, without adjustment for covariates. Subsequently, four different backward (multiple) linear regression models were used to examine the independent effects of the polymorphisms on HDL-C concentration. The first (main effects) model included only the main effects of the 25 polymorphisms. The second (combined effects) model included the main effects and a selection of two-way interactions (gene–gene, gene–environment, and environment–environment interactions). All two-way interaction terms were assessed, and those that were significant after adjustment for the main effects of all polymorphisms were included. Backward selection was then performed on the main effects of the polymorphisms not included in the selected interactions. The remaining two models consisted of the above-mentioned models with adjustment for a set of five covariates, that is: sex, smoking, alcohol use, BMI, and concomitant beta-blocker use. A P-value of <0.05 was considered significant. For all four models, the percentage

explained (R^2) variation in baseline HDL-C concentration was calculated. Statistical analyses were performed using SPSS software (version 11.0, Chicago, IL, USA).

Results

From the 2400 patients who comprised the study population, a total of 1002 individuals could be completely genotyped for 25 selected polymorphisms in addition to having complete clinical data. These 1002 subjects did not differ significantly from the original cohort of 2400 patients with regard to any of the clinical characteristics. The characteristics of the study population are summarized in Table 1. The average HDL-C concentration without lipid-lowering medication was 1.20 ± 0.35 mmol/l. Among patients, beta-blocker use was more prevalent than diuretic use (11 *vs* 4%). Furthermore, beta-blocker use had a significant ($P < 0.0001$) effect on HDL levels, whereas diuretic use did not influence HDL levels ($P = 0.99$). Therefore, beta-blocker use alone was chosen as a covariate in the statistical models (see below). The presence of diabetes mellitus did not contribute to plasma HDL levels in this population. Therefore, diabetes mellitus was not included as a covariate in the statistical models (see below).

A total of 193 patients (19%) had experienced a cardiovascular event before plasma HDL determination.

Table 1 Clinical characteristics of the genotyped FH patients

Number of subjects	1002
Demographics	
Male sex (%)	49.0
Age at first visit to lipid clinic	44.1 ± 12.3
Cardiovascular event prior to first clinic visit (%)	19
Risk factors	
Smoking, ever (%)	73.9
Alcohol use at first visit to lipid clinic (%)	75.7
Hypertension (%)	9.4
Diabetes mellitus (%)	4.8
Physical examination	
BMI (kg/m^2)	25.1 ± 3.5
Systolic blood pressure (mmHg)	134 ± 18
Diastolic blood pressure (mmHg)	82 ± 10
Tendon xanthomas (%)	44%
Laboratory parameters	
Total cholesterol (mmol/l)	9.36 ± 1.94
LDL cholesterol (mmol/l)	7.33 ± 1.95
HDL cholesterol (mmol/l)	1.20 ± 0.35
Triglycerides (mmol/l)	1.57 (1.11–2.23)

Values are given as mean levels ± standard deviation, except where given as percentages. Conversion factor for mmol/l to mg/dl: for cholesterol multiply by 39, for triglycerides multiply by 89. Triglycerides are given as median with the interquartile range between brackets. BMI = body mass index; Lp(a) = lipoprotein(a).

However, due to confounding with the other covariates in the model (sex, smoking, body mass index, alcohol use, and beta-blocker use), the presence of a prior CVD event (as a covariate) did not remain significant in the statistical model. These risk factors have been studied extensively in this study population,[8] and are confirmed classical risk factors for the development of CVD in FH. Therefore, in a statistical model, the presence of CVD cannot function as a statistically independent predictor of plasma HDL.

Genotype counts and mean HDL-C plasma levels according to genotype are presented for the 23 biallelic SNPs in Table 2. All but two ($ApoAIV_{Thr347Ser}$ and $ApoC\text{-}III_{T3206G}$) of the 23 polymorphisms studied were in Hardy–Weinberg equilibrium (HWE). We also considered the triallelic *ApoE* polymorphism that is determined by arginine/cysteine variations at codons 112 and 158. The E3E3, E3E4, E2E3, E2E4, E4E4, and E2E2 genotypes were defined in 574, 286, 64, 30, 46, and two subjects, respectively. The *ApoE* polymorphism was in HWE. HDL-C plasma levels for these genotypes were 1.20 (±0.35), 1.20 (±0.35), 1.21 (±0.37), 1.02 (±0.21), 1.24 (±0.37), and 1.12 (±0.09) mmol/l, respectively.

Effect of multiple polymorphisms and their interactions on HDL-C plasma levels

After the backward selection procedure, the main-effects model consisted of five polymorphisms that together accounted for 3.9% of the population variability in HDL-C plasma levels (Table 3). These five polymorphisms were $CETP_{TaqIB}$, $ABCA1_{Arg219Lys}$, $LPL_{Asn291Ser}$, $ApoCIII_{C1100T}$, and $ApoAIV_{Thr347Ser}$.

The combined-effects model incorporated 12 main effects and five gene–gene interaction effects, and explained 12.5% of HDL-C variation. The interactions were ($ABCA1_{C69T} \times ApoAIV_{Thr347Ser}$), ($ABCA1_{C69T} \times ApoCIII_{T3206G}$), ($ApoCIII_{C(-482)T} \times CETP_{TaqIB}$), ($ApoCIII_{T(-455)C} \times LPL_{Ser447Stop}$), and ($ApoE \times CETP_{TaqIB}$), with individual R^2-values of 1.0, 1.9, 1.8, 2.0, and 1.6%, respectively. In addition to the main effects of the polymorphisms in the interaction terms, the main effects of $ABCA1_{Arg219Lys}$, $ApoCIII_{C1100T}$, $HL_{C(-514)T}$, and $LPL_{Asn291Ser}$ remained significant.

When the five covariates (sex, smoking, alcohol use, BMI, and concomitant beta-blocker use) were incorporated into the model, four polymorphisms ($ABCA1_{Arg219Lys}$, $HL_{C(-514)T}$, $LPL_{Asn291Ser}$, and $CETP_{TaqIB}$) remained in the main-effects model, explaining 20.2% of HDL-C variation. The covariate-adjusted combined-effects model could even explain 32.5% of HDL-C variation, while containing 18 polymorphism main effects and eight gene–gene, two gene–environment, and one environment–environment interactions. The most important gene–gene interactions were ($ABCA1_{C69T} \times ApoCIII_{T3206G}$), ($ApoCIII_{C(-482)T} \times CETP_{Taq1B}$), ($LPL_{Ser447Stop} \times PON2_{Ser311Cys}$), and ($PON2_{Ser311Cys} \times CETP_{Ile405Val}$), with individual R^2-values of 2.1, 1.8, 1.1, and 1.1%, respectively. The most significant gene–environ-

Table 2 Genotype counts and HDL-C plasma levels for all investigated SNPs

		Common allele homozygotes		*Heterozygotes*		*Rare allele homozygotes*			
Gene	*SNP*	*Mean ± SD*	*n*	*Mean ± SD*	*n*	*Mean ± SD*	*n*	*P-value*	*R^2 (%)*
ABCA1	C69T	1.20 ± 0.37	451	1.20 ± 0.33	429	1.20 ± 0.33	122	0.981	0.01
ABCA1	ins319	1.21 ± 0.34	778	1.17 ± 0.35	210	1.17 ± 0.36	14	0.337	0.2
ABCA1	Arg219Lys	1.18 ± 0.32	544	1.24 ± 0.38	391	1.15 ± 0.29	67	0.011	0.9
ApoA4	Thr347Ser	1.21 ± 0.35	679	1.18 ± 0.35	271	1.11 ± 0.32	52	0.073	0.5
ApoA4	Gln360His	1.20 ± 0.35	847	1.19 ± 0.32	152	1.00 ± 0.43	3	0.569	0.1
ApoC3	C(−641)A	1.21 ± 0.33	373	1.20 ± 0.36	468	1.17 ± 0.34	161	0.455	0.2
ApoC3	C(−482)T	1.20 ± 0.33	524	1.20 ± 0.36	395	1.17 ± 0.36	83	0.749	0.1
ApoC3	T(−455)C	1.20 ± 0.33	388	1.21 ± 0.36	460	1.17 ± 0.35	154	0.575	0.1
ApoC3	C1100T	1.22 ± 0.35	535	1.18 ± 0.36	378	1.17 ± 0.32	89	0.165	0.4
ApoC3	T3206G	1.19 ± 0.32	396	1.21 ± 0.37	428	1.18 ± 0.34	178	0.507	0.1
ApoC3	C3175G	1.20 ± 0.34	782	1.18 ± 0.36	206	1.15 ± 0.23	14	0.640	0.1
CETP	C(−629)A	1.16 ± 0.32	262	1.20 ± 0.34	513	1.25 ± 0.39	227	0.010	0.9
CETP	C(−631)A	1.20 ± 0.35	865	1.17 ± 0.34	131	1.35 ± 0.40	6	0.413	0.2
CETP	Ile405Val	1.20 ± 0.35	454	1.20 ± 0.35	436	1.19 ± 0.33	112	0.931	0.01
CETP	Taq1B	1.15 ± 0.32	320	1.21 ± 0.34	504	1.26 ± 0.41	178	0.005	1.1
HL	C(−514)T	1.18 ± 0.34	602	1.23 ± 0.36	339	1.23 ± 0.34	61	0.094	0.5
LPL	T(−93)G	1.20 ± 0.35	959	1.09 ± 0.26	42	1.21	1	0.109	0.4
LPL	Asp9Asn	1.20 ± 0.35	965	1.10 ± 0.26	36	1.21	1	0.208	0.3
LPL	Asn291Ser	1.20 ± 0.35	934	1.08 ± 0.29	67	0.96	1	0.011	0.9
LPL	Ser447X	1.20 ± 0.35	828	1.23 ± 0.33	160	1.17 ± 0.32	14	0.554	0.1
PON_1	Met55Leu	1.20 ± 0.35	427	1.19 ± 0.34	449	1.19 ± 0.32	126	0.762	0.1
PON_1	Gln192Arg	1.20 ± 0.34	470	1.20 ± 0.35	432	1.18 ± 0.33	100	0.820	0.01
PON_2	Ser311Cys	1.19 ± 0.35	590	1.22 ± 0.34	348	1.17 ± 0.36	64	0.432	0.2

HDL-C levels are presented as mean (in mmol/l) ± SD (conversion factor for mmol/l to mg/dl: multiply by 0.002586). ABCA1 indicates ATP-binding cassette A1; ApoA4 = apolipoprotein A4; ApoC3 = apolipoprotein C3; CETP = cholesteryl ester transfer protein; HL = hepatic lipase; LPL = lipoprotein lipase; PON = paraoxonase.

Table 3 Explained variation of HDL-C plasma concentration

Variables	*Main effects model (main effects only)*	*Combined effects model (main effects+selected two-way interactions)*
Genotypes	3.9% (10)	12.5% (42)
Genotypes plus covariates	20.2% (13)	32.5% (72)

R^2-values for multiple linear regression models incorporating all genetic variants only, and genetic variants plus covariates (sex, smoking, alcohol, BMI, and concomitant beta-blocker use). Values are presented for models that incorporate main effects only, and main effects plus two-way interactions that contributed significantly to the explained HDL-C variation. The number of variables per model is given between parentheses.

ment interactions were ($CETP_{Ile405Val} \times$ alcohol use) and ($PON_{1Leu55Met} \times sex$), with individual R^2-values of 0.9 and 0.6%, respectively.

Discussion

The present investigation provides evidence that, indeed, the combined effects of polymorphisms involved in HDL metabolism can explain 12.5% of the variation of HDL-C plasma levels. Moreover, when sex and several environmental factors were taken into account, a striking 32.5% of the variation in HDL-C levels could be explained. To our knowledge, this is the largest exploratory study to date to investigate the contribution of genetic variants to plasma HDL-C levels in FH.

It is assumed that the combined effects of multiple common genetic variants might explain a large part of HDL-C variation.[13] Accordingly, we found that the effect of individual polymorphism genotypes on HDL-C concentration was not impressive. In particular, the $CETP_{TaqIB}$ polymorphism showed the strongest association with HDL-C plasma level, but only accounted for 1.1% of this variation. Indirect evidence has been provided by twin and family studies reporting that genetic background explains 50–70% of the variation in HDL-C concentration.[13,22–24] This heritability may be caused by a combination of common genetic variants with a small effect (as shown in our study) and rare genetic variants with a strong effect, as shown recently by Cohen *et al*.[25]

Several other studies have used a multigenetic approach to explain the variability of plasma HDL-C levels. Previous studies with FH patients that used a similar approach exhibit important differences with ours.[26,27] The first study by Miltiadous *et al* was performed in a small ($n = 84$) cohort of FH patients, where the individual effects of only five polymorphisms were studied ($CETP_{Taq1B}$, $ApoAIV_{Thr347Ser}$, $ApoAIV_{Gly360His}$, *ACE ins/del,* and *ApoE*), and, importantly, interactions between genotypes were not taken into account. The second study by Bertolini *et al* was performed in a larger cohort of FH patients ($n = 221$ index cases and

$n = 349$ relatives with FH), but, again, only eight polymorphisms that could potentially affect HDL variation were studied (*ApoE*, $LPL_{Asn291Ser}$, $LPL_{Ser447Stop}$, $HL_{C(-514)T}$, $HL_{G(-250)A}$, $FABP\text{-}2_{A54T}$, *Apo* AV_{T1131C}, and $ABCA1_{Arg219Lys}$) and interactions between genotypes were not considered. Other comparable studies performed in the general population, such as a subanalysis of the Northwick Park Heart Study with 2773 healthy middle-aged men and a study performed in the Stanislas cohort, demonstrated that polymorphisms explained only 2.5 and 11.3% (in men; 16.4% in women) of the variation of HDL-C plasma levels, respectively.[28,29] In summary, none of the multigenetic studies performed thus far have examined such a large number of polymorphisms in such a large cohort of FH patients, nor have they demonstrated a similarly large percentage of the variation in plasma HDL-C levels.

Several aspects must be taken into account when interpreting the results of the present study. To begin with, the present study focuses on variation in HDL-C levels and the contribution thereof to the clinical expression in FH. Although the inverse relationship between HDL-C levels and CVD risk is well founded, alterations in HDL-regulating genes that result in high HDL-C levels have not consistently been associated with cardiovascular protection. Several genetic variants have been reported to be associated with a lower risk of CVD, but independent of HDL-C levels.[30,31] Therefore, studying the contribution of polymorphisms to HDL-C variation should ultimately be translated into their contribution to CVD risk. Moreover, it has been suggested that not only the level of HDL-C but also the composition contributes to CVD risk.[32] Unfortunately, HDL-C subclasses were not determined in these patients. Future studies in this cohort will be aimed at the association of polymorphisms with CVD risk, as prospectively assessed over time. Secondly, two of the 23 polymorphisms studied were not in HWE. The exact reason for the deviation is not known and we can only speculate on this. Importantly, the deviation from HWE is not due to mixed ethnic groups, as the Dutch population is known to be a homogenous one. Over 99% of our patients were Caucasian and patients were randomly selected from all over the country. In addition, most deviations were caused by an excess of heterozygotes, which makes genotyping errors unlikely. Furthermore, the accuracy of genotyping in 500 randomly selected DNA samples was assessed by re-analysis of several polymorphisms in three genes, revealing that less than 0.5% of the results were discordant. Thirdly, due to the retrospective design and reliance on documentation in the medical records, no standardized information was available regarding dietary habits and physical activity. Therefore, we cannot estimate the contribution of these environmental factors that are known to modulate HDL-C plasma levels. Finally, statistical aspects of our study may require some explanation. Statistical analysis of genetic population studies is still in development. A major issue in this field is the interpretation of data sets with a large number of genetic variables. These analyses have a tendency to be 'overfitted', that is, the number of explaining variables approaches or even surpasses the number of observations. In the current analysis, we used 'conventional' multiple linear regression. We incorporated the main effects of genotypes and, subsequently, we selected only those interaction terms that contributed significantly to the explained HDL-C variation after adjustment for all other variables in the model. Our data set comprised 1002 individuals, and the total number of variables in the combined-effects model (with adjustment for covariates) was 72, resulting in a predictor variable to patient ratio of 1:14. This ratio is lower than the 1:10 ratio that is used as a rough rule of thumb to prevent overfitting. This ratio is based on Harrell *et al*,[33] who showed that, for a regression model to have predictive discrimination, the number of predictor variables in a linear regression model should not exceed the number of patients divided by 10.

Modulation of the expression of the investigated genes to increase HDL-C levels is anticipated to decrease the risk for CVD. This is supported by evidence that a 1% increase in HDL-C with the use of gemfibrozil can yield a 3% risk reduction of CVD.[34] Our results indicate, however, that gene–gene and gene–environment interactions account for a substantial proportion of the overall 32.5% variation in HDL-C found in this study. Therefore, targeting specific genes may only be efficacious in the context of a specific metabolic background. Nevertheless, pharmacological inhibition of CETP is currently being tested in FH patients to assess their ability to raise HDL-C levels and to induce regression of carotid intima-media thickness. Shortly, ApoA1 mimetics and ABCA1 agonists will follow and will hopefully supplement our armamentarium in the fight against CVD in this high-risk disorder.

Conclusion

In FH patients, the variation in HDL-C levels is thought to contribute significantly to the overall risk for CVD in these individuals. However, little is known about the actual contribution of genes and environment to HDL in FH. The present study demonstrates that, in combination with sex and environmental factors, the combined effects of polymorphisms involved in HDL metabolism can explain up to 32.5% of the variation of HDL-C plasma levels. A better understanding of HDL metabolism may lead to improved cardiovascular risk assessment of FH patients. Furthermore, this may lead the way to the identification of future targets for intervention to improve CVD outcome in these high-risk patients.

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