A Combination of Single Nucleotide Polymorphisms is Associated with the Interindividual Variability of Cholesterol Bioavailability in Healthy Adult Males

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A combination of single nucleotide polymorphisms is associated with the interindividual variability of cholesterol bioavailability in healthy adult males.

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Abbreviations: CVD, cardiovascular diseases; D7, heptadeuterated; NPC1L1, NPC1 like intracellular cholesterol transporter 1; PLS, partial least squares; SNP, single nucleotide polymorphism; TC, total cholesterol; VIP, variable importance in the projection. A complete list of gene names and symbols can be found in **Supplemental Table 1**.

Keywords: cardiovascular diseases; blood lipids; genetic variation; absorption; nutrigenetics.

ABSTRACT

Scope: Cholesterol bioavailability displays a high interindividual variability, partly due to genetic factors. Existing studies have focused on single nucleotide polymorphisms (SNPs) analysed individually, which only explained a minor fraction of the variability of this complex phenotype. We aimed to identify a combination of SNPs associated with a significant part of the variability in cholesterol bioavailability.

Methods and results: Thirty-nine healthy adult males were given a standard test snack containing 80 mg heptadeuterated (D7) cholesterol. The plasma D7-cholesterol concentration was measured at equilibrium 40 h after test snack intake. The D7-cholesterol response (D7-cholesterol/total cholesterol concentration) exhibited a relatively high interindividual variability (CV=32%). The association of exonic SNPs in candidate genes (188 genes involved in or related to cholesterol metabolism) with the plasma D7-cholesterol response was assessed by univariate statistics followed by partial least squares regression. Data obtained allowed us to generate a significant model (p-value after cross-validation ANOVA = 1.64×10^{-7}) that included 8 SNPs (*SOAT2*-rs9658625, *DNAH11*-rs11768670, *LIPC*-rs690, *MVK*-rs2287218, *GPAM*-rs10787428, *APOE*-rs7412, *CBS*-rs234706 and *WRN*-rs1801196) which explained 59.7% of the variance in cholesterol bioavailability (adjusted R^2).

Conclusion: We here report a combination of SNPs associated with a significant part of the variability in dietary cholesterol bioavailability.

This trial was registered at http://www.clinicaltrials.gov as NCT02100774.

1. INTRODUCTION

Prolonged exposure to high blood cholesterol concentrations has been associated with an increased risk to develop cardiovascular diseases (CVD) later in life [1, 2], underlining the need for early detection of hypercholesterolemia and reasoned choice of relevant dietary and pharmacological treatment strategies. Patients with high intestinal cholesterol absorption, who might represent as much as 25% of the population, respond poorly to statin treatment, which inhibits the rate-limiting enzyme of cholesterol synthesis, and have a higher risk of recurrence of major coronary events [3]. In order to improve their outcome and minimize treatment cost [4], these patients should receive tailored dietary recommendations, *e.g.* plant sterols and stanols [5, 6] and/or lower dietary cholesterol intake [7], and if insufficient, pharmacological treatments [8], *e.g.* ezetimibe [9] or bile acid sequestrants [10]. Targeting of intestinal cholesterol absorption has recently been shown to be a valuable approach in CVD risk reduction. Indeed, the addition of ezetimibe, which inhibits NPC1 like intracellular cholesterol transporter 1 (NPC1L1), the main protein involved in the apical transport of cholesterol in the small intestine [11], to a statin therapy resulted in a further reduction of LDL-cholesterol (LDL-C) concentrations and cardiovascular event risk in patients with a recent acute coronary syndrome [12]. Recently, ezetimibe monotherapy was shown to lead to a decrease in the incidence of a composite of sudden cardiac death, myocardial infarction, coronary revascularization, or stroke in patients aged ≥75 years with elevated LDL-C concentration, together with a decrease in LDL-C concentration [13].

Although a healthy diet is widely acknowledged as a cornerstone of disease prevention and management [14], its optimal composition, in terms of specific foods or isolated nutrients, such as cholesterol, is still a matter of great debate. This is notably due to the fact that intestinal cholesterol absorption efficiency displays a high interindividual variability, with values ranging between 30 and 80% [15], assumed to be due to both dietary factors (*e.g.* dietary fat and cholesterol, fibres, plant sterols) and host-related factors [16], including the gut microbiota [17] and genetic factors [18, 19]. Although some SNPs involved in this process have been identified, the explained variability remains low since existing studies have focused on few candidate SNPs [20] or rare mutations [21]. Yet, this process is a complex phenotype, which is likely to involve numerous genes. We thus assumed that a significant part of its variability is explained by the additive effect of several genotypes which, taken individually, may have a small, barely significant effect. Our aim was thus to identify a combination of SNPs significantly associated with the interindividual variability in cholesterol bioavailability using D7-cholesterol as an acknowledged marker of cholesterol absorption.

2. SUBJECTS AND METHODS

2.1 Reagents

D7-cholesterol (25,26,26,26,27,27,27-H^7; 99% enrichment) was supplied by CDN (Pointe Claire, QC, Canada); epicoprostanol and methylsilyltrifluoroacetamide were from Sigma (Saint-Quentin-Fallavier, France); absolute ethanol, hexane and pyridine were from Carlo Erba Reagents S.A.S. (Peypin, France). All chemicals used in this study were of analytical grade.

2.2 Participants

Forty healthy, non-obese (BMI <30 kg/m²), non-smoking male adults were recruited for the study. Participants reported normal energy intake with <2% alcohol as total energy intake. Participants had no history of chronic disease, hyperlipidaemia, or hyperglycaemia and were not taking any medication known to affect lipid metabolism the month before the study or during the study period. Because of the relatively large volume of blood collected during the study, blood haemoglobin concentration >13 g/dL was an inclusion criteria. The study was approved by the regional committee on human experimentation (N°2008-A01354-51, Comité de Protection des Personnes Sud Méditerranée I, France). Procedures followed were in accordance with the Declaration of Helsinki of 1975 as revised in 1983. Objectives and requirements of the study were fully explained to all participants before beginning the study, and written informed consent was obtained from each subject. One subject left the study for personal reasons before he participated in the cholesterol-feeding experiment, which left 39 participants whose baseline characteristics are reported in **Table 1**.

2.3 Cholesterol-feeding trial

In order to compare cholesterol bioavailability between participants, plasma D7-cholesterol concentrations were measured 40 h after consumption of a standardized test snack containing D7-cholesterol [22]. After an 8 h fast, a baseline blood sample was taken from the participants via glass tubes containing EDTA-K_3 at the local Center for Clinical Investigation (Hôpital de la Conception, Marseille, France). Plasma was separated from whole blood by centrifugation (2000 *g* for 10 min at 20°C) and frozen at -20°C until further analysis. Eighty mg D7-cholesterol was incorporated as a powder in 25 g butter melted at 45°C in a microwave oven and the mixture was homogenized until solidification at room temperature. Participants then consumed a standardized test snack that comprised 25 g butter spread on 80 g of bread slices,

to which 30 g jam could be added, and were allowed to drink only water. Two days later, they were invited to return in the fasting state at the Center for Clinical Investigation where a blood sample was collected 40 h following the ingestion of D7-cholesterol. Indeed, it has been previously shown that the area under the curve of the plasma free cholesterol enrichment of a single isotope labelled cholesterol tracer was well correlated with the cholesterol absorption rate as measured by the plasma dual stable-isotope ratio method over a 24–96 h period following its intake [23]. Moreover, we have shown that the plasma D7-cholesterol concentration plateaued 24 h after consumption of a standardized test snack containing D7-cholesterol and that it remained at this equilibrium level up to 48 h after consumption of the standardized snack [22].

2.4 Chemical analyses

Fasting plasma total cholesterol (TC), HDL-cholesterol, LDL-C, triglyceride, glucose and haemoglobin concentrations were measured before consumption of the test snack with standard methods: analyte concentrations were measured on a Modular® PP instrument (Roche Diagnostics, Meylan, France), except for haemoglobin which was measured on an ADVIA 120® instrument (Siemens Healthcare Diagnostics, Saint-Denis, France) at La Conception Hospital (Biochemistry Laboratory, Marseille). All analyses were performed according to the manufacturer's instructions.

D7-cholesterol concentration was measured as previously described [24]. Briefly, 200 µL epicoprostanol (5 µmol/L), used as internal standard, was added to 200 µL plasma. After alkaline hydrolysis using 6.4 M KOH and extraction (water/hexane; 2/5 mL), samples were derivatised with a methylsilyltrifluoroacetamide/pyridine mixture (40/50 µL) for 60 min at 70°C. After evaporation, the dry residue was dissolved in 500 µL hexane and a volume of 2 µL was injected in a gas chromatography-mass spectrometer (Hewlett-Packard 6890 GC-5973 MS). Samples were analysed on a quadrupole mass spectrometer in Single Ion Monitoring mode (ions m/z 336 and m/z 370 were chosen for D7-cholesterol and epicoprostanol quantification respectively). Plasma D7-cholesterol enrichment was calculated by comparison to a standard curve with internal standard and was expressed as µmol D7-cholesterol/mmol cholesterol, as usual [25].

2.5 DNA preparation and genotyping methods

An average of 25 µg of DNA was isolated from saliva samples using the Oragene kit (DNA Genotek, Ottawa, Ontario, Canada). DNA preparation and genotyping methods were performed as previously described [26]. The whole genome was genotyped using

HumanOmniExpress BeadChips (Illumina), which allow for the analysis of ≈ 7.33×10^5 SNPs/DNA sample.

2.6 Choice of candidate genes and SNPs

Literature search in PubMed for genes whose SNPs have been shown to be associated with cholesterolemia variability and analysis of pathways involved in cholesterol absorption or metabolism resulted in the selection of 188 genes (**Supporting Information Table S1**). SNP selection is summarized in **Supporting Information Figure S1**. Due to the relatively small size of our study population, we narrowed our analysis to exonic SNPs (360 SNPs expressed on the arrays, out of 11747 in total; National Center for Biotechnology Information annotation as provided by Illumina). SNPs for which the genotype call rate was <95% and SNPs that presented a significant departure from the Hardy-Weinberg equilibrium (p<0.05; Chi-squared test) were excluded from all subsequent analyses (20 SNPs excluded), leaving 340 SNPs. SNPs were tested under both additive and dominant models [27-29]. The additive model assumes that there is a uniform, linear increase or decrease in the quantitative trait for each copy of the minor allele. For SNPs under the dominant model, participants homozygous for the lesser frequent allele were grouped with heterozygous participants and were compared with participants homozygous for the more frequent allele. SNPs with fewer than 5 observations in the minor group were excluded from further analysis, leaving 46 and 172 SNPs in the additive and dominant model, respectively. Moreover, for each candidate gene, when two SNPs were in perfect linkage disequilibrium (R^2=1), one was randomly excluded from further analysis, leaving 44 and 156 SNPs (in 76 genes) for statistical analysis in the additive and dominant model, respectively. Linkage disequilibrium information was retrieved using the LD TAG SNP Selection tool from the SNPinfo Web Server (https://snpinfo.niehs.nih.gov/snpinfo/snptag.html) considering genotype data from dbSNP in the European population.

2.7 Calculations and statistical analysis

Data were expressed as mean ± SEM. Normality was assessed using the Kolmogorov-Smirnov and the Shapiro-Wilk tests. Statistical dependence between two variables was assessed by Pearson's correlation coefficient. Differences in fasting TC concentration before and 40h after intake of the D7-cholesterol test snack were tested by paired t-test. For all tests, the bilateral alpha risk was α=0.05. Statistical analyses were performed using IBM SPSS Statistics (Version 20.0; Armonk, NY, U.S.A.).

Statistics: PLS regression

To identify SNPs associated with the variability observed in cholesterol bioavailability, we calculated the D7-cholesterol/TC ratio (*see* Results for an explanation), hereafter named the D7-cholesterol response. In a first approach, we performed univariate analyses to compare the D7-cholesterol response between subgroups of participants who bore different genotypes at the candidate SNPs, using Plink (v1.07, http://pngu.mgh.harvard.edu/purcell/plink/) [30]. SNPs that exhibited a p-value <0.05 (Wald test asymptotic p-value) were selected for partial least squares (PLS) regression analysis, which is the multivariate regression extension of principal component analysis [31], following previously published rationale and model assumptions [32, 33]. First, a PLS regression model including all selected SNPs coded in units of variance was built. Variables were ranked according to their variable importance in the projection (VIP) value and several PLS regression models were then built using increasing VIP threshold values. The model maximizing the adjusted explained variance (adjusted R^2) was selected (32). The adjusted R^2 was calculated according to Equation 1:

$$Adjusted\ R^2 = 1 - \frac{(1-R^2)(n-1)}{n-k-1} \quad \text{(Equation 1)}$$

With *n* the sample size and *k* the number of predictors in the model (excluding the constant). SIMCA-P13 software (Umetrics, Umeå, Sweden) was used for all multivariate data analyses and modelling. Additional validation criteria and procedures of the PLS regression models are described in **Supporting Information Methods**.

3. RESULTS

3.1 Plasma D7-cholesterol response of the participants

Fasting plasma TC concentration of the participants did not change over the course of the experiment (4.33 ± 0.16 *vs* 4.31 ± 0.14 mmol/L at t0 and t+40 h respectively; p=0.949, paired t-test). The plasma D7-cholesterol concentration of the participants at t+40 h did not significantly correlate with any of the reported characteristics of the participants, except with TC (Pearson's r=0.513; p=0.001) and LDL-C concentration (Pearson's r=0.638; p<0.001). Thus, in order to normalize the D7-cholesterol concentration to the cholesterol pool of each subject, we calculated the D7-cholesterol response as the D7-cholesterol/TC ratio (µmol/mmol) [25] (**Figure 1**). It followed a normal distribution (p=0.20 and p=0.91 following the Kolmogorov-Smirnov and the Shapiro-Wilk test respectively; skewness=0.221 and kurtosis=-0.209). The CV of the D7-cholesterol response was 32% and the ratio between the highest and the lowest responder was ≈ 4.9.

3.2 Genetic variants associated with the D7-cholesterol response

We measured the association between exonic SNPs and the interindividual variability in the D7-cholesterol response. Five and nine SNPs exhibited a significant p-value under the additive and dominant model, respectively (**Table 2**). Under the additive model, *WRN*-rs1801195 and *WRN*-rs1800392 were in strong linkage disequilibrium (R^2=0.945). Under the dominant model, the three SNPs in *DNAH11* were not in linkage disequilibrium (maximum R^2=0.307). Unstandardized regression coefficients (*B* coefficients), which represent the mean change in the D7-cholesterol response for each additional copy of the minor allele under the additive model and in the presence of the minor allele under the dominant model are also provided in **Table 2**.

3.3 Combination of genetic variants associated with the D7-cholesterol response

We first entered all fourteen significant SNPs into PLS regression analysis. From the first model thus obtained, SNPs were ranked by decreasing variable importance in the projection (VIP) value, which estimates the contribution of each SNP in the projection used in the PLS regression model, and thus in the studied phenotype. When two SNPs were in linkage disequilibrium (*i.e.* WRN-rs1801195 and WRN-rs1800392, R^2=0.95), the SNP exhibiting the lowest VIP value was excluded. When the same SNP was entered coded under both additive and dominant models (*i.e.* LIPC-rs690), the SNP under the genetic model with the lowest VIP value was excluded. The model obtained explained 63.8% of the variance (R^2), *i.e.* of the

interindividual variability of the D7-cholesterol response, with an adjusted R^2 of 0.491 and the R^2 after cross-validation was 0.521 (**Supporting Information Table S2** and **Supporting Information Methods**). To improve the model and find a combination of SNPs explaining a higher percentage of the variance in the D7-cholesterol response, we generated new models by sequentially excluding the SNP that displayed the lowest VIP value and we finally selected the model maximising the adjusted R^2 and the R^2 after cross validation (**Supporting Information Table S2**). The retained model included 8 SNPs, *SOAT2*-rs9658625, *DNAH11*-rs11768670, *LIPC*-rs690, *MVK*-rs2287218, *GPAM*-rs10787428, *APOE*-rs7412, *CBS*-rs234706, and *WRN*-rs1801196 (**Table 3** and **Figure 2**). It explained 68.2% of the variance (R^2) and had an adjusted R^2 of 0.597 and a R^2 after cross-validation of 0.580 (p-value after cross-validation ANOVA=1.64×10^{-7}) (**Table 3).** The robustness and the stability of the model was validated by three additional methods (**Supporting Information Methods, Table S3, Figure S2 and Figure S3**). With the knowledge of a participant genotype at the 8 SNPs in the selected model, it was possible to calculate the participant D7-cholesterol response using the following equation:

$$\mathrm{D7} = 1.222 + \sum_1^8 ri \times \text{number of minor allele} \quad \text{(Equation 2)}$$

with D7 the plasma D7-cholesterol response, r_i the unstandardized regression coefficient of the i^{th} SNP in the PLS regression model (provided in **Table 3**). When SNPs were entered under the dominant model, participants homozygous for the lesser frequent allele were grouped with heterozygous participants and the number of minor alleles for both these groups was considered to be 1.

4. DISCUSSION

In this study in 39 healthy adult males, using a multivariate statistical approach, we showed that the relatively high interindividual variability in intestinal cholesterol absorption efficiency (CV=32%) was significantly associated with a combination of 8 exonic SNPs in genes involved in lipid transport and metabolism (R^2=0.597). In order to estimate cholesterol bioavailability we measured the plasma concentration of a stable isotope-enriched cholesterol moiety, D7-cholesterol, ingested together with a standardized test snack. To reach the general circulation, cholesterol is first packed by the small intestine into chylomicrons, which are secreted into the lymph during several sequential meals [22]. To account for this kinetics, plasma D7-cholesterol concentration was measured 40 h after its intake, *i.e.* at its steady state in the plasma, as previously shown [15, 23, 24, 34]. Several methods exist to assess intestinal cholesterol absorption, as reviewed elsewhere [35]. The use of a single stable isotope [23, 36] offers the advantage of being direct (which is not the case when using plant sterol as a marker), safe (especially against radioisotopic methods), relatively non-invasive (a single blood collection is required), it does not require stool collection and its steady-state plasma concentration correlates well with intestinal cholesterol absorption when compared with sophisticated radioisotopic methods [23]. However, its relatively high price limits its applicability to small studies.

The first noteworthy observation was the partial positive correlation between the D7-cholesterol concentration of the participants and their fasting LDL-C concentration, an independent risk factor for CVD [37]. This is in agreement with results from a random sample in the Finnish population, where cholesterol fractional absorption was measured by the peroral double-isotope continuous cholesterol-betasitosterol feeding method [38], and with results from the *Ludwigshafen Risk and Cardiovascular health* study, where cholesterol absorption was estimated by measuring plasma plant sterol concentrations [39]. Nonetheless, the effect of a high cholesterol absorption phenotype on CVD is not solely mediated by its effect on LDL-C concentration since other studies have shown a significant association between high cholesterol absorption and CVD while LDL-C concentrations remained unchanged [3, 40]. This highlights the relevance of the identification of the absorber/synthesizer phenotype in primary or secondary prevention of CVD, beyond simple CVD risk estimation based on LDL-C concentration [8].

The complexity of the relationship between intestinal cholesterol absorption and LDL-C concentration might be due to the complexity of cholesterol metabolism. Indeed, circulating cholesterol originates both from *de novo* synthesis and from dietary intakes, which vary widely between individuals according to their own *de novo* synthesis and their dietary habits. In addition, cholesterol *de novo* synthesis and cholesterol absorption exhibit a reciprocal relationship [38, 41]. It should also be stressed that cholesterol absorbed at the intestinal level has two origins, namely dietary cholesterol and cholesterol from bile, which is actually the main contributor to the intraluminal cholesterol pool [18]. Finally, cholesterol absorption displays a high interindividual variability [15]. Our study confirms this key trait since the CV of the D7-cholesterol response was 32% herein, in line with that observed by Bosner *et al.* in thirty-two healthy males (CV of 21% as calculated by the present authors) [15]. Although it is well established that an increase in dietary cholesterol leads on average to a relatively modest increase in LDL-C [42, 43], whether it translates into increased CVD risk remains a matter of great debate. A meta-analysis of forty studies and nineteen trials did not reveal any significant association with CVD risk [42]. Recently, two large studies reported opposing results concerning the association between egg intake and LDL-C concentrations or CVD risk [44, 45]. Although the discrepancy in results can be due to errors inherent to food frequency questionnaires in epidemiological studies, another possible explanation is unadjusted residual confounding. It is fairly conceivable that there could be a significant effect of the interaction of the cholesterol absorber/synthesizer phenotype with the impact of dietary cholesterol intakes on LDL-C concentration and thus CVD risk. If verified, this hypothesis could lead to personalized dietary recommendations, based on this phenotype, targeting either cholesterol absorption or synthesis in order to decrease CVD risk.

We showed that the interindividual variability in intestinal cholesterol absorption efficiency is notably due to genetic variations and that a combination of 8 exonic SNPs is significantly associated with this variability. Following candidate SNPs approaches, a few genetic variations have been previously reported [46], namely SNPs in *ABCG5* and *8* [20, 47], *ABO* [20], *NPC1L1* [48] or *APOE* [49]. Since cholesterol bioavailability is a complex process that involves numerous genes [18], a thorough study of the association between candidate genetic variants and this phenotype should ideally include SNPs in all genes that are assumed to be involved in this phenotype. Following thorough pathway analysis and literature search, we selected 188 candidate genes (**Supporting Information Table S1**), which allowed us to investigate the effect of 156 exonic SNPs on cholesterol absorption efficiency. The results of

our statistical analysis first allowed the identification of 14 SNPs associated individually with the variability of the D7-cholesterol response between the study participants. We then applied a multivariate regression analysis, PLS regression, to determine which combination of these SNPs could maximize the explained variance of the studied phenotype. Our analysis, whose validity was checked by several tests, showed that a combination of 8 SNPs was significantly associated with the interindividual variability in the D7-cholesterol response (adjusted R^2 = 0.597). For *LIPC*-rs690, *GPAM*-rs10787428, *APOE*-rs7412 and *WRN*-rs1801196, the presence of the minor allele was associated with an increase in intestinal cholesterol absorption efficiency, as illustrated by a positive regression coefficient, while for *SOAT2*-rs9658625, *DNAH11*-rs11768670, *MVK*-rs2287218 and *CBS*-rs234706, the presence of the minor allele was associated with a decrease in intestinal cholesterol absorption efficiency, as illustrated by a negative regression coefficient (**Table 2**).

SOAT2 encodes for sterol O-acyltransferase 2 but is also known under the alternative name *ACAT2* (acyl-Coenzyme A: cholesterol acyltransferase 2; which is different from acetyl-Coenzyme A acetyltransferase 2, *ACAT2*). SOAT2 is a membrane-bound protein, mainly localized in enterocytes, that converts free cholesterol to cholesteryl esters for they packaging into chylomicrons [50] and whose deficiency in mice has been shown to lead to a decrease in cholesterol absorption efficiency and resistance to diet-induced hypercholesterolemia [51]. Yoshida *et al.* reported that the minor allele of *SOAT2*-rs9658625 was protective against chronic kidney disease in Japanese individuals with hypertension [52]. Since patients with chronic kidney disease have been shown to exhibit higher cholesterol absorption efficiency compared to healthy individuals [53], the negative association we find here between *SOAT2*-rs9658625 and cholesterol absorption is in agreement with results from Yoshida *et al.*. In addition, this SNP has been reported to be protective against dyslipidaemia in healthy Chinese [54].

For *DNAH11* and *GPAM*, no precise biological mechanisms in relation to cholesterol metabolism are known but these genes were included as candidate genes following the association of some of their variants with circulating cholesterol concentration in a genome-wide association study [55].

Hepatic lipase, encoded by the *LIPC* gene, is involved in the metabolism of HDL: by hydrolysing HDL triglycerides and phospholipids, it promotes the generation of smaller, denser HDL particles, which increase cholesterol efflux [56] and could thus also increase cholesterol reverse transport [57, 58]. There is no previous report of an association of *LIPC*-rs690 with circulating TC or LDL-C concentrations.

MVK is involved in *de novo* cholesterol synthesis [59]. One case-control study showed that *MVK*-rs2287218 is associated with an increased risk of coronary heart disease and ischemic stroke in a Chinese population, together with a slight decrease in HDL-cholesterol concentration in the controls [60]. Since there is an inverse relationship between cholesterol absorption and synthesis [41], we can hypothesize that the increase in *de novo* cholesterol synthesis suggested by this study could be related to a concomitant decrease in cholesterol absorption, as shown by the negative regression coefficient in our regression model.

APOE encodes for apolipoprotein E, which plays a critical role in the intravascular transport and blood clearance of lipids. ApoE exists as three major isoforms, ApoE2, ApoE3 and ApoE4, which are the products of two SNPs, rs7412 and rs429358 [61]. Here, we show that *APOE*-rs7412 SNP, which determines the APOE2 isoform, is associated with an increase in the D7-cholesterol response. Results from other studies have yielded fairly conflicting results concerning the effect of APOE on cholesterol absorption [39, 49, 62].

CBS encodes for cystathionine beta-synthase, an enzyme that catalyses the conversion of homocysteine to cystathionine, the first step in the transsulfuration pathway. *CBS*-rs234706 was found to be associated with an increase in TC and LDL-C concentrations in participants from the *Malmö Diet and Cancer Cardiovascular Cohort* [63]. There is no known biological mechanism in relation to cholesterol metabolism although it has been linked to altered lipoprotein metabolism in mice [64] and expression levels of key enzymes involved in cholesterol synthesis in ovarian cancer cell lines [65].

WRN was selected as a candidate gene because a SNP in this gene was reported to affect cholesterol efflux *in vitro* and to correlate with cholesterol concentrations *in vivo* [66].

Of note, we did not find any associated SNP in *NPC1L1*, which encodes for the main intestinal apical transporter of cholesterol and for which inactivating mutations have recently been associated with lower LDL-C concentrations and protection from CVD [21]. In fact, these mutations are rare, the most frequent having a minor allele frequency of only 0.02% (meaning one in every 5000 individuals is a heterozygous carrier), and were thus not present on the Beadchips (which usually have SNPs with minor allele frequency >1%).

We acknowledge some limitations of the study. It is likely that other SNPs that have a significant effect on cholesterol bioavailability were not entered in the statistical analysis because they are not present on the BeadChips, they were excluded from the analysis, or simply because they are located in other genes than the selected ones. Moreover, these findings need to be tested in other populations of greater size (based on *e.g.* sex, age, or genetic backgrounds).

To conclude, our results show that, in this sample of healthy adult males, the relatively high interindividual variability in cholesterol bioavailability, is significantly associated with a combination of 8 exonic SNPs (*SOAT2*-rs9658625, *DNAH11*-rs11768670, *LIPC*-rs690, *MVK*-rs2287218, *GPAM*-rs10787428, *APOE*-rs7412, *CBS*-rs234706 and *WRN*-rs1801196) located in genes involved in lipid metabolism and transport. Future studies based on the present data and model, as well as identification of other genes/SNPs involved in cholesterol bioavailability, will contribute to improve the quality of this association, with the ultimate objective to provide clinicians with an accurate and validated genetic tool to predict cholesterol bioavailability and thus provide them with additional information in order to personalize dietary/pharmacological treatment strategy to prevent CVD.

Author contributions

PB, EW and DL designed the research; MN and EW conducted the clinical research; EW and AN analysed D7-cholesterol by GC-MS; CDes, RV, MCA, PEM and PB analyzed data; CDes and CDef performed statistical analyses; CDes and PB wrote the paper and had primary responsibility for final content of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors have declared no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Plasma D7-cholesterol response 40 h after the ingestion of a D7-cholesterol-rich test snack.

Participants were sorted by increasing plasma D7-cholesterol response, *i.e.* the ratio plasma D7-cholesterol concentration/fasting plasma TC concentration.

Figure 2. D7-cholesterol response according to the genotype at the 8 SNPs in the selected Partial Least Squares regression model.

Corresponding p-values can be found in **Table 2**.

TABLES

Table 1. Participant characteristics.[a]

Characteristic	Mean
Age (y)	33.1 ± 2.1
Weight (kg)	73.5 ± 1.3
BMI (kg/m²)	22.9 ± 0.3
Total cholesterol (mmol/L)[b]	4.3 ± 0.1
HDL-cholesterol (mmol/L)[b]	1.2 ± 0.0
LDL-cholesterol (mmol/L)[b]	2.8 ± 0.1
Triglycerides (mmol/L)[b]	0.8 ± 0.1
Glucose (mmol/L)[b]	4.8 ± 0.1
Haemoglobin (g/dL)[b]	15.1 ± 0.1

[a]n = 39.

[b]Fasting plasma variables.

Table 2. Genes and SNPs significantly associated with the plasma D7-cholesterol response following univariate analysis.[a,b]

Gene[c]	SNP rs	Alleles	Minor allele Frequency[d]	Coding status	Unstandardized regression coefficient[e]	p-value[e]
Additive model[f]						
LIPC	rs690	G>T	G=0.427		0.213 ± 0.083	0.015
GPAM	rs10787428	T>C	T=0.417	NonSyn[g]	0.229 ± 0.100	0.028
WRN	rs1801195	G>T	T=0.449	NonSyn	0.205 ± 0.097	0.042
WRN	rs1800392	G>T	T=0.452		0.194 ± 0.095	0.049
Dominant model[h]						
SOAT2	rs9658625	A>G	G=0.073	NonSyn	-0.468 ± 0.165	0.007
DNAH11	rs11768670	G>A	A=0.223	NonSyn	-0.320 ± 0.121	0.012
MVK	rs2287218	C>T	T=0.176		-0.299 ± 0.128	0.025
LIPC	rs690	G>T	G=0.427		0.307 ± 0.133	0.027
APOE	rs7412	C>T	T=0.087	NonSyn	0.340 ± 0.153	0.032
CBS	rs234706	G>A	A=0.328		-0.278 ± 0.126	0.033
WRN	rs1801196	C>T	T=0.291		0.271 ± 0.124	0.036
DNAH11	rs6965750	G>A	A=0.140	NonSyn	-0.274 ± 0.130	0.041
DNAH11	rs9639393	A>G	A=0.396		-0.255 ± 0.125	0.048

[a] Plasma D7-cholesterol response represents the ratio plasma D7-cholesterol concentration/fasting plasma TC concentration.

[b] SNPs are ranked by decreasing p-values.

[c] See **Supporting Information Table S1** for gene names.

[d] Minor allele frequency were retrieved from dbSNP (https://www.ncbi.nlm.nih.gov/snp/) using the Allele Frequency Aggregator (ALFA) dataset (pooled allele frequency data from dbSNP and the dbGaP) in the global population.

[d] Unstandardized regression coefficients (B coefficients) represent the mean change in the D7-cholesterol response for each additional copy of the minor allele under the additive model and in the presence of the minor allele under the dominant model.

[e] Wald test asymptotic p-value.

[f] The additive model assumes that there is a uniform, linear increase or decrease in the quantitative trait for each copy of the minor allele.

[g] NonSyn: non synonymous variation. All SNPs were otherwise coding variants (*i.e.* located in exons).

[h] For SNPs under the dominant model, participants homozygous for the lesser frequent allele were grouped with heterozygous participants and were compared with participants homozygous for the more frequent allele.

Table 3. Combination of SNPs associated with the plasma D7-cholesterol response.[a]

Gene[b]	**SNP**	**VIP value**	**Regression coefficient**[c]
SOAT2	rs9658625	1.143	-0.292
DNAH11	rs11768670	1.084	-0.200
LIPC	rs690[d]	1.051	0.133
MVK	rs2287218	0.971	-0.187
GPAM	rs10787428[d]	0.955	0.143
APOE	rs7412	0.931	0.213
CBS	rs234706	0.927	-0.174
WRN	rs1801196	0.913	0.169

[a] Variables were ranked by decreasing variable importance in the projection (VIP) value, which estimates the contribution of each SNP in the projection used in the PLS regression model, and thus in the studied phenotype.

[b] See **Supporting Information Table S1** for gene names.

[c] Regression coefficient are for untransformed variables and represent the mean change in the D7-cholesterol response for each additional copy of the minor allele under the additive model and in the presence of the minor allele under the dominant model.

[d] These SNPs were entered under the additive model.