Papers in Press. Published November 20, 2014 as doi:10.1373/clinchem.2014.231365



# Refinement of Variant Selection for the LDL Cholesterol Genetic Risk Score in the Diagnosis of the Polygenic Form of Clinical Familial Hypercholesterolemia and Replication in Samples from 6 Countries

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BACKGROUND: Familial hypercholesterolemia (FH) is an autosomal-dominant disorder caused by mutations in 1 of 3 genes. In the 60% of patients who are mutation negative, we have recently shown that the clinical phenotype can be associated with an accumulation of common small-effect LDL cholesterol (LDL-C)-raising alleles by use of a 12–single nucleotide polymorphism (12-SNP) score. The aims of the study were to improve the selection of SNPs and replicate the results in additional samples.

METHODS: We used ROC curves to determine the optimum number of LDL-C SNPs. For replication analysis, we genotyped patients with a clinical diagnosis of FH from 6 countries for 6 LDL-C-associated alleles. We compared the weighted SNP score among patients with no confirmed mutation (FH/M–), those with a mutation (FH/M+), and controls from a UK population sample (WHII).

RESULTS: Increasing the number of SNPs to 33 did not improve the ability of the score to discriminate between FH/M– and controls, whereas sequential removal of SNPs with smaller effects/lower frequency showed that a weighted score of 6 SNPs performed as well as the 12-SNP score. Metaanalysis of the weighted 6-SNP score, on the basis of polymorphisms in *CELSR2* (cadherin, EGF LAG 7-pass G-type receptor 2), *APOB* (apolipoprotein B), *ABCG5/8* [ATP-binding cassette, sub-family G (WHITE), member 5/8], *LDLR* (low density lipoprotein receptor), and *APOE* (apolipoprotein E) loci, in the independent FH/M– cohorts showed a consistently higher score in comparison to the WHII population ($P < 2.2 \times 10^{-16}$). Modeling in individuals with a 6-SNP score in the top three-fourths of the score distribution indicated a >95% likelihood of a polygenic explanation of their increased LDL-C.

CONCLUSIONS: A 6-SNP LDL-C score consistently distinguishes FH/M– patients from healthy subjects. The hypercholesterolemia in 88% of mutation-negative patients is likely to have a polygenic basis.



Familial hypercholesterolemia (FH),[16] in its classic form, appears to be an autosomal-codominant disorder, characterized by increased plasma concentrations

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Received October 9, 2014; accepted October 16, 2014.
Previously published online at DOI: 10.1373/clinchem.2014.231365

[16] Nonstandard abbreviations: FH, familial hypercholesterolemia; LDL-C, LDL cholesterol; CHD, coronary heart disease; PFH, possible FH; DFH, definite FH; GWAS, genome-wide association study; SNP, single nucleotide polymorphism; WHII, Whitehall II; FH/M–, mutation-negative familial hypercholesterolemia; AIC, Akaike information criterion; BIC, Bayesian information criterion; FH/M+, mutation positive familial hypercholesterolemia; TC, total cholesterol; MEDPED, Make Early Diagnosis Prevent Early Death.

of LDL cholesterol (LDL-C) and premature symptoms of coronary heart disease (CHD) *(1)*. The prevalence of heterozygous FH is 0.2%–0.5% *(1, 2)*, with a higher prevalence in some populations due to founder effects *(2)*. Worldwide, 14–34 million people are thought to be affected with heterozygous FH, of whom at least 95% are undiagnosed *(2)*. Clinical diagnostic systems for FH have been developed in the UK *(3)*, the US *(4)*, and the Netherlands *(5)*. On the basis of the degree of increase in LDL-C concentrations (typically >189 mg/dL or >4.9 mmol/L in adults) and a family history of early CHD and/or increased cholesterol concentrations, such patients are given a diagnosis of possible FH (PFH). The additional presence of clinical features, such as tendon xanthomas, results in a diagnosis of definite FH (DFH). When patients carry variants deemed to be pathogenic, they also receive DFH as diagnosis. The usefulness of a molecular test to provide an unequivocal diagnosis is becoming increasingly appreciated, in particular to enhance unambiguous identification of affected relatives *(6)*. Early identification of at-risk individuals allows changes in lifestyle including dietary intervention and drug treatment, usually with one of the statin class of lipid-lowering agents, which have been shown to significantly reduce coronary atherosclerosis *(7)* and improve life expectancy *(8, 9)*.

Since 2008, several guidelines for the identification and management of patients with FH have been published *(10–13)*. Although they differ in detail and emphasis, there are several common threads (reviewed in *(14)*), including the utility of genetic testing to confirm the diagnosis and apply it in cascade testing of the relatives, which is a cost-effective approach to find new cases *(15–17)*. Cascade testing has been used extensively in several countries in Europe, most notably in the Netherlands *(6)*, where it has resulted in the identification of 67% of FH patients with an assumption of 1:450 prevalence *(18)*, which is probably underestimated *(19, 20)*. The UK guidelines *(10)* state that cascade testing of first-degree relatives of every FH proband should be carried out where a mutation has been identified in the proband, or if no mutation can be identified, on the basis of LDL-C measures. However, in the Netherlands, cascade testing is carried out only in families in which a mutation has been identified *(6)*, and this approach is also being adopted in Wales *(21)*.

FH is caused by mutations in *LDLR* (low density lipoprotein receptor),[17] *APOB* (apolipoprotein B), or *PCSK9* (proprotein convertase subtilisin/kexin type 9) *(1, 2)*. The most common class of genetic defect is a mutation in *LDLR*, and currently >1200 mutations have been reported worldwide (http://www.ucl.ac.uk/fh) *(22)*. Even with exhaustive screening, in a small proportion of DFH subjects (10%–15%) with tendon xanthomas and a larger proportion of PFH patients (60%–75%), no mutation can be found (e.g., *(23)*). This may be for several reasons, for instance: failure to detect all DNA changes present by use of current methods, the mutations being in genetic regions that are not currently covered (e.g., introns), or the mutations being in genes that are yet to be identified as causing FH. However, the most likely reason is the inclusion of non-FH patients (i.e., a clinical false-positive diagnosis).

In 2010, metaanalysis of genome-wide association study (GWAS) data identified 95 loci involved in determining lipid concentrations *(24)*, and we have used a 12–single nucleotide polymorphism (12-SNP) LDL-C genetic risk score (the weighted sum of the LDL-C-raising alleles, where weights are the effect sizes from GWAS) as an unbiased genetic instrument for Mendelian randomization studies *(25)*. Compared with >3000 subjects from the UK population–based Whitehall II (WHII) study, the weighted LDL-C-raising SNP score frequency distribution among UK FH patients with no identified mutation (FH/M–) was significantly higher ($P = 4.5 \times 10^{-16}$), an effect that was confirmed in a cohort of similar patients from Belgium. This strongly suggests that a substantial proportion of FH/M– patients (up to 20%) are likely to have a polygenic cause of the increase in LDL-C rather than an as yet unknown single-gene mutation. Cascade testing is likely to be less effective in such cases, since fewer than the predicted 50% of first-degree relatives will have inherited enough of the polygenes to have concentrations of LDL-C above the diagnostic threshold *(26)*.

In the current article, we have examined the possibility of using additional SNPs to improve discrimination and fewer SNPs to reduce genotyping costs; we have examined the utility of the LDL-C SNPs score in an additional 7 cohorts (from 6 countries) of patients with a clinical diagnosis of FH; and we have estimated the likelihood of having a polygenic (as opposed to a monogenic) cause of hypercholesterolemia.

## Methods

### SELECTION AND GENOTYPING OF LDL-C GENETIC RISK SCORE SNPs

We performed LDL-C genetic score variant selection analysis with the WHII cohort *(25)* and the FH/M– patients (n = 175) (Oxford familial hypercholesterol-

[17] Human genes: *LDLR*, low density lipoprotein receptor; *APOB*, apolipoprotein B; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *CETP*, cholesteryl ester transfer protein, plasma; *CELSR2*, cadherin, EGF LAG 7-pass G-type receptor 2; *ABCG5/8*, ATP-binding cassette, sub-family G (WHITE), member 5/8; *APOE*, apolipoprotein E.

emia study, *(27)* and *(28)*). We added 21 additional meta-GWAS LDL-C-associated SNPs (see Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue1) *(24)* to the original 12-SNP score. Genotypes were obtained with the Metabochip (Illumina) genotyping array. We included all SNPs in regression models for LDL-C and used the best-fitting sets of SNPs determined by the lowest Akaike information criterion (AIC) and Bayesian information criterion (BIC) to construct 2 additional scores. We assessed the discriminatory ability of these scores using the area under the ROC. The original 12 SNPs in the score were ranked by their frequency and effect size, and the top 6 SNPs were selected for score calculations. We used ROC curves to evaluate the sensitivity/specificity of using a 12- vs 6-SNP score in discriminating between a general population and FH patients with no mutation (performed on cohorts from the original study *(25)*).

We performed genotyping with KASPar PCR TaqMan assays (Life Technologies) and genotype calling with an automated system, the results of which were checked manually by study personnel with SNPviewer software. One SNP (rs4299376) could not be genotyped by TaqMan, and a proxy was used instead (rs6544731).

#### PATIENT COHORTS

We collected 7 independent cohorts of patients diagnosed with FH. Informed written consent was obtained from all subjects, and the study was approved by ethics committees in each county. The biggest cohort comprised 638 Dutch adults, which included 66 mutation-positive (FH/M+) and 572 FH/M– patients. Other cohorts included 128 Greek children (68 FH/M+, 60 FH/M–), 22 Dutch children (all mutation FH/M–), 76 adults from Canada (39 mutation FH/M+, 37 FH/M–), 202 adults from Italy (144 FH/M+, 58 FH/M–), 29 adults from Poland (14 FH/M+, 15 FH/M–), and 63 adults from Israel (20 FH/M+, 43 FH/M–). All individuals were of white background. All subjects had an autosomal-dominant mode of inheritance of hypercholesterolemia in the family; the presence of primary hypercholesterolemia [total cholesterol (TC) ≥290 mg/dL or ≥7.5 mmol/L (or TC ≥259 mg/dL or ≥6.7 mmol/L for children <16 years of age)] in the proband or proband's first-degree relative; plasma or serum LDL-C ≥189 mg/dL or ≥4.9 mmol/L; and family history of coronary artery disease at <55 years for men and <60 years for women in a first-degree relative. In addition, some subjects had a personal or a family history of tendon and cutaneous xanthomas. Patients from Israel were clinically diagnosed with the Make Early Diagnosis Prevent Early Death (MED-PED) criteria *(4)*. The FH mutation detection methods varied slightly; however, they all included screening of the entire coding region of *LDLR*. The samples were also tested for *APOB* p.R3527Q (apart from the Greek cohort, since the mutation has never been found in Greece) and *PCSK9* p.D374Y.

#### SNP SCORE CALCULATIONS IN REPLICATION COHORTS

The LDL-C SNP score was calculated with weighted sums for the 6 selected SNPs. We used a group of 3020 healthy individuals (participants of the WHII study *(29)*) for comparison (baseline characteristics of WHII are shown in online Supplemental Table 2).

#### ESTIMATING THE PROBABILITY OF A POLYGENIC CAUSE

Given an individual who is diagnosed with FH but for whom no causal mutation has been found in the known FH genes, we assume that the LDL-C is >189 mg/dL or 4.9 mmol/L either because of an unknown single-gene mutation or a polygenic cause. For such individuals, we can use the equation below to calculate the probability of a polygenic cause (explained further in online Supplemental Methods):

$$P(x = -ve|LDL > 189, g, m = -ve) = \frac{P(LDL > 189|x = -ve, g) \cdot P(x = -ve|m = -ve)}{\Sigma_x P(LDL > 189|x, g) \cdot P(x|m = -ve)}$$

The relative probability of these 2 causes depends on the frequency of unknown single gene mutations and the probability distribution of the polygenic effects. Given these, it is straightforward to work out the probability of a polygenic cause given an individual's mutational status at the known FH genes, LDL-C measurement, and polygenic score. However, we do not know either the true polygenic score (since not all the LDL-C genes have been found) or the frequency of unknown single-gene mutations (by definition). Here we approximate the polygenic term by use of the effects of the 6-SNP score in WHII individuals and calculate the probability for several different unknown mutation frequencies (0, 0.001, 0.005, 0.01). Note that, if we assume the frequency of confirmed FH is 1/500 = 0.002, when we have found all of the *LDLR*/*APOB*/*PCSK9* mutations, the prevalence of undetected monogenic mutations must be <0.002. Also note that use of the 6-SNP genetic risk score underestimates the role of the polygenic component and so will underestimate the probability of a polygenic cause.

## Results

#### PATIENT BASELINE CHARACTERISTICS

The baseline characteristics of the FH patients included in this study are shown in online Supplemen-

tal Table 3. Overall, in all cohorts where data was available, FH/M+ patients had higher pretreatment TC and LDL-C than FH/M– patients from the same cohort.

### VARIANT SELECTION

We first attempted to improve the performance of the SNP score by including 21 additional SNPs (online Supplemental Table 1), previously identified by the Global Lipid Genetics Consortium GWAS metaanalysis as influencing LDL-C *(24)*. To maintain a high specificity for LDL-C, we had originally included SNPs whose only or major effect was on LDL-C and not on another lipid trait, but for this analysis the additional genes [e.g., *CETP* (cholesteryl ester transfer protein, plasma)] included affected lipid traits other than LDL-C.

Addition of these 21 LDL-C-raising SNPs did not significantly improve the ability of the SNP score to discriminate between FH/M– and healthy subjects ($A_{ROC}$ = 0.673, 95% CI 0.632–0.715, $P$ = 0.98) (see online Supplemental Fig. 1). BIC analysis selected 13 SNPs for the score (6 SNPs from the original 12-SNP score and 7 GWAS SNPs of the additional 21) (see online Supplemental Table 4). AIC analysis selected a 25-SNP set (composed of 8 SNPs from the original 12 SNPs and 17 SNPs from the additional 21) (see online Supplemental Table 5). Neither BIC nor AIC SNP selections improved the performance of the 12-SNP score (see online Supplemental Fig. 1). After this, the sequential removal of SNPs of smaller effects and/or lower minor allele frequencies showed that a weighted score of 6 SNPs performed as well as the 12-SNP score ($P$ = 0.16) (Fig. 1). Thus, to improve the cost-efficiency of the study, the SNP score calculations in the replication cohorts were based on genotypes of 6 SNPs [nearby gene]: rs629301 [*CELSR2* (cadherin, EGF LAG 7-pass G-type receptor 2)], rs1367117 [*APOB*], rs6544713 [proxy of rs4299376, *ABCG5/8* (ATP-binding cassette, sub-family G (WHITE), member 5/8)], rs6511720 [*LDLR*], rs429358 [*APOE* (apolipoprotein E)], and rs7412 [*APOE*], summarized in online Supplemental Table 6. Genotypes for the 6-SNP score were available in a total of 351 FH/M+ and 807 FH/M– patients.

### LDL-C SNP SCORE

Overall, the FH/M– group had the highest mean LDL-C SNP score (0.708), followed by the FH patients with an identified mutation (FH/M+) (0.656). The control WHII cohort had the lowest weighted score (0.632), which was significantly lower than the FH/M– ($P < 2.2 \times 10^{-16}$) and the FH/M+ ($P$ = 0.04) cohorts (see online Supplemental Fig. 2). Among the FH/M– patient cohorts, the highest LDL-C SNP score was observed in Dutch children (0.782) followed by Greek children (0.731) (see online Supplemental Table 7). Among FH/M– patients, 707 (88%) of had a score above the first quartile, of whom 288 (36% of the whole FH/M– cohort) had a score that fell within the top quartile of the WHII LDL-C SNP score distribution.

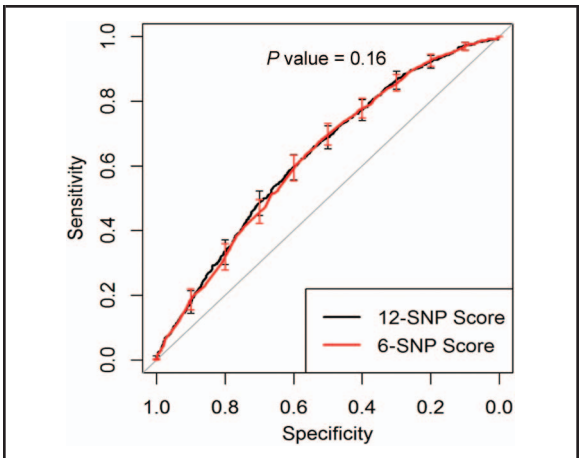


**Fig. 1. $A_{ROC}$ analysis of the discrimination between healthy individuals and FH-M/– patients by use of 12-SNP vs. 6-SNP LDL-C score.**

There was no significant specificity/sensitivity difference between the scores.

The FH/M+ patients were divided into *LDLR* mutation carriers (n = 323), *APOB* p.R3527Q (n = 13) carriers, and *PCSK9* p.D374Y carriers (n = 2). Patients who had the *APOB* p.R3527Q mutation had significantly lower LDL-C SNP score than patients with other mutations (0.521 vs 0.661, $P$ = 0.05) (Fig. 2).

LDL-C SNP score results for each of the 7 cohorts genotyped in this study were combined with 2 large cohorts (from the UK Simon Broome register and from Belgium) analyzed in the original study *(25)*, for a metaanalysis, shown in Fig. 3. Again the effect in all cohorts was highly consistent, and the overall standardized mean difference for all FH/M– groups compared with the WHII sample was 0.381 (95% CI 0.328–0.433).

### ESTIMATION OF THE PROPORTION OF FH/M– SUBJECTS LIKELY TO BE POLYGENIC BY SNP SCORE

For clinical utility, it would be valuable to estimate the probability that the increased LDL-C seen in an FH/M– individual can be explained by their weighted 6-SNP score. The first estimate needed for this calculation is the underlying rate of undetected monogenic mutations in FH/M– subjects. On the basis of the lack of novel genes causing FH reported to date, and in our whole exome next-generation sequencing data of 70

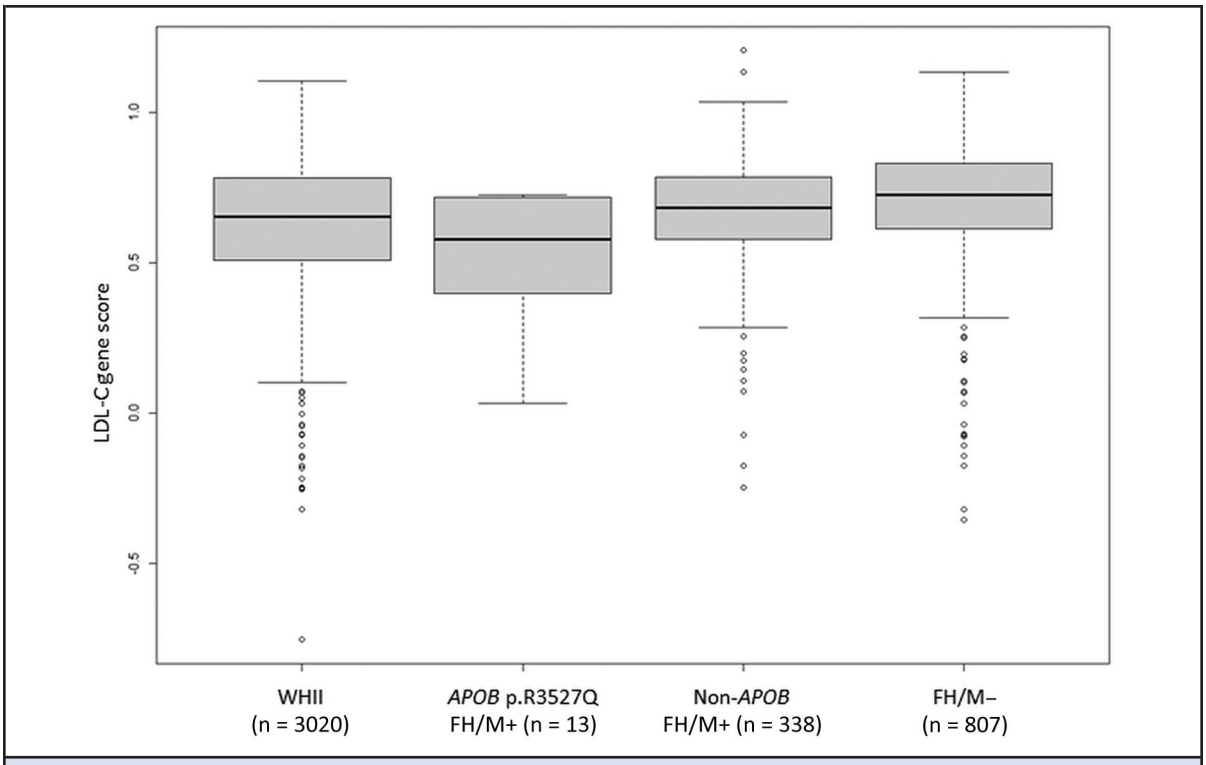


**Fig. 2. Boxplot of the LDL-C-weighted SNP score in WHII control cohort and patients groups.**
The patients who carried the *APOB* p.R3527Q mutation (n = 13) had significantly lower SNP scores than patients with *LDLR* and *PCSK9* mutations (0.521 vs 0.661, $P = 0.05$).

FH no-mutation patients, which also failed to identify a novel common FH-causing gene *(30)*, this is a reasonable estimate. By contrast, if we have identified only 75% of all mutations to be found, the frequency of the remaining undetected mutations would be 0.0005, and this seems likely to be the upper limit of undetected mutations. At an undetected mutation frequency of 0.0005, our analysis, shown in Fig. 4, suggests that the probability of a polygenic cause for LDL >189 mg/dL (4.9 mmol/L) in all the assessed FH/M– individuals is >95%, and it goes down when the frequency of undetected monogenic cause increases (see online Supplemental Fig. 3).

## Discussion

The LDL-C SNP score analysis in 7 independent cohorts consistently confirmed the findings reported by Talmud et al. *(25)*, that patients with a clinical diagnosis of FH but with no identified mutation (FH/M–) have a significantly higher mean LDL-C-raising SNP score than individuals from the general population (combined sample $P < 2.2 \times 10^{-16}$), which suggests that their high plasma LDL-C concentrations are considerably influenced by polygenes. In addition, as previously reported, FH patients who carry an FH-causing mutation (FH/M+) also had higher mean LDL-C SNP scores than the WHII cohort ($P = 0.04$), which confirms results from Talmud et al. *(25)* and suggests that in at least some cases the FH phenotype is being caused by the combination of a single mutation of large effect and several LDL-C-raising alleles of modest effect. This result could help to explain the variability in penetrance of certain FH mutations in the relatives of FH probands. When analyzing the mutation-positive patients by the mutated gene, patients with the defective *APOB* (due to the p.R3527Q mutation) had the lowest SNP score (0.521) among all studied groups. This suggests that the *APOB* mutation is highly penetrant, which is contradictory to what has been shown previously *(31)* and may reflect sample bias in this selected group of FH patients. Another explanation is that not all *LDLR* variants identified in the FH/M+ group are truly pathogenic, which leads to misclassification. The highest LDL-C SNP score was observed in the 2 mutation-negative hypercholesterolemic children cohorts (1 from the Netherlands and 1 from Greece), showing for the first time that the SNP score discrimi-

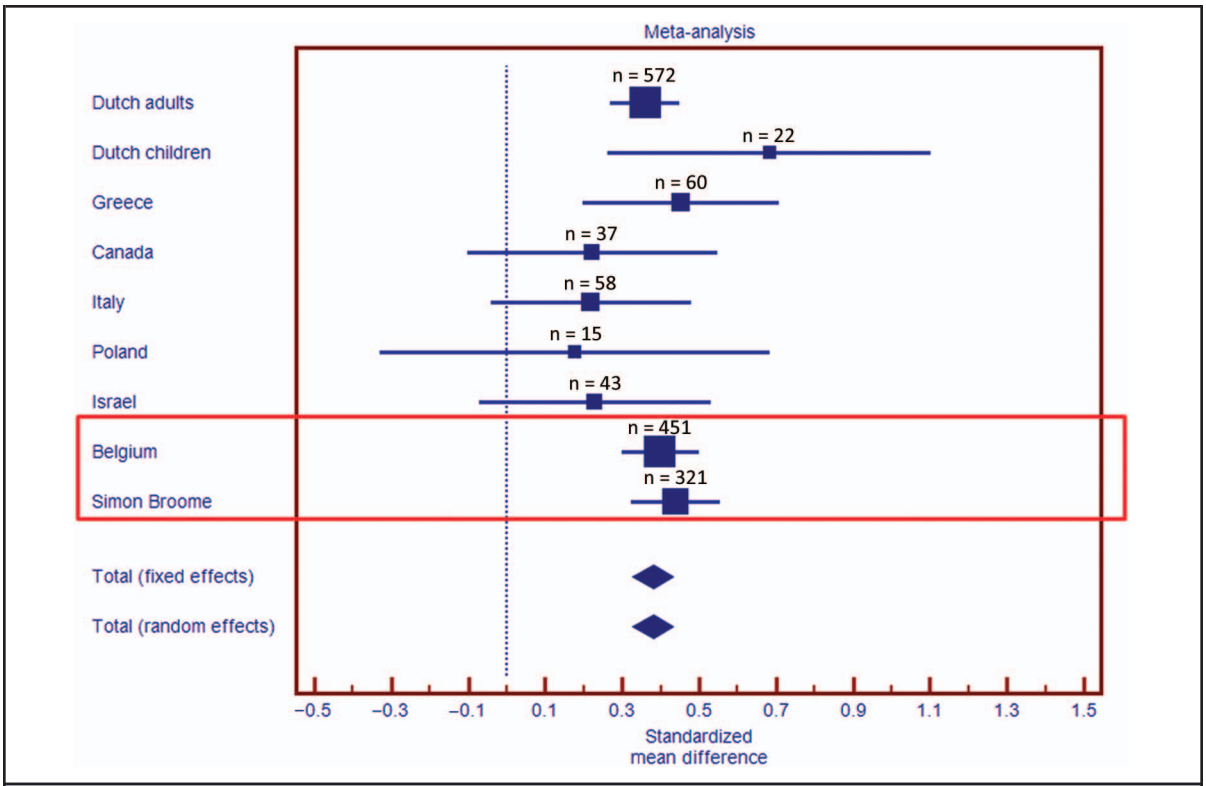


**Fig. 3. Meta-analysis of the LDL-C SNP score in 9 independent FH mutation–negative cohorts in comparison to the WHII population.**

Highlighted in red box are 2 cohorts studied in the original report *(25)*. The overall standardized mean difference was 0.381.

nates well in children as well as adults. In general, the mutation detection rate in children with a clinical diagnosis of FH is higher than in adults *(32)*, and this is because, when comparing the LDL-C distribution in FH patients and their unaffected siblings, the false-positive and false-negative rate is smaller in childhood than in adulthood *(26)*, where secondary environmental causes for high LDL-C concentrations may have an influence. Our data suggest that in a child, once a single-gene cause for having highly increased LDL-C is ruled out, a polygenic cause is highly likely. However, to confirm this result, the child cohorts should be compared against a control of country-matched children with homogeneous lifestyle backgrounds, which is currently unavailable and remains a limitation to this study.

One of the limitations in the 6-SNP score we have used here is that it does not contain all of the information on the genetic determinants of LDL-C concentrations available following the recent GWAS studies. If patient samples are being tested with next-generation sequencing approaches, it is technically and financially feasible to include all 12 SNPs and indeed to include all SNPs that have been associated with LDL-C even if they also influence other lipid traits. From a diagnostic point of view, we have shown that the 6-SNP score is as good at discriminating between FH/M– and WHII control subjects as the 12-SNP score, and a smaller number of SNPs would clearly have cost benefits. We show here that the 6-SNP score discriminates well in FH patients from an additional 6 countries, but all samples are from white patients and we currently have no data to allow us to extrapolate the utility of this score to patients from other ethnic backgrounds, in whom the minor allele frequency will differ considerably and in whom the raising effect of the SNPs on LDL-C may not be consistent. Another limitation may be that the probability of having LDL-C >189 mg/dL (4.9 mmol/L) given the LDL-C genetic risk score was estimated in the FH patients on the basis of a model with observed LDL-C concentrations in the WHII cohort. However, given the high mean LDL-C concentration in WHII, the estimated probability of having LDL-C >189 mg/dL or >4.9 mmol/L is likely to be higher than

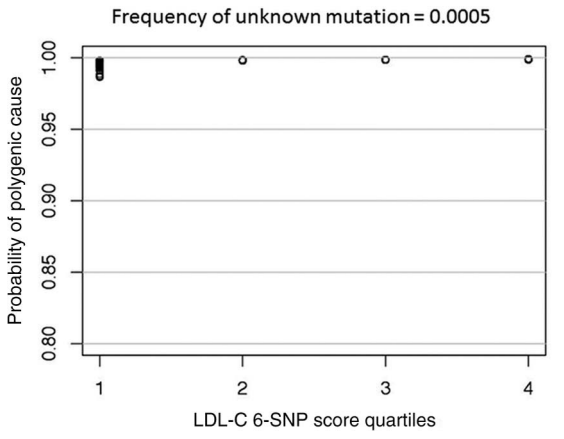


**Fig. 4. Probability of having a polygenic cause of hypercholesterolemia on the basis of predicted LDL-C (assuming population mean 135 mg/dL or 3.5 mmol/L) assuming that the frequency of unknown mutations in the population is 0.0005.**

that estimated in a younger, healthier sample. This will translate into a lower probability of a polygenic cause, especially for those in the lowest quartile of the genetic risk score.

The question remains whether the mutation-negative patients do indeed carry an unidentified FH-causing mutation and, if so, what proportion this represents. Although we accept that this is a possibility, we believe it will be a very rare event. The prior probability that a patient with a clinical diagnosis of FH has a mutation in 1 of the 3 known FH-causing genes is approximately 80% (i.e., in those with the clinical diagnosis of definite FH this is the mutation detection rate previously reported *(3)*). To date, there have been no reports of any identified fourth gene where mutations cause autosomal-dominant FH. Once the presence of a mutation in known genes is ruled out by comprehensive molecular genetic diagnostic methods, the second most likely probable cause, as we show here, is a polygenic inheritance. Our analysis here indicates that, assuming an undetected mutation frequency of 0.0005, the probability of a polygenic cause for LDL-C >189 mg/dL (4.9 mmol/L) in the assessed FH/M– individuals is >95%. There is also a possibility that FH/M– patients who have the LDL-C genetic risk score in the lower quartiles of the score distribution have an intermediate phenotype between FH and familial combined hyperlipidemia, hence the slightly higher TG in FH/M– patients. This could be due to inheritance of higher numbers of LDL-C- and TG-raising alleles. Therefore, as before *(25)*, we believe that the clinical diagnosis of FH should be used only for patients with a DNA-identified genetic cause.

All recent guidelines for the diagnosis and cascade testing of FH, except in the US, have recommended the utility of DNA testing when the family mutation is known and of LDL-C measures where the mutation is not available *(14)*. On the basis of the data we present here, only in those probands with a confirmed monogenic cause will cascade testing be cost effective, because in the remainder there is most likely a polygenic cause. In countries where DNA testing is not available (for reasons of availability or willingness to fund such genetic tests), cascade testing, on the basis of LDL-C measures, will prove to be less effective than it could be. These data support the approach taken in the Netherlands and Wales of using costly cascade testing resources only in the families in which the proband has an identified mutation, since in the majority (at least 75%, i.e., the top 3 quartiles of the 6-SNP score) of the no-mutation patients, the most likely explanation for their clinical diagnosis of FH is a polygenic cause. In individuals with a clinical diagnosis of FH with a SNP score in the lowest quartile, however, it is unlikely that there is a polygenic cause, and although a mutation in 1 of the 3 known FH genes may have been missed for technical reasons, research to identify whether the individuals have a mutation in a yet-to-be-identified gene would be valuable.

**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

**Authors' Disclosures or Potential Conflicts of Interest:** *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

**Employment or Leadership:** V. Mollaki, Athens University; J. Whittaker GSK.
**Consultant or Advisory Role:** J. Defesche, Regeneron.
**Stock Ownership:** J. Whittaker, GSK.
**Honoraria:** None declared.
**Research Funding:** The WHII study has been supported by grants from the Medical Research Council; British Heart Foundation; Health and Safety Executive; Department of Health; National Heart, Lung, and Blood Institute (grant number NHLBI: HL36310) and National Institute on Aging (AG13196), US NIH; Agency for Health Care Policy Research (grant number HS06516); and the John D. and Catherine T. MacArthur Foundation Research Networks on Successful Midlife Development and Socio-economic Status and Health. M. Futema, BHF (PG08/008), National Institute for Health Research University College London Hospitals Biomedical Research Centre; R.A. Whittall, BHF (PG08/008), National Institute for Health Research University College London Hospitals Biomedical Research Centre; E. Drogari, Athens University; V. Mollaki, Athens University; E. Sijbrands, The Nether-

lands Heart Foundation (2006B190); P. Talmud, BHF (PG08/008), National Institute for Health Research University College London Hospitals Biomedical Research Centre; S.E. Humphries, chair funded by the British Heart Foundation, BHF (PG08/008), National Institute for Health Research University College London Hospitals Biomedical Research Centre.
**Expert Testimony:** None declared.

**Patents:** None declared.

**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

**Acknowledgments:** We thank Biobank of CEINGE S.C.ar.l. Advanced Biotechnology, Naples, Italy.

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