A meta-analysis of Italian and Estonian individuals shows an effect of common variants in HMGCR on blood apoB levels

Martina Rostici‡, Natalia Pervjakova‡, Marika Kaakinen‡, Arrigo F Cicero†, Arne P Feufer§, Letizia Marullo†, Krista Fischer§, Longda Jiang§, Sergio D’Addato†, Elisabetta Rizzoli†, Gianmichele Massimo§, Marina Giovannini†, Sabrina Angelini§, Patrizia Hrelia§, Chiara Scapoli§, Claudio Borghi§ & Inga Prokopenko*.
†Medicine & Surgery Sciences Department, Alma Mater Studiorum University of Bologna, Bologna, Italy
‡Estonian Genome Center, Institute of Genomics, University of Tartu, Tartu, Estonia
§Genomics of Common Disease, Division of Diabetes, Endocrinology & Metabolism, Department of Medicine, Imperial College London, London, UK
*Author for correspondence: Tel.: +44 0 207 594 6501; i.prokopenko@imperial.ac.uk
‡Co-first authors
§Co-last authors

Aim: The aim of the study was to explore the effects of variants at HMGCR-KIF6 loci on a range of cardio-metabolic phenotypes. Methods: We analyzed the range of variants within Genetics in Brisighella Health Study and KIF6 genes using an additive genetic model on 18 cardiometabolic phenotypes in a sample of 1645 individuals from the Genetics in Brisighella Health Study and replicated in 10,662 individuals from the Estonian Genome Center University of Tartu. Results: We defined directly the effects of rs3846662:C>A at HMGCR on apoB levels. The analysis also confirmed effects of on low-density lipoprotein-cholesterol and total cholesterol levels. Variants in KIF6 gene did not reveal any associations with cardiometabolic phenotypes. Conclusion: This study highlights effect of HMGCR locus on assay-determined apoB levels, an infrequent measure of blood lipids in large studies.

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Cardiovascular disease (CVD) encompasses a wide range of pathological conditions, including raised blood pressure, hypertension, ischemic and coronary heart disease, cerebrovascular disease and heart failure [1]. To date, CVD is the leading cause of mortality and morbidity globally with an estimated number of 17.5 million deaths per year in the world, projected to reach 23.3 million by 2030 [2,3]. Several lines of evidence from epidemiological and biochemical studies have widely established that high level of LDL-C, low concentration of HDL-C and high level of total cholesterol (TC) play a pivotal role for the development of CVD [4], and moreover can be considered as important factors in the neurodegenerative pathology. In particular, high levels of LDL-C and triglycerides are causally related to the risk of coronary artery disease (CAD) [5–7]. Apolipoproteins are molecules that may function as the regulator of lipoprotein metabolism through their maintenance of the structure of the lipoprotein particles [8]. Apolipoproteins are known to bind lipids to form lipoproteins such as LDL, HDL, VLDL and iLDL that play an important function in recognition of these complexes by other molecules’ receptors. While the number and type of apolipoproteins vary between lipoproteins, the LDL metabolite is mostly attached to apoB [9,10]. ApoB molecules, mainly apoB-100, are particles responsible for a transport function for cholesterol and triglycerides in the blood stream [10]. It has been estimated that 90–95% of apoB molecules circulating in plasma provide a good measure
of the total number of LDL particles, reflecting the atherogenicity of LDL-C [11–15]. The genetic background and predictive value of LDL-fractions such as apoB has so far only been investigated in a limited number of relatively small studies [7,13,15].

Over the past decade, more than 40 loci in human genome have been described with respect to the differential effect of statins on decreasing the risk of clinical end points including cardiovascular death and myocardial infarction, and on modulating lipid levels [16–19]. One of the most well-known genes in this respect is HMGCR, also known as HMG-CoA reductase. HMGCR is a transmembrane glycoprotein of the endoplasmic reticulum that serves as a rate-limiting enzyme in cholesterol biosynthetic pathway [20,21]. Statins, the most used lipid-lowering drugs worldwide, act as HMG-CoA inhibitors, by actively occupying binding sites and thus blocking the association of enzyme with substrate [22]. Despite the fact that this mechanism of action proved its effectiveness in reducing the risk of CVD event and overall inflammation, there is a wide range of inter-individual variability to statin response [23]. This variation could be caused by alternative splicing occurring in exon 13 and other single nucleotide polymorphisms (SNP) located outside of HMGCR that contributes to differences in LDL cholesterol level [24].

KIF6 is a relatively recent candidate for CVD [25–27], initially suggested as contributing to cardiovascular risk within the European population [28]. KIF6 protein is a homodimeric molecule belonging to the KIF9 family of kinesins and is known to be ubiquitously expressed in the coronary arteries and vascular cells [29]. The function of KIF6 gene is yet poorly understood, although it is reported to mediate intracellular transport of organelles, protein complexes and mRNAs [30]. Variants at KIF6 gene are characterized by controversial observations. Specifically, a common SNP, rs20455 (c.2104:T>C; p[Trp719Arg]) resulting in a missense tryptophan to arginine substitution at position 719 of the codified polypeptide chain has been widely studied since 2007 [27,31,32]. In a meta-analysis of eight prospective studies, it has been demonstrated that carriers of the KIF6 719Arg variant were at an increased risk to develop CVD [33] and received a significant benefit from the statin therapy compared with 719Trp wild-type individuals [32]. However, variants within the KIF6 locus have not been reported for their effects on lipids levels in genome-wide association studies (GWAS) [34], and their role in regulating statin therapy response is not defined.

In this study, we aimed to investigate the role of HMGCR and KIF6 variants in the body metabolism through analysis of association with cardiometabolic phenotypes, including 14 quantitative traits and four categorical outcomes in two European studies in up to 12,307 individuals.

### Materials & methods

**Study populations & sample collection**

The Genetics in Brisighella Health Study (GBHS) is a prospective, population-based longitudinal epidemiological investigation of genetic factors influencing a range of cardiometabolic phenotypes involving 2939 randomly selected Caucasian individuals (1491 men and 1448 women), aged 14–84 years, free of cardiovascular disease at enrolment, resident in the northern Italian rural town of Brisighella. The study started in 1972 and it is still ongoing. The town of Brisighella was originally selected as the site for the study, given a homogeneous life-style of its residents, with a very low rate of migration. Subjects were clinically evaluated at baseline and every 4 years thereafter by collecting an extensive amount of clinical and laboratory data. For the purpose of this study, we selected 1645 subjects from the 2008/2012 GBHS population survey, with available blood samples [35–38]. The sample was selected in order to avoid direct familial connections between volunteers, such as parent–child or brother–sisters pairs. Only one individual from each pair was selected for further analyses.

The Estonian Genome Center University of Tartu (EGCUT) is a prospective, volunteer-based sample of the Estonian resident adult population (aged ≥18 years). The current number of participants – close to 52,000 – represents about 5% of the Estonian adult population, making it ideally suited to population-based studies. General practitioners and medical personnel in the special recruitment offices have recruited participants throughout the country. At baseline, the general practitioners performed the standardized health examination of the participants, who also donated blood samples and filled out a 16-module questionnaire on health-related topics such as lifestyle, diet and clinical diagnoses described in WHO ICD-10. In total, 10,662 individuals were selected with available blood samples according to the requested protocols to complement the GBHS population data. In analogy with GBHS data, only one individual from each pair of relatives was selected for the analyses.

All participants provided written informed consent. The Ethics Committee of Human Studies, University of Tartu, Estonia and Ethical Board of the University of Bologna approved the studies. All experiments in all cohorts were performed in accordance with relevant guidelines and regulations.
We analyzed 18 cardiometabolic phenotypes (Supplementary Table 1) which consisted of 14 quantitative traits and four binary outcomes, including Type 2 diabetes (T2D), coronary artery disease (CAD), hypertension and hypercholesterolemia. Four disease statuses were defined following the standard international guidelines by ICD-10 codes between I20 and I25, and between I60 and I71 [39–41].

Quantitative phenotypes included total cholesterol (TC), HDL-C, LDL-C, triglycerides (TG), apoA1, apoB, fasting glucose, body mass index (BMI), waist circumference, systolic and diastolic blood pressure (SBP, DBP), heart rate, serum uric acid (UR) and creatinine (CREA). Laboratory methods for the measurement of each quantitative trait are summarized in Supplementary Table 2. Following indication reported elsewhere, in the analysis of DNA variant effects on lipids, we excluded individuals undergoing lipid-lowering therapy, while for individuals undergoing anti-hypertension therapy we added 15 mmHg to SBP and 10 mmHg to DBP [34,42].

To check the assumption about normally distributed quantitative traits, we performed a Shapiro–Wilk test on all phenotypes. Since normality test significantly deviated from normal distribution (p < 0.05) for all phenotypes, we applied an inverse normal transformation of residuals on all traits. Residuals for lipids and BMI were adjusted for sex, age and age², while fasting glucose, CREA, UR were adjusted for sex and age only. Anthropometric traits, DBP, SBP and heart rate were adjusted for sex, age, age² and BMI. Residuals for categorical phenotype were calculated as follows: T2D was adjusted for sex, age and BMI; CAD was adjusted for sex and age; hypercholesterolemia for sex, age and age²; and hypertension for sex, age, age² and BMI. Normality test and transformation of data were run using R software version 3.3.1 [43].

Correlation between phenotypes were calculated using the polycor package implemented in R software [43]. Hetcor function allowed to perform a Pearson product–moment correlation test between quantitative traits, a polyserial correlation test between quantitative-discrete traits and a polychoric correlation test between discrete–discrete traits.

Genotyping, quality control & imputation
Genomic DNA of GBHS samples was isolated from EDTA-anticoagulated whole blood using the QIAamp DNA Blood kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. DNA samples were diluted and stored as 10 ng/μl aliquots at -20°C. Five SNPs were genotyped: three in the KIF6 gene on chromosome 6, including one missense variant rs20455 (c.2104:T>C; p[Trp719Arg]) and two highly correlated intronic variants; and two in the HMGCR gene on chromosome 5, including one upstream variant (rs3761740:C>A) and another in intron 12. Linkage disequilibrium (LD) between the variants was calculated using Haploview software [44].

Genotyping was performed by real time using the 5′-nuclease allelic discrimination assay (TaqMan®, Applied Biosystems, CA, USA), according to the manufacturer instruction. Negative controls were included to each reaction as a quality control.

Two independent sets containing 2589 and 8073 randomly selected individuals from EGCUT were used for replication. Samples were genotyped with commercially available Illumina Human370CNV and Illumina OMNI-Express arrays, respectively.

Sample and SNP quality control was undertaken within each contributing study. Sample quality control covered exclusions on the basis of call rate (<95%); in the EGCUT additional exclusions were based on the extreme heterozygosity (±3 SD), sex discordance, cryptic relatedness (pihat ≥0.1), and outlying ethnicity. SNP quality control covered exclusions on the basis of call rate (<95%) across samples, minor allele frequency (MAF; <1%) and extreme deviation from the Hardy–Weinberg equilibrium (p < 1 × 10⁻⁸).

Within the EGCUT, the autosomal markers were imputed up to the 1000 Genomes project multiethnic reference panel (Phase III, March 2012 release). Imputation was performed using IMPUTEv2 software [45]. Poorly imputed variants (IMPUTE info <0.8) were excluded from further analysis.

Association analysis
For quantitative traits, we assumed an additive genetic model of effects and applied a linear regression and for categorical phenotypes – log-additive genetic effects and implemented a logistic regression. All single variant association analyses were run using R software [43] and PLINKv1.07 software in parallel [46,47]. Association analysis in two Estonian samples was run using SNPTESv2.5 software using maximum likelihood genotype estimates on imputed SNP array data [48]. Meta-analysis of results from three studies was performed using GWAMA software [49]. Association tests were Bonferroni corrected for multiple testing, accounting for 19 non-highly (r < 0.5) correlated
Results

We performed the association analysis of two HMGCR and three KIF6 variants and 18 cardiometabolic phenotypes. Genotyping data for these five SNPs (Supplementary Table 3) were available in up to 12,307 individuals of European descent from two studies, including discovery performed in GBHS and replication undertaken in the EGCUT. All five SNPs passed quality control measures, and were then tested for association in each study and combined across the two studies by a fixed-effect meta-analysis.

Prior the analysis, we calculated linkage disequilibrium (LD) between two sets of genotyped SNPs – rs3761740:C>A and rs3846662:C>A for HMGCR and rs20455 (c.2104:T>C; p.[Trp.719Arg]), rs9462535:A>C and rs9471077:G>A for KIF6 tested in this study for the GBHS. The two variants within the HMGCR locus are only mildly correlated (Supplementary Figure 2A) and showed slightly lower LD ($r^2_{rs3761740-rs3846662} = 0.15$) than that reported for 1000 Genomes project data for Europeans ($r^2_{rs3761740-rs3846662} = 0.22$). The variants within the KIF6 gene, instead, were in high LD ($r^2_{rs20455-rs9462535} = 0.801$, $r^2_{rs20455-rs9471077} = 0.801$, $r^2_{rs9471077-rs9462535} = 1$; Supplementary Figure 2B), consistent with LD patterns for Europeans from the 1000 Genomes project ($r^2_{rs20455-rs9462535} = 0.86$, $r^2_{rs20455-rs9471077} = 0.85$, $r^2_{rs9471077-rs9462535} = 0.98$).

The power for our analysis was calculated using the Quanto software [50,51] given the different sample sizes for studied phenotypes, level of significance and minor allele frequency of the variants. The analysis revealed 80% power for HMGCR variant rs3846662 (MAF = 0.45) at $p = 8.7 \times 10^{-4}$ to detect effects of $\beta > 0.12$ for the smallest sample size ($n = 2400$) and effect of $\beta > 0.06$ for the maximum sample size ($n = 11,400$) available for this variant. With the MAF = 0.35 (KIF6 variants, MAFrs20455 = 0.365; MAFrs96462535 = 0.381; MAFrs9471077 = 0.384) we have about 80% power at $p = 8.7 \times 10^{-4}$ to detect effects of $\beta \geq 0.13$ for the smallest sample size ($n = 2400$) and $\beta \geq 0.06$ for the maximum sample size ($n = 11,409$). With the MAF = 0.10 (HMGCR variant MAFrs3761740 = 0.096) we have about 80% power at $p = 8.7 \times 10^{-4}$ to detect effects of $\beta > 0.20$ for the smallest sample size ($n = 2400$) and $\beta \geq 0.09$ for maximum sample size ($n = 11,400$).

The discovery analysis in the GBHS confirmed the established effects of HMGCR locus variant rs3846662:C>A on LDL cholesterol levels ($\beta_{[SE]} = 0.13[0.04]$, $p = 0.001$; Supplementary Tables 1 & 4). Meta-analysis of GBHS and EGCUT results confirmed the effect of the rs3846662:C>A on LDL cholesterol ($\beta_{[SE]} = 0.13[0.03]$; $p = 1.4 \times 10^{-5}$). Our study reveals LDL cholesterol strong correlation with apoB levels ($r = 0.67$ in our epidemiological data (Supplementary Figure 1), which also supports the finding of our analysis highlighting an effect of the previously suggested rs3846662:C>A variant at HMGCR on assay-determined apoB levels ($r = 0.67$) in our epidemiological data (Supplementary Figure 1), which also supports the finding of our analysis highlighting an effect of the previously suggested rs3846662:C>A variant at HMGCR on assay-determined apoB levels ($r = 0.67$) in our epidemiological data (Supplementary Figure 1), which also supports the finding of our analysis highlighting an effect of the previously suggested rs3846662:C>A variant at HMGCR on assay-determined apoB levels ($r = 0.67$). The apoB level increasing allele was associated with increased LDL cholesterol, in accordance with previously reported epidemiological correlation [52], which confirms direct relationship of these two molecules. Meta-analysis also confirmed an association of the HMGCR locus rs3846662:C>A with TC ($p = 4.02 \times 10^{-5}$). HMGCR rs3761740:C>A variant showed an additional associations with SBP ($p = 0.003$) and DBP ($p = 0.007$) in GBHS, but was not replicated in EGCUT and did not result in a significant overall effect in the meta-analysis of all the cohorts.

Association analysis of KIF6 did not reveal any associations with cardiometabolic traits in the meta-analysis. The KIF6 rs20455 (c.2104T>C; p.[Trp.719Arg]) variant was associated with HDL-C ($p = 0.001$) and apoB levels ($p = 0.008$) in GBHS but not in EGCUT (Table 1). The other two analyzed KIF6 variants (rs9471077:G>A and rs9462535:A>C), both intronic, were associated with higher levels of TG ($p_{rs9471077} = 0.004$; $p_{rs9462535} = 0.003$) and apoB ($p_{rs9471077} = 0.001$; $p_{rs9462535} = 0.003$), and lower HDL-C ($p_{rs9471077} = 0.009$; $p_{rs9462535} = 0.007$) in GBHS but were not replicated in EGCUT (Table 1).

Discussion

We have explored the role of HMGCR and KIF6 variants in the body metabolism and response to statin treatment through analysis of association with cardiometabolic phenotypes in two European population-based studies in up to 12,307 individuals. In this study, we report a confirmation for association of HMGCR locus variants with apoB levels. The HMGCR gene itself is a well-established locus associated with TC and LDL cholesterol levels. The first report about HMGCR effects on cardiometabolic phenotypes was based on a comprehensive GWAS study.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Variant</th>
<th>Meta-analysis of discovery and replication</th>
<th>Replication (EGCUT cohort)</th>
<th>Meta-analysis of discovery and replication</th>
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<td>0.13 (0.04)</td>
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<td>0.16 (0.06)</td>
<td>0.007</td>
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<tr>
<td>KIF6</td>
<td>rs9471077</td>
<td>G/A</td>
<td>0.13 (0.04)</td>
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The phenotype showed a statistically significant association in the discovery sample and was selected for replication.

The p-values were adjusted for multiple testing using the false discovery rate (FDR) method.

DBP: Diastolic blood pressure; EGCUT: Estonian Genome Center University of Tartu; SBP: Systolic blood pressure; TC: Total cholesterol; TG: Triglyceride.
conducted in 16 population-based studies from up to 22,562 individuals [53]. This study reported rs3846662:C>A as a lead variant at HMGCR (p = 2.5 × 10^{-19} for TC; p = 1.5 × 10^{-11} for LDL cholesterol) [53]. Another GWAS in more than 100,000 individuals of European ancestry, published a year later, also revealed a new association of genetic variants located in HMGCR with coronary artery disease [54]. In parallel with this discovery, the SNP rs3846662 was for the first time suggested to be associated with apoB levels in the gene-centric analysis [55]. Moreover, additional evidence that pointed to the importance of this signal came from the study performed by Kettunen et al., where the downstream gene variant rs4703667, which is in high LD with intronic rs3846662, was also associated with the nuclear magnetic resonance-based metabolomics platform apoB levels [56]. Our study results are completely in line with previous reports and show the impact of the variant on assay-determined apoB levels in blood. The rs3846662 variant located within intron 13 of HMGCR gene is not only associated with a modest increase of LDL cholesterol levels, but also modulates efficiency of HMGCR pre-mRNA molecules by decreased level of naturally occurring nonfunctional transcripts [57]. This observation provides great support for our finding about the HMGCR locus association with apoB molecules that mainly transport LDL cholesterol molecules in human bloodstream.

ApoB levels are rarely measured within large-scale studies. Our study and association of the HMGCR genetic variant rs3846662:C>A with apoB levels, thus bring a new highlight to the discussion of adding apoB to the standard clinical lipid measurements. It is important to note that our study has shown that the direction of genetic effect to apoB was similar to epidemiological correlation with LDL cholesterol, which proves the use of apoB molecule as a valuable predictor of severe CVD outcomes. In contrast to other major lipid groups, UK Biobank performed apoB measurement in hundreds of thousands of individuals to provide a better prediction of CVD risk and evaluate the efficacy of statin treatment. This hypothesis, if confirmed by further studies, may help in setting up novel approaches of diagnosis and treatment for hyperlipidemia and CVD. We believe that adding apoB to the standard clinical measurements would contribute to enhance the accuracy and outcome prediction of CVD and thus would help in choosing the right dose of a medication, resolving a problem of over-prescription of statins.

In our study, we did not observe any single-variant effect of the KIF6 locus on 18 cardiometabolic phenotypes. KIF6 has been reported in association with differential benefits, in terms of CVD events. For the first time, an association of genetic variants located in the KIF6 gene with coronary heart disease was demonstrated in a meta-analysis of seven prospective studies, including three prospective studies of individuals without coronary heart disease events at baseline and the placebo groups of four statin trials [58]. In particular, no association has been detected between the KIF6 p. Trp719Arg polymorphism and coronary heart disease in the Wellcome Trust Case Control Consortium [59]. It was suggested that the negative results may arise due to the case–control study design if statin therapy was not included in the selective criteria, therefore reducing the power of the study [58]. Another study that took into account statin therapy in 19 case–control studies comprised from a total of 17,000 cases and 39,369 controls also revealed no increased risk of nonfatal coronary artery disease by the p. Trp719Arg polymorphism [60]. In our study, we also did not find any significant associations between the three most studied polymorphisms at the KIF6 locus in terms of risk of CVD outcomes. The null findings from our study about the role of KIF6 variants may be due to a lack of power, given the sample size, as the power analysis showed. Our analyses may also be biased by the type of data used for the analyses. Additionally, other studies have suggested that the KIF6 gene may reveal unexpected associations due to chance, when studied in single cohorts [61]. Further studies, particularly based on meta-analysis of the literature and stratification for the type of statin would be better suited to overcome these limitations.

Our study also has some limitations. First, the sample sizes of our discovery and replication samples are relatively small, and further replication analyses in larger samples are required to confirm our novel findings. Moreover, the biological validity and clinical usefulness of the obtained results has to be verified using functional approaches [62]. Functional validation might involve multiple independent siRNA duplexes to validate the set of genes affecting apoB secretion distinguished from the effect caused by cell viability [62]. Finally, the set of cardiometabolic diseases used in our analysis has been chosen arbitrarily, even if the diagnostic cut-offs applied are those suggested by the main specific international guidelines. We expect that further comprehensive multiphenotype analyses might improve our understanding of the effects of specific loci on groups of related traits, thus to dissect better their pathophysiological impact on human health. Beyond that, the data are reliable and obtained from two European population-based studies, well characterized and defined [63,64].
Conclusion
In conclusion, this study did not reveal any association between the KIF6 and cardiometabolic traits, but highlighted a novel effect of HMGCR variants on apoB levels, which might be predictive of CVD risk. Further investigation of apoB levels variability in relation to CVD would be important. Additionally, evaluation of biological mechanisms underlying this relationship between the HMGCR gene and lipids metabolism will facilitate the advance of our knowledge and help in implementation of findings from this study in more personalized approaches for CVD prevention and therapy.

Summary points
- Cardiovascular diseases (CVDs) are the number one cause of death globally. According to the WHO data, an estimated 17.7 million people died from CVDs in 2015, representing 31% of all global deaths.
- Lipoproteins, such as LDL-C, HDL-C and total cholesterol, play a pivotal role for the development of CVD.
- We conducted the meta-analysis for cardiometabolic phenotypes, including 14 quantitative traits and four categorical traits, in two European studies in up to 12,307 individuals.
- Meta-analysis of Genetics in Brisighella Health Study and Estonian Genome Center University of Tartu results confirmed the effect of the variant rs3846662 on LDL cholesterol.
- The epidemiological data reveals LDL cholesterol strong correlation with apoB molecule.
- Meta-analysis confirmed previously suggested effect of the variant rs3846662 to apoB molecule.
- Association analysis of KIF6 variants did not reveal any associations with cardiometabolic traits in the meta-analysis.
- The variant rs3761740 in HMGCR gene showed an associations with systolic and diastolic blood pressure in Genetics in Brisighella Health Study, but not in Estonian Genome Center University of Tartu.

Supplementary data
To view the supplementary data that accompany this paper please visit the journal website at: https://www.futuremedicine.com/doi/suppl/10.2217/bmm-2017-0431

Author contributions
M Rosticci, L Marullo, C Borghi and I Prokopenko conceived and designed the experiments. M Rosticci, N Pervjakova, M Kaakinen, L Marullo, L Jiang, S D’Addato, C Borghi and I Prokopenko performed the experiments. M Rosticci, N Pervjakova, AF Cicero, RMägi, K Fischer and C Borghi contributed data. N Pervjakova, M Kaakinen, L Marullo, AP feufer, M Rosticci, AF Cicero, RMägi, K Fischer, L Jiang, S D’Addato, E Rizzoli, G Massimo, M Giovannini, S Angelini, P Hrelia, C Scapoli, C Borghi and I Prokopenko wrote the paper. RMägi, C Scapoli, Borghi and I Prokopenko supervised the work.

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No writing assistance was utilized in the production of this manuscript.
Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Papers of special note have been highlighted as: ● of interest; ●● of considerable interest


●● Shows the strategic importance of population-wide primary prevention of unfavorable serum cholesterol levels.


●● The prospective study shows the importance of apolipoprotein B as a risk factor for ischemic heart disease.


● Shows that variants located in 3-hydroxy-3-methylglutaryl-CoA reductase gene can be used as predictors of lipid-lowering response to atorvastatin therapy.


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