Exploring genetic determinants of plasma total cholesterol levels and their predictive value in a longitudinal study

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Abstract

Background: Plasma total cholesterol (TC) levels are highly genetically determined. Although ample evidence of genetic determination of separate lipoprotein cholesterol levels has been reported, using TC level directly as a phenotype in a relatively large broad-gene based association study has not been reported to date.

Methods and results: We genotyped 361 single nucleotide polymorphisms (SNPs) across 243 genes based on pathways potentially relevant to cholesterol metabolism in 3575 subjects that were examined thrice over 11 years. Twenty-three SNPs were associated with TC levels after adjustment for multiple testing. We used 12 of them (rs7412 and rs429358 in APOE, rs646776 in CELSR2, rs1367117 in APOB, rs6756629 in ABCG5, rs662799 in APOA5, rs688 in LDLR, rs10889353 in DOCK7, rs2304130 in NCAN, rs3846662 in HMGCR, rs2275543 in ABCA1, rs7275 in SMARCA4) that were confirmed in previous candidate association or genome-wide-association studies to define a gene risk score (GRS). Average TC levels increased from 5.23 ± 1.11 mmol/L for those with 11 or less cholesterol raising alleles to 6.03 ± 1.23 mmol/L for those with 18 or more (P for trend < 0.0001). The association with TC levels was slightly stronger when the weighted GRS that weighted the magnitude of allelic effects was used.

Conclusion: A panel of common genetic variants in the genes pivotal in cholesterol metabolism could possibly help identify those people who are at risk of high cholesterol levels.

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1. Introduction

Mammalian cells require cholesterol for maintenance of membrane integrity and multiple cellular functions. Cells obtain cholesterol by either de novo synthesis in the endoplasmic reticulum or receptor-mediated uptake of lipoproteins, processes that are tightly controlled by feedback regulation to prevent the toxicity of excess unesterified cholesterol in membranes. Only 9% of the body cholesterol pool is easily accessible in plasma, the remaining 91% being found in tissues. Plasma total cholesterol (TC) levels represent a combination of cholesterol in structurally and metabolically heterogeneous groups of lipoprotein, such as very low density lipoprotein (VLDL), low density lipoprotein (LDL), high density lipoprotein (HDL) and cholesterol in other lipoproteins, among which cholesterol (and other lipids) and certain apolipoproteins are constantly exchanged [1–3]. It is known that about two-thirds of the blood TC is attributable to LDL and the remaining mainly to HDL and VLDL. Ample evidence for a genetic basis of separate lipoprotein cholesterol levels has been reported so far [4–7]. The heritability of TC levels has been estimated to be around 40–60% [8]. However, plasma TC levels, as a comprehensive index of our body cholesterol pool, have rarely been explored directly as a phenotype in a relatively large, broad-gene based association study despite its being more sensitive in predicting cardiovascular disease risk compared with LDL cholesterol levels [9].

In this study, we followed 3575 subjects over 11 years during which their plasma TC levels were measured three times, which enabled us to better account for intra-individual variation. In order to get a broad list of genes potentially relevant to cholesterol metabolism, we adopted a pathway-driven approach to select genes known to be involved in the regulatory pathways that control fatty acid, glucose, cholesterol and bile acid homeostasis. We
emphasized on the intricate links within lipid metabolism and between glucose and lipid metabolism through common transcriptional factors [10]. By surveying genetic variants in this broad list of candidate genes, we want to provide some new insights into the genetic determinants of plasma TC levels, and investigate whether common genetic variants in genes involved in cholesterol metabolism could predict the plasma cholesterol levels.

2. Materials and methods

2.1. Study population

Our study was conducted in Doetinchem, a town in a rural area in the east of the Netherlands. The design and detailed methods have been reported earlier [11]. At baseline (round 1, 1987−1991), an age-and-sex stratified random sample of the population was drawn. A total of 12,405 subjects aged 20−59 years were willing to participate and underwent a first measurement. A random two-third of those measured in round 1 were invited for round 2 (1993−97), and 6118 subjects participated. They were invited for a third measurement round (1998−2002) and 4917 subjects participated. For 4662 participants, all three measurements were available. Participants who changed their smoking habits, had missing data on smoking status or were pregnant at the time of measurement were excluded. Finally, 3779 participants met the inclusion criteria for this study. Informed consent was obtained from all participants. In brief, the subjects were surveyed on demographic, anthropometric and lifestyle information, disease history and medication by questionnaires. A validated semi-quantitative food frequency questionnaire was used in the second and third surveys to assess the habitual consumption of 178 food items during the previous year [12]. A non-fasting blood sample was taken after being sent to the Lipid Reference Laboratory of the University Medical Center using the Sequenom iPLEX platform.

2.2. Gene and SNP selection and genotyping

Genomic DNA was extracted from the buffy coat fraction with a salting out method. For 3639 subjects high throughput SNP genotyping was performed with the Illumina Golden Gate assay using the Sentrix Array Matrix platform (Illumina Inc., San Diego, California). Gene selection started from the master regulator genes (encoding the nuclear receptors [PPARs, LXR, and FXR] or transcription factors [SREBPs]), and continued by selecting their associated co-activators, co-repressors and target genes. Several candidate genes, described in literature to be associated with blood lipids or blood pressure were also added. The SNPs in each gene were selected based on either published associations or the web-based program SNPSelector [17]. Detailed information on the gene and SNP selection procedures, quality control and the full gene and SNP list have been reported before [18]. Two SNPs that failed in the Illumina assay (rs7412 and rs429358 in APOE) were successfully re-genotyped with a Taqman assay. In addition, 6 SNPs associated with blood total cholesterol levels from the latest genome-wide association (GWA) study that was published after our initial gene selection (rs646776 in CELSR2, rs3846662 in HMCR, rs2304130 in NTCAN, rs10889353 in DOCK7, rs6756629 in ABCG5, and rs174570 in FADS2) [4] were genotyped by KBioscience (Hoddesdon, Hertfordshire, UK) using the KASPar chemistry, a competitive allele specific PCR SNP genotyping system using FRET quencher cassette oligonucleotides (http://www.kbioscience.co.uk). After exclusion of subjects for whom all genotypes failed, exclusion of failed genotypes and quality control, data were available of 361 SNPs in 243 genes for a total of 3575 participants. APOC3 SNPs (rs2854116 and rs4520) were genotyped in the replication sample at the Leiden University Medical Center using the Sequenom iPLEX platform.

2.3. Statistical analysis

Statistical analyses were performed with SAS version 9.1 software (SAS Institute, Cary, NC), unless indicated otherwise. The paired Student’s t test and Chi-square test were used for comparisons of means and proportions between measurement rounds, respectively. The Wilcoxon Signed-Rank test was used for comparisons of alcohol consumption between two rounds of survey. Testing of deviation from Hardy Weinberg Equilibrium (HWE) and calculation of linkage disequilibrium (LD) were done with PROC ALLELE. For testing of deviation from HWE, the chi-square goodness-of-fit test was used (p > 0.05). The TC levels of participants who took lipid-lowering medication at the time of survey were treated as missing values (Table 1). The intake of total fat and cholesterol was averaged over the second and third surveys and the medians (34.9 energy% and 224.9 mg/day, respectively) were used to categorize the subjects into low or high intake of fat or cholesterol. Since inheritance patterns of the causal alleles are unknown, an additive model was used, which gives good overall performance in any of the three potential modes of inheritance [19]. Random coefficient models (multi-level modeling, PROC MIXED) were used to study the relationship between SNPs and repeated measurements of TC levels, which accounts for correlation between repeated measurements within subjects [20]. Men and women were analyzed separately for the five X-linked SNPs (rs2073115, rs3213451, rs5969919, rs1403543, and rs3048). When the overall difference was statistically significant, the Tukey-Kramer method was used to identify significant differences between the genotype groups. To adjust for potential confounding effects and to improve model fitting, age, sex, current smoking status (yes or no), alcohol consumption, and body mass index (BMI) were added to the model as covariates. The exact follow-up time in years was put into the model as a continuous variable. Intercept and time were treated as random effects allowing unique baseline levels and unique changes of TC level over time for each individual. Gene × time interactions were explored by including interaction terms into the MIXED models. The odds ratio (OR) of having an average cholesterol level over the three rounds ≥6.5 mmol/L (hypercholesterolemia) in the subjects carrying ≥18 total cholesterol raising alleles was calculated using a logistic regression model adjusting for age, sex, current smoking habits, alcohol use and BMI. Subjects with <18 total cholesterol raising alleles were used as reference. All reported p values were two-tailed, and statistical significance before adjustment for multiple testing was defined at the α = 0.05 level. The false discovery rate (FDR, PROC MULTTEST) was applied to take multiple testing into account. To date, there is no conventional q-value threshold to categorize a discovery as significant. As in similar research, a q-value threshold of 0.20 was used to define significance [21].

2.4. Genetic risk score computation and analysis

A genetic risk score (GRS) was calculated on the basis of those SNPs associated with TC levels after adjustment for multiple testing that were replicated in other studies. If SNPs were in LD (r2 > 0.9),
only the SNP with the most significant main effect in our study was included in the score. As reported before [22], two methods were used to create the GRS: a simple count method (count GRS) and a weighted method (weighted GRS). Both methods assume each SNP to be independently associated with TC levels. We assumed an additive genetic model for each SNP, applying a linear weighting of 0, 1, and 2 to genotypes containing 0, 1, or 2 risk alleles (raising total cholesterol levels), respectively. The count method assumes that each SNP in the panel contributes equally to the cholesterol increasing effect and was calculated by summing the total number of risk alleles, producing a maximal score of 24. For the weighted GRS, each SNP was weighted by the β-coefficients (Supplementary Table 4) obtained from the linear regression of individual SNP on average TC levels over the three rounds of measurements by using an additive model in a randomly selected half of the total subjects with no missing value in any of the SNPs included in GRS calculation (n = 1668). The weighted GRS was calculated by multiplying each β-coefficient by the number of corresponding risk alleles (0, 1 or 2) and then summing the products. This produces a score out of 3.4 (twice the sum of the β-coefficients), which was then divided by 3.4 and multiplied by 24 to facilitate interpretation and comparison with the count GRS. The weighted GRS was associated with plasma TC levels in the other half of the subjects (n = 2022) and was also assessed in the same subjects (Supplementary Table 5). To assess possible discriminative improvement for hypercholesterolemia attributable to the GRS, we calculated the areas under the receiver-operating characteristic curves (AUCs or c-index) from a logistic regression model including conventional risk factors only (age, sex, current smoking habit, alcohol use, and BMI) and a model which additionally included the GRS. This analysis was done in Stata 11 (StataCorp, Texas).

3. Results

Mean age of the subjects at the first survey was 40.8 years, ranging from 20 to 60 years (Table 1). The average TC levels increased from the first survey (5.47 ± 1.03 mmol/L) to the second survey (5.52 ± 1.02 mmol/L), and the third survey (5.74 ± 1.03 mmol/L). More people took lipid-lowering medication in the second (5.52 ± 1.03 mmol/L) and third surveys (5.74 ± 1.03 mmol/L). The average TC levels increased from 20 to 60 years (Table 1). The average TC levels increased from the first survey (5.47 ± 1.03 mmol/L) to the second survey (5.52 ± 1.02 mmol/L), and the third survey (5.74 ± 1.03 mmol/L). More people took lipid-lowering medication in the second (5.52 ± 1.03 mmol/L) and third surveys (5.74 ± 1.03 mmol/L). The average TC levels increased from 20 to 60 years (Table 1). The average TC levels increased from the first survey (5.47 ± 1.03 mmol/L) to the second survey (5.52 ± 1.02 mmol/L), and the third survey (5.74 ± 1.03 mmol/L).

Thirty-five SNPs were found to be significantly associated with TC levels (p < 0.05, Table 2) after adjustment for age, sex, current smoking status, alcohol consumption and BMI. Twenty-three of them remained statistically significant after adjustment for multiple testing (FDR,q < 0.2). Eleven of them (rs7412 and rs429358 in APOE [23], rs646776 in CERSLR2 [4], rs1367117 in APOB [6,7], rs6756629 in ABCG5 [4,24], rs627799 in APOA5 [6], rs688 and rs5925 in LDLR [25,26], rs10889353 in DOCK7 [4,24], rs2304130 in NCAN [4,24], rs3846662 in HMGCGR [4]) were reported to be associated with blood TC or LDL levels in candidate genetic association studies.
studies or GWA studies. For the other 12 SNPs, we explored the association with plasma cholesterol levels in the publicly available results of a meta-analysis of seven GWA studies on LDL and HDL cholesterol levels [5]. In this meta-analysis, rs2275543 in ABCA1 was associated with both LDL (p = 0.004) and HDL (p = 1.437E-7) cholesterol levels. Also, rs7275 in SMARCA4 was associated with LDL cholesterol levels (p = 1.921E-6, Supplementary Table 1). For the two SNPs in APOC3 (rs2854116 and rs4520) that were associated with TC levels in our study, no corresponding SNPs or tagging SNPs were genotyped or imputed in the meta-analysis dataset. Therefore, we carried out a replication study in 2221 subjects randomly selected from another population study using the same methodology as the Doetinchem cohort (details in Supplementary Table 2). Rs2854116 was not associated with TC levels (p > 0.05). Despite a larger difference in TC levels between genotypes of rs4520 in the replication samples than in the Doetinchem cohort, the association did not reach statistical significance (p > 0.05).

As an explorative analysis, we investigated whether TC levels changed differentially over the 11 years of follow-up for the genotypes of the above identified 13 significant SNPs (11 replicated in literature, 2 replicated in the meta-analysis of GWA studies). Four SNPs (rs7412 in APOE, rs646776 in CELSR2, rs662799 in APOA5, and rs7275 in SMARCA4) interacted with follow-up time on TC levels (p for interaction < 0.05, Supplementary Table 3). No interaction between the above identified significant SNPs and gender was detected (data not shown).

In order to evaluate to what extent the 13 identified SNPs in aggregate explained the variation in TC levels, we constructed a gene risk score for each subject by using a simple count (count GRS) or a weighted (weighted GRS) approach. One SNP (rs5925) was excluded from the GRS calculation because of its LD with rs688 in LDLR (r² = 0.99). Both the count and weighted GRSs ranged from 7 to 21. The median count and weighted GRSs were 14 and 15.5, respectively. Demographic and lifestyle characteristics (age, gender, current smoking status, alcohol consumption and BMI) did not differ significantly across quartiles of the count or weighted GRS (data not shown). Based on the count GRS, the average increase of TC levels per risk allele was 0.11 ± 0.01 mmol/L after adjustment of age, sex, current smoking status, alcohol consumption and BMI. Average TC levels increased from 5.23 ± 0.82 mmol/L for subjects with a score of 11 or less to 6.03 ± 1.11 mmol/L for subjects with a score of 18 or more (p for trend < 0.0001, Table 3). Estimates were slightly higher (0.14 ± 0.01 mmol/L per 1 unit increase in weighted GRS) and the association between GRS and TC levels was slightly stronger when using the weighted GRS (Table 3 and Supplementary Table 5). In total, the variance in average TC levels explained by these 12 SNPs was 6.9% (7.2% and 6.7% in the low and high-fat intake group, respectively; 7.6% and 6.1% in the low and high-cholesterol intake group, respectively). Subjects in the top 5% of the distribution of the number of TC raising alleles (≥ 18 risk alleles) had an increased odds of having hypercholesterolemia using 6.5 mmol/L as a cut-off point (OR: 2.4; 95% CI: 1.7–3.5) as compared to the subjects with < 18 risk alleles. The GRS improved the discriminative accuracy of hypercholesterolemia, measured by the AUC, from 0.705 (95% CI: 0.684–0.727) to 0.734 (95% CI: 0.713–0.755) when the count GRS was adjusted (p < 0.0001) (Fig. 1). Similar improvement was obtained when the weighted GRS was included (data not shown).

4. Discussion

In this longitudinal study, out of 361 SNPs in 243 genes, 23 SNPs were associated with plasma total cholesterol levels. Eleven of them in 9 genes were reported in previous genetic association or GWA studies on associations with blood total or LDL cholesterol levels. Two (rs2275543 in ABCA1 and rs7275 in SMARCA4) were found to be associated with cholesterol levels in a meta-analysis of seven GWA studies. A gene risk score based on these significant SNPs was strongly associated with TC levels and the prevalence of hypercholesterolemia. Four of the SNPs (rs7412 in APOE, rs646776 in CELSR2, rs662799 in APOA5, and rs7275 in SMARCA4) interacted with follow-up time on TC levels.

Rs2275543 in ABCA1 was in high LD with the recently reported rs3905000 (r² = 0.89) known to be strongly associated with total and HDL cholesterol levels [4]. Compared to the major T allele of rs2275543, the C allele was associated with lower TC levels (Supplementary Table 3). This cholesterol decreasing effect (both LDL and HDL cholesterol levels) associated with the C allele was also observed in the results of a meta-analysis of seven GWA studies (Supplementary Table 1) [5]. Rare mutations in ABCA1 in humans, causing Tangier disease, were reported to affect not only plasma HDL cholesterol levels (6% of normal), but also total cholesterol levels (32% of normal) and LDL cholesterol levels (37% of normal) [27]. SMARCA4 genetic variation (rs1529729) was recently reported to be associated with serum LDL cholesterol levels (r² = 0.38 between rs1529729 and rs7275 in SMARCA4) [6]. However, we think that rs7275 might tag certain functional SNPs in the LDLR gene due to its proximity to this gene (29 kb upstream). This may explain the association between rs7275 and TC levels observed in our study.

Among the 4 SNPs that interacted with follow-up time on TC levels, rs662799 in APOA5 was recently reported to interact with dietary fat intake. G allele carriers had higher TC levels compared to AA homozygotes only in the high fat intake group [28]. Therefore, we explored whether fat intake explained the strong TC increase over time in GG homozygotes observed in our study. However, adjustment for fat intake did not alter our results and no statistically significant interaction between dietary fat intake and rs662799 was detected (data not shown). Higher TC levels with alcohol consumption [29] and stronger positive associations between HDL cholesterol levels and alcohol consumption [30] have been reported in APOE e2 carriers compared to e3 or e4 carriers. However, although alcohol consumption increased over the follow-up in our study, no statistically significant interaction between APOE genotype and alcohol consumption was observed (data not shown). Therefore these environmental factors could not explain our gene × time interactions. They may represent a differential effect of aging on TC levels according to genotype, but we cannot rule out the possibility that our results are due to chance findings. This should be investigated further in other studies.
We investigated whether the replicated SNPs in aggregate could predict plasma cholesterol levels. A statistically significant positive trend was consistently observed in each round of survey with each count GRS point associated with an increase of 0.11 mmol/L in average TC levels. This effect is similar in size to the effect per count GRS point based on 21 independent SNPs in 9 genes or loci on LDL cholesterol levels recently observed by Talmud et al. [6]. These 21 SNPs could explain 14.6% of the LDL cholesterol variance. In our study, the variance in TC levels explained by the 12 SNPs was smaller (6.9%) and slightly higher in the low-fat cholesterol intake group compared to the high-fat (cholesterol) intake group.

### Table 3

<table>
<thead>
<tr>
<th>Count gene risk score (N)</th>
<th>p for trend</th>
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</thead>
<tbody>
<tr>
<td><strong>TC (1st measurement)</strong></td>
<td></td>
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<tr>
<td>≤11 (230)</td>
<td>0.0001</td>
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<tr>
<td>12 (316)</td>
<td>0.0001</td>
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<tr>
<td>13 (509)</td>
<td>0.0001</td>
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<tr>
<td>14 (661)</td>
<td>0.0001</td>
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<tr>
<td>15 (672)</td>
<td>0.0001</td>
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<tr>
<td>16 (495)</td>
<td>0.0001</td>
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<tr>
<td>≥17 (184)</td>
<td>0.0001</td>
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<tr>
<td>TC (2nd measurement)</td>
<td></td>
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<tr>
<td>&lt;12 (86)</td>
<td>0.0001</td>
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<tr>
<td>12–13 (95)</td>
<td>0.0001</td>
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<tr>
<td>13–14 (177)</td>
<td>0.0001</td>
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<tr>
<td>14–15 (313)</td>
<td>0.0001</td>
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<td>15–16 (364)</td>
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<td>16–17 (356)</td>
<td>0.0001</td>
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<tr>
<td>17–18 (177)</td>
<td>0.0001</td>
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<tr>
<td>TC (3rd measurement)</td>
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<tr>
<td>TC over three measurements</td>
<td></td>
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<tr>
<td>Weighted gene risk score (N)</td>
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<td>&lt;12 (86)</td>
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<td>12–13 (95)</td>
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<td>13–14 (177)</td>
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<td>17–18 (177)</td>
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</table>

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**TC:** plasma total cholesterol levels in mmol/L; values presented as mean ± SD.

*The count gene risk score represents the number of unfavorable alleles (raising total cholesterol levels) at 12 SNPs. These 12 SNPs were rs7412 and rs429358 in APOE, rs646776 in CELSR2, rs1367117 in APOB, rs662799 in APOA5, rs688 in LDLR, rs10889353 in DOCK7, rs2304130 in NCAN, rs3846662 in HMGCR, rs2275543 in ABCA1, and rs72725 in SMARCA4. The SNPs included in the weighted gene risk score computation were the same as in the count gene risk score. See Section 2 for detailed weighted gene risk score computation. The subjects who took lipid-lowering medication at the time of survey were excluded from the analysis.

*The analyses of association between weighted gene risk score and TC levels were conducted in a random half of the subjects (n = 1669, see Section 2 for the details).

*The explained variation in average TC levels: 5.3% for the count gene risk score and 7.2% for the weighted gene risk score.

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It is well understood that for a particular disease or trait, most individuals will have inherited mainly variants that confer risk of increased risk and some variants that provide protection, resulting in an overall risk around the average. However, a small proportion of people will have inherited mainly variants that confer risk of developing disease [32]. In our study, carrying ≥18 TC raising alleles (5% of the studied population) confers an OR of 2.4 of the carrier having hypercholesterolemia. This indicates that occupancy of extreme distributions of certain traits (hypercholesterolemia in this case) is achieved in some individuals by carriage of a large repertoire of common alleles of modest effect [32,34]. In others, it will result perhaps, from carrying a small number of rare alleles of large effect, e.g. in LDLR. Addition of the GRS significantly improved the discriminative accuracy of hypercholesterolemia beyond that afforded by conventional risk factors with a 3% increase of the AUC. However, the actual predictive ability might have increased even more, as the increase in AUC may be an insensitive measure of the improvement in risk prediction when a novel risk factor is considered [22]. Unfortunately, our current design prohibited us from estimating the predictive power of GRS more precisely by using other methods, such as net reclassification improvement [35]. Nevertheless, our results suggest that a gene risk score based on a panel of comprehensive and independent risk alleles could be used to help identify those people who are at risk of high plasma cholesterol levels and enable early preventive strategies. Such scores may be more relevant to cardiovascular disease risk prediction because it may reflect life-time exposure better compared to a single-time-point measurement of cholesterol levels [26,34]. A recent study demonstrated that a GRS composed of 11 SNPs (including most of our top SNPs, such as rs10889353, rs646776, rs3846662, rs2304130, rs6756629, etc.) was significantly associated with coronary heart disease and intima media thickness (even after adjusting for blood TC levels for intima media thickness) [4].

We adopted the false discovery rate method to adjust for multiple testing. As we took a candidate gene approach (and not random markers on the genome), Bonferroni correction would have been too stringent and we consider it justified to take a more liberal threshold of FDRq-value < 0.2. A number of factors could have resulted in a type II error, leading to the inability to detect a true underlying association. Firstly, only a limited number of SNPs within a candidate gene have been studied. Failure to find an association with a SNP does not exclude the possibility that other SNPs in the gene are related to TC levels. Secondly, our candidate gene list may still not be broad enough, as some genes that are recently found to be involved in the cholesterol metabolism were not taken into account, such as PCSK9 [5,7]. Our blood samples were taken from the subjects in a non-fasting condition. However, TC levels at most change minimally in response to normal food intake in individuals in the general population [36]; therefore, we think the non-fasting state has not influenced our results. In summary, in this relatively large, broad-gene based association study, we found that the common variants in genes in regulating cholesterol biosynthesis (HMGCR), VLDL metabolism (APOE, APOA5), LDL metabolism (APOB, LDLR), HDL metabolism (ABCA1, APOA5), intestinal or hepatic cholesterol efflux (ABC5) affect plasma TC levels. We also demonstrated that a panel of SNPs in genes pivotal in cholesterol metabolism could possibly help identify those people who are likely to have high plasma cholesterol levels.
Conflicts of interest

None declared.

Acknowledgements

The Doetinchem Cohort Study was financially supported by the Ministry of Health, Welfare and Sport of The Netherlands and the National Institute for Public Health and the Environment. The authors thank the epidemiologists and fieldworkers of the Municipal Health Service in Doetinchem for their contribution to the data collection for this study. Logistic management was provided by J Steenbrink and P Vissink, and administrative support by EP van der Wolf. Data management was provided by A Blokstra, AWV van Kessel and PE Steinberger. The authors would also like to thank B Hoebee for her role in the conception of the study, C Strien for their role in genotyping, and K Siezen for her assistance in SNP selection, and also E Reiling for critically reading the manuscript. Y Lu and A Vaarhorst are funded by grant 2006B195 of the Netherlands Heart Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2010.08.053.

References