Causal Relevance of Blood Lipid Fractions in the Development of Carotid Atherosclerosis

Mendelian Randomization Analysis

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**Background**—Carotid intima–media thickness (CIMT), a subclinical measure of atherosclerosis, is associated with risk of coronary heart disease events. Statins reduce progression of CIMT and coronary heart disease risk in proportion to the reduction in low-density lipoprotein cholesterol. However, interventions targeting triglycerides (TGs) or high-density lipoprotein cholesterol (HDL-C) have produced inconsistent effects on CIMT and coronary heart disease risk, making it uncertain whether such agents are ineffective for coronary heart disease prevention or whether CIMT is an inadequate marker of HDL-C or TG-mediated effects. We aimed to determine the causal association among the 3 major blood lipid fractions and common CIMT using mendelian randomization analysis.

**Methods and Results**—Genetic scores specific for low-density lipoprotein cholesterol, HDL-C, and TGs were derived based on single nucleotide polymorphisms from a gene-centric array in >50000 individuals (Cardiochip scores) and from a genome-wide association meta-analysis in >100000 individuals (Global Lipids Genetic Consortium scores). These were used as instruments in a mendelian randomization analysis in 2 prospective cohort studies. A genetically predicted 1 mmol/L higher low-density lipoprotein cholesterol concentration was associated with a higher common CIMT by 0.03 mm (95% confidence interval, 0.01–0.04) and 0.04 mm (95% confidence interval, 0.02–0.06) based on the Cardiochip and Global Lipids Genetic Consortium scores, respectively. HDL-C and TGs were not causally associated with CIMT.

**Conclusions**—Our findings confirm a causal relationship between low-density lipoprotein cholesterol and CIMT but not with HDL-C and TGs. At present, the suitability of CIMT as a surrogate marker in trials of cardiovascular therapies targeting HDL-C and TGs is questionable and requires further study.


Key Words: carotid intima–media thickness • lipids • mendelian randomization

Higher low-density lipoprotein cholesterol (LDL-C) concentration is associated with a higher risk of coronary heart disease (CHD), and the relationship is considered causal because randomized trials using LDL-lowering interventions such as statins have shown to reduce CHD risk in proportion to the LDL-C reduction. Interventions to elevate high-density lipoprotein cholesterol (HDL-C) or reduce triglycerides (TGs)
might also confer incremental protection against CHD, but thus far randomized trials of drugs directed at these 2 lipid fractions have been unable to confirm or refute such effects.

**Clinical Perspective on p 72**

Conclusive demonstration of the benefit and safety of new lipid-modifying interventions requires evaluation in large,
Methods

Study Participants

WHII recruited 10,308 participants (70% men) between 1985 and 1989 from 20 London-based Civil Service departments. The study was approved by the University College London (UCL) Research Ethics Committee, and participants gave informed consent to each aspect of the study. Clinical measurements were taken at 5-year intervals. Clinical data were available from 4 phases (phase 1, 1985–1988; phase 3, 1991–1993; phase 5, 1997–1999; and phase 7, 2003–2004). Phase 3 (1991–1993) provided the first comprehensive phenotyping and is considered the baseline phase.

The IMPROVE study recruited 3711 individuals (48% men) between March 2004 and April 2005 from 7 centers in 5 European countries with a median age of 64.4 years. Eligibility criteria included age between 55 and 79 years, presence of at least 3 vascular risk factors, absence of symptoms of cardiovascular diseases, and any conditions that might limit longevity or visualization of the carotid intima. The study was designed in accordance with the rules of Good Clinical Practice and with the ethical principles established in the Declaration of Helsinki. Informed consent was obtained from all participants. Baseline measures were available for this analysis.

Lipid and CIMT Measurements

In WHII, lipid measurements from phase 3 were used in this analysis because few participants were on lipid-modifying medication compared with follow-up phases, whereas ultrasound vascular measurements were only available at phase 7 (2003–2004). Measurement of serum lipids and common CIMT in WHII and IMPROVE is described in detail in the online-only Data Supplement Methods section.

Genotyping and Quality Control

For WHII participants, DNA was extracted from whole blood samples as previously described. Five thousand five hundred ninety-two samples were genotyped using the Illumina Human Cardiochip, and 3413 were later genotyped using the Illumina Metabochip. After quality control (filtering for duplicates, cryptic relatedness, ambiguous sex, self-reported nonwhites, outliers based on the genome-wide identity-by-state analysis implemented in PLINK, sample call rate <80%, and SNP call rate <98%), 5059 Cardiochip genotyped samples and 3126 Metabochip genotyped samples individuals were available for the analysis.

In the IMPROVE study, DNA was extracted as described by Baldassarre et al. Total, 3695 samples were genotyped using the Illumina Metabochip. After quality control (using call rate <95% and removing individuals for relatedness [confirmed or cryptic], reported non-European descent, outliers identified by multidimensional scaling, estimated inbreeding [excessive homozygosity], and mismatch between recorded and genotype-determined sex), 3430 individuals remained for this analysis.

Derivation of Lipid Genetic Scores

Two lipid genetic scores were derived: 1 from a discovery–analysis using the Illumina Human Cardiochip in WHII, and 1 based on lipid-associated variants reported by the GLGC.

Cardiochip Score

SNPs previously found to be associated with baseline LDL-C, HDL-C, or TGs in WHII were included in a stepwise variable selection scheme with the Bayesian Information Criterion, implemented separately for each chromosome, to select the best genetic predictors for each lipid trait. For each lipid fraction, the risk allele counts (LDL-C and TG-raising alleles and HDL-C–reducing alleles) for the selected SNPs were weighted using the risk-allele β-coefficient obtained from a ridge regression in WHII using the Lawless and Wang estimate of the ridge constant. The ridge regression is a variant of ordinary multiple linear regression that shrinks the β-coefficients of redundant SNPs, thereby circumventing issues that may arise if highly correlated SNPs are retained by the variable selection model. In both the variable selection and ridge regression, sex and age were added in the baseline model. The final genetic score for each participant was the sum of the weighted risk allele count. Apolipoprotein E (APOE) is a major determinant of LDL-C levels, and the effect of the APOE haplotype was included in the LDL score calculation. Of the
2 SNPs that determine the major APOE isoforms (APOE2, APOE3, and APOE4), only 1 (rs7412) is represented on the genome-wide and gene-centric platforms used in WHII and GLGC. In both studies, the 2 SNPs (rs7412 and rs429358) had been separately genotyped, and haplotypes determined.\textsuperscript{22,27} We used the previously reported effect of the APOE haplotypes on LDL-C, from a meta-analysis in 61,463 healthy participants, to generate a weighted APOE score. Based on the latter study, using ε3/ε3 individuals as reference,\textsuperscript{29} the APOE haplotype was scored as follows: ε2/ε2=-0.9, ε2ε3=-0.4, ε2ε4=-0.2, ε3ε3=0, ε3ε4=0.1, and ε4ε4=0.2. The SNPs used in the Cardiochip scores are shown in online-only Data Supplement Tables I–III. The same weights were used to calculate lipid genetic scores for individuals in the IMPROVE dataset.

GLGC Score
A threshold of $P<5\times10^{-8}$ was used by the GLGC to denote association between SNPs and lipid traits.\textsuperscript{19} For the purpose of the genetic score calculation, only the lead SNP from each locus was selected, and if a SNP was associated with >1 lipid fraction, it was only used in the genetic score calculation for the trait with which it had the most significant association $P$ value. Risk allele counts were calculated in WHII and IMPROVE and weighted using the reported univariate $\beta$-coefficients. Because the discovery of these SNPs was performed in an independent dataset and only a single SNP was selected at each locus, the issues of inflated instrument strength because of discovery bias and linkage disequilibrium were minimized. The SNPs used in the GLGC scores are shown in online-only Data Supplement Tables I–III.

For both sets of scores, SNPs not present in the data (because they were not represented on the genotyping platform or failed quality control) were excluded from the genetic score calculations. Individuals with missing genotypes were also excluded from the analysis.

Strength and Association of Lipid Levels With Lipid Genetic Scores
We evaluated the association of lipid levels with their respective genetic scores using linear regression, with no adjustment for covariates. The proportion of variance explained ($R^2$) and the F-statistic were reported as measures of the strength of each genetic score as an instrument.\textsuperscript{29} To show the benefit of using a combined genetic score as an instrument over a single SNP, we compared the $R^2$ of the GLGC scores (because these are not affected by discovery bias) with the most strongly associated SNPs for each trait, namely, rs51821 (proxy for the APOA5 SNP rs662799, previously reported to be associated with TG levels and CHD),\textsuperscript{19} rs17231506 (proxy for the CETP Taq1B polymorphism associated with HDL-C), and the APOE haplotypes that create the APOE2/ε3/ε4 isoforms.

Direct Associations of Blood Lipid Fractions With CIMT
Association of CIMT with lipid levels was determined using linear regression, with and without adjustment for sex, age, smoking, diabetes mellitus status, and statin use.

Instrumental Variable Analysis
To evaluate the causal association of each lipid fraction with CIMT, we performed an instrumental variable (IV) analysis using the 2-stage least squares method,\textsuperscript{11} whereby each genetic score was used as an instrumental variable for the unconfounded and unbiased effect of the respective lipid fraction on CIMT. No adjustment was made for covariates. A meta-analysis of the effect estimates was also performed using a fixed-effect model. We repeated the 2-stage least squares method analysis using lipid levels that were corrected for statin use. For statin users, the recorded lipid values were multiplied by a constant: LDL-C by 1.352, HDL-C by 0.949, and TG by 1.210. The multiplicative correction factors were based on analysis of repeatedly measured lipid levels, including levels measured before and after lipid-lowering treatment, in WHII. This methodology has been used in the most recent large-scale lipid meta-analysis.\textsuperscript{32}

## Results

### Study Characteristics
Population characteristics and sample sizes with both genotype and phenotype data are shown in Table 1. The mean age of IMPROVE participants in this analysis was 64.2 years (SD=5.4), similar to the mean age of WHII participants at the follow-up phase when CIMT measurements were taken (60.9 years [SD=6.0]). Mean CIMT in IMPROVE and WHII was 1.17 mm (±0.33) and 0.79 mm (±0.15), respectively. The lower mean LDL-C level in IMPROVE (3.55 mmol/L; SD=1.00) compared with WHII (4.37 mmol/L; SD=1.01) may partly be explained by the larger proportion of participants on statin medication (40% versus 0.9%, respectively).

### Cardiochip Lipid Genetic Scores
Seventeen SNPs (including the 2 APOE SNPs genotyped separately) were used for the LDL genetic score, and 12 and 13 SNPs, respectively, for the HDL and TG genetic scores (online-only Data Supplement Tables I–III). After applying quality control filters, all SNPs were available in the WHII dataset. In the IMPROVE dataset, 13 LDL (including 2 APOE SNPs), 11 HDL, and 9 TG SNPs were available for the score calculation.

### GLGC Lipid Genetic Scores
Of the lead SNPs reported by the GLGC meta-analysis, 12 (including 2 APOE SNPs) were used for the GLGC LDL score, and 29 and 16 SNPs, respectively, were used for the HDL and TG genetic scores (online-only Data Supplement Tables I–III). In WHII, all LDL SNPs, 28 of 29 HDL SNPs, and all TG SNPs were present. In IMPROVE, 10 (including 2 APOE SNPs) of 12 LDL SNPs, 28 of 29 HDL SNPs, and all TG SNPs were present in the genotype data after quality control filters were applied.

### Table 1. Cohort Characteristics

<table>
<thead>
<tr>
<th></th>
<th>WHII</th>
<th>IMPROVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>5059</td>
<td>3430</td>
</tr>
<tr>
<td>Men %</td>
<td>74</td>
<td>48</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>49.1 (5.9)</td>
<td>64.2 (5.4)</td>
</tr>
<tr>
<td>Follow-up</td>
<td>60.9 (6.0)</td>
<td>...</td>
</tr>
<tr>
<td>Mean CIMT (SD), cm</td>
<td>0.79 (0.15)</td>
<td>1.17 (0.33)</td>
</tr>
<tr>
<td>Baseline mean LDL-C (SD), mmol/L</td>
<td>4.37 (1.01)</td>
<td>3.55 (1.00)</td>
</tr>
<tr>
<td>Baseline mean HDL-C (SD), mmol/L</td>
<td>1.43 (0.41)</td>
<td>1.26 (0.36)</td>
</tr>
<tr>
<td>Baseline mean triglyceride (SD), mmol/L</td>
<td>1.44 (1.11)</td>
<td>1.59 (1.24)</td>
</tr>
<tr>
<td>Baseline % on statins</td>
<td>0.87</td>
<td>40.3</td>
</tr>
<tr>
<td>Number of participants with CIMT measurement</td>
<td>3617</td>
<td>3430</td>
</tr>
<tr>
<td>Cardiochip data</td>
<td>5059</td>
<td>0</td>
</tr>
<tr>
<td>Cardiochip data and CIMT</td>
<td>3256</td>
<td>0</td>
</tr>
<tr>
<td>Metabochip data</td>
<td>3126</td>
<td>3430</td>
</tr>
<tr>
<td>Metabochip data and CIMT</td>
<td>2138</td>
<td>3430</td>
</tr>
</tbody>
</table>

CIMT indicates carotid intima–media thickness; HDL-C, high-density lipoprotein cholesterol; IMPROVE, IMT Progression as Predictors of Vascular Events in a High Risk European Population; and LDL-C, low-density lipoprotein cholesterol.
Association of Lipid Levels With Lipid Genetic Scores

A 1 SD higher Cardiochip LDL genetic score was associated with 0.37 (95% CI = 0.34, 0.39) and 0.16 mmol/L (95% CI = 0.13, 0.2) higher LDL-C in WHII and IMPROVE, respectively. A 1 SD higher HDL genetic score was associated with -0.11 (95% CI = -0.12, -0.1) and -0.08 mmol/L (95% CI = -0.09, -0.07) lower HDL-C in WHII and IMPROVE, respectively. A 1 SD higher TGs genetic score was associated with 0.25 (95% CI = 0.22, 0.28) and 0.25 mmol/L (95% CI = 0.21, 0.29) higher TGs in WHII and IMPROVE, respectively. Differences in lipid levels associated with the GLGC genetic scores were in the same direction but were slightly lower in magnitude in both studies (Figure 1B).

Lipid Genetic Scores as Instruments for MR Analysis

The Cardiochip genetic scores explained 13% and 3% of the total variance in LDL-C, 7% and 5% of the variance in HDL-C, and 5% and 4% of the variance in TGs in WHII and IMPROVE, respectively. The GLGC genetic scores explained 11% and 2% of the total variance in LDL-C, 4% and 5% of the variation in HDL-C, and 2% and 2% of the variation in TGs levels in WHII and IMPROVE, respectively. All scores had large F-statistics (Table 2). The WHII $R^2$ and F-statistics for the Cardiochip scores are likely to be inflated because of discovery bias, and the higher sample size in WHII for the Cardiochip scores will also contribute to a higher F-statistic. The considerably lower $R^2$ values for the LDL genetic score in IMPROVE may reflect the large number of individuals on
Table 2. Strength of Genetic Instruments

<table>
<thead>
<tr>
<th></th>
<th>WHII IMPROVE</th>
<th>WHII IMPROVE</th>
<th>WHII IMPROVE</th>
<th>WHII IMPROVE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-Statistic</td>
<td>Sample Size</td>
<td>F-Statistic</td>
<td>Sample Size</td>
</tr>
<tr>
<td><strong>Cardiochip genetic scores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>0.13</td>
<td>697</td>
<td>90</td>
<td>4635</td>
</tr>
<tr>
<td>HDL</td>
<td>0.07</td>
<td>371</td>
<td>181</td>
<td>4745</td>
</tr>
<tr>
<td>TG</td>
<td>0.05</td>
<td>259</td>
<td>137</td>
<td>4760</td>
</tr>
<tr>
<td><strong>GLGC genetic scores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>0.11</td>
<td>366</td>
<td>75</td>
<td>3005</td>
</tr>
<tr>
<td>HDL</td>
<td>0.04</td>
<td>143</td>
<td>194</td>
<td>3052</td>
</tr>
<tr>
<td>TG</td>
<td>0.02</td>
<td>76</td>
<td>78</td>
<td>3062</td>
</tr>
</tbody>
</table>

F and F-statistic obtained from the first stage regression between lipid levels and the respective genetic scores.

GLGC indicates Global Lipids Genetic Consortium; HDL, high-density lipoprotein; IMPROVE, IMT Progression as Predictors of Vascular Events in a High Risk European Population Study; LDL, low-density lipoprotein; TG, triglyceride; and WHII, Whitehall II study.

In all cases, the genetic scores were much stronger instruments than individual SNPs. Comparison of the GLGC genetic scores with single SNPs in WHII is shown in online-only Data Supplement Figure I, which also shows that the genetic scores have improved specificity as instruments over single SNPs with respect to the 3 lipid fractions.

Direct Association of Blood Lipid Fractions With CIMT

After adjustment for age, sex, smoking, diabetes mellitus status, and statin use, only LDL-C and HDL-C were associated with CIMT (Table 3). A 1 mmol/L higher LDL-C was associated with 0.01 mm (95% CI, 0.006–0.02) and 0.02 mm (95% CI, 0.005–0.03) higher CIMT in WHII and IMPROVE, respectively. A 1 mmol/L higher HDL-C was associated with 0.02 mm (95% CI, 0.01–0.04) and 0.05 mm (95% CI, 0.02–0.08) lower CIMT in WHII and IMPROVE, respectively.

Instrumental Variable Analysis

Based on the meta-analysis of the estimates derived from the instrumental variable analysis, a 1 mmol/L higher LDL-C was associated with 0.03 mm (95% CI, 0.01–0.04; P=0.0002) and 0.04 mm (95% CI, 0.02–0.06; P<0.0001) higher CIMT, when using the Cardiochip and GLGC LDL genetic scores, respectively, as instruments (Figure 2). HDL-C and TGs were not found to be associated with CIMT using instrumental variable analysis (Figure 2). There was no change in the overall IV estimate when using lipid levels corrected for statin use (Figure 3).

Discussion

LDL-C, HDL-C, and TG genetic scores were used in a MR analysis to assess the causal relationship between each lipid fraction and CIMT. Although there was a positive association between directly measured LDL-C and CIMT and a negative association between directly measured HDL-C and CIMT, the results from the MR analysis support a causal association with LDL-C only. Despite differences in cohort characteristics (ie, healthier individuals and much smaller proportion on lipid-lowering medication in WHII compared with IMPROVE), the different effect of genetic score on lipid levels (smaller in the IMPROVE study) and the different SNPs used in the 2 genetic scores (Cardiochip versus GLGC), the association between LDL-C and CIMT was found to be consistent in both studies. Although the genetic scores were comparable instruments for HDL-C and TGs, no effect on CIMT was found for these 2 genetic scores.

One criterion for causality is assessing the concordance of effect estimates from different study designs. In our study, using GLGC genetic variants as instruments for LDL-C yielded a 0.04 mm difference in CIMT per mmol/L difference in LDL-C. To contextualize these findings, a meta-analysis of 11 statin trials found that after treatment with statins (mean treatment duration of 25.6 months), there was a significant reduction in the mean LDL-C (pretreatment, 168.6±33.3 mg/dL; posttreatment, 102.33±27.9 mg/dL; P<0.05; n=2132) and also a 0.04 mm (95% CI, 0.028–0.052) difference in mean CIMT between statin therapy arm and placebo arm (Bedi et al 2010). This is roughly equivalent to a 0.02 mm decrease in CIMT per mmol/L decrease in LDL-C, and therefore reasonably consistent with the genetic data.

Clinical Perspective

Randomized controlled drug trials with hard clinical end points require a large number of participants and follow-up

Table 3. Associations of the Major Lipid Fractions With Carotid IMT in the Whitehall II and IMPROVE Studies

<table>
<thead>
<tr>
<th>Lipid Phenotype</th>
<th>Study</th>
<th>(\beta) (Confidence Interval)</th>
<th>(P)</th>
<th>Adj. for Sex, Age, Smoking, Diabetes Mellitus Status, and Statin Use</th>
<th>(\beta) (95% Confidence Interval)</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL-C</td>
<td>WHII</td>
<td>0.02 (0.02 to 0.03)</td>
<td>(1 \times 10^{-15})</td>
<td>0.01 (0.006 to 0.02)</td>
<td>1 \times 10^{-05}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IMPROVE</td>
<td>0.002 (−0.009 to 0.01)</td>
<td>0.7</td>
<td>0.02 (0.005 to 0.03)</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>HDL-C</td>
<td>WHII</td>
<td>−0.03 (−0.04 to −0.01)</td>
<td>0.0001</td>
<td>−0.02 (−0.04 to −0.01)</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IMPROVE</td>
<td>−0.08 (−0.1 to −0.05)</td>
<td>(4 \times 10^{-07})</td>
<td>−0.05 (−0.08 to −0.02)</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>WHII</td>
<td>0.009 (0.004 to 0.01)</td>
<td>0.001</td>
<td>0.005 (−0.001 to 0.01)</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IMPROVE</td>
<td>−0.004 (−0.01 to 0.005)</td>
<td>0.50</td>
<td>−0.005 (−0.01 to 0.004)</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

Effect sizes are shown as mm change in CIMT per mmol/L change in lipid level. For IMPROVE, association is shown for the unadjusted analysis and adjusted for sex, age, smoking, diabetes status, and statin use.

CIMT indicates carotid intima–media thickness; HDL-C, high-density lipoprotein cholesterol; IMPROVE, IMT Progression as Predictors of Vascular Events in a High Risk European Population Study; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; and WHII, Whitehall II study.
over a long period, making them technically and financially challenging. As a result, clinically related, laboratory-derived surrogate end points have been used as alternative end points in these situations. CIMT has been used as a surrogate end point in many lipid-modifying drug trials to help inform later cardiovascular disease end-point trials.33–36 However, the suitability of CIMT as a surrogate marker in cardiovascular drug trials has come under debate because of conflicting trial results, for example, the Stop Atherosclerosis in Native Diabetics Study (SANDS)37 and Fenofibrate Intervention and Event Lowering in Diabetes (FIELD)38 trials. The underlying assumption in randomized trials using CIMT as a surrogate marker is that the rate of change in CIMT over time in response to drug therapies reflects the change in the risk of cardiovascular outcomes. The majority of CIMT trials have short follow-up periods and modest sample sizes and, therefore, lack power to identify associations with cardiovascular outcomes. Rather, they are designed to provide inferences on cardiovascular outcomes based on a presumed inverse relationship between atherosclerosis progression and cardiovascular benefit.39

A recent large-scale meta-analysis of 41 randomized trials assessing CIMT at baseline and follow-up after treatment,40 including 18,307 participants, concluded that regression of CIMT induced by cardiovascular drug therapies was not
associated with a reduction in cardiovascular events. Although the meta-analysis was technically sound, the heterogeneity in the interventions evaluated, the methods used for CIMT measurement, the outcome definition, study design, population characteristics, and follow-up time among the 41 trials may have reduced the ability to detect an association between CIMT and cardiovascular event reductions in such trials.

The aim of the Action to Control Cardiovascular Risk in Diabetes (ACCORD) lipid trial was to test whether treatment of patients with type 2 diabetes mellitus with fenofibrate, to increase plasma HDL-C levels and reduce plasma TG concentrations, would result in additional cardiovascular benefit compared with simvastatin (LDL-lowering) therapy alone. Although the addition of fenofibates to statin treatment did not show any significant reduction in clinical events in the placebo versus treatment groups, subgroup analyses suggested benefits of fenofibrate therapy in mixed dyslipidemia individuals. Similar results were reported in post hoc analyses performed in other fibrate studies. Therefore, an MR analysis to determine causality among HDL-C, TGs, and CIMT in a sufficiently large mixed dyslipidemia sample would be worthwhile.

Our findings suggest that CIMT is likely to be a reliable surrogate outcome measure in randomized trials of LDL-lowering therapy, but because of the lack of evidence for an

![Figure 3](http://circgenetics.ahajournals.org/)

**Figure 3.** Instrumental variable analysis using lipid levels corrected for statin use. Association of lipid fractions with carotid intima–media thickness obtained from the instrumental variable analysis in which lipid genetic scores act as instruments for the nonconfounded effect of each lipid fraction. Effect sizes and 95% confidence intervals in each study and summary estimates from a fixed-effect model are shown as millimeter change in carotid intima–media thickness per mmol/L change in lipid level. FE indicates fixed-effect; GLGC, Global Lipids Genetic Consortium; HDL, high-density lipoprotein; IMPROVE, IMT Progression as Predictors of Vascular Events in a High Risk European Population Study; LDL, low-density lipoprotein; and WHII, Whitehall II study.
association of HDL-C and TGs genetic scores, and CIMT, they cast doubt on the use of CIMT in trials of HDL-C- and TG-modifying therapies.

**Previous MR Studies Based on Lipid-associated SNPs**

To date, there have been few MR studies addressing association of lipids with CIMT or CHD. A study by Aulchenko et al.\(^2\) generated genetic scores for total cholesterol, LDL-C, HDL-C, and TGs based on 28 SNPs (identified before the GLGC study). They looked at the direct association of the genetic scores with CIMT and found only the total cholesterol genetic score to be associated with CIMT. However, they did not use an instrumental variable approach to estimate the causal effect. The GLGC\(^3\) found 4 lipid-associated SNPs to also be associated with CAD, but they did not attempt to quantify the causal effect of the lipid fractions using an instrumental variable approach. The Triglyceride Coronary Disease Genetics Consortium and emerging Risk Factor Collaboration compared the risk of genetically (using the APOA5 SNP rs662799) elevated TGs levels among more than 20,000 CHD cases and 35,000 controls.\(^4\) They concluded that there was a causal role for TG-mediated pathway(s) in CHD. However, the APOA5 variant was also associated with HDL-C levels in their study. The association of genetically determined TG levels with CHD was also attenuated to the null after adjusting not only for HDL, but also for non-HDL-C and other variables. This nonspecific effect of rs662799 compromises 1 key assumption for a valid MR analysis of a biomarker and complicates the interpretation of the results. The latter 2 studies did not look at the association of lipid-associated SNPs with CIMT, which is the objective of this study.

**Limitations**

The validity of the MR analysis may be compromised by (1) population stratification, where allele frequencies and disease rates differ between population subgroups; (2) pleiotropy, where genetic instruments affect the outcome through >1 intermediate risk factor, though this is not an issue for cis-acting SNPs used as instruments for a protein biomarker; (3) linkage disequilibrium, where another polymorphism in close proximity (and in linkage disequilibrium) to the variant of interest, is causing disease through another pathway; and (4) weak instrument bias. Analysis in the WHII was restricted to whites, and principal component analysis revealed no substantial population stratification. In the IMPROVE study, although all individuals were whites, there was population stratification that reflected the geographical location from which the samples were obtained.\(^2\) However, the SNPs used to generate the GLGC genetic scores were also discovered in individuals of European descent from the United States, Europe, or Australia. Therefore, the scores should be applicable to the general European population, and stratification is less likely to be an issue in this MR analysis.

In WHII, lipid measures used in the IV analysis were those from an earlier phase to CIMT measurements because the proportion of statin users at this earlier phase was very low. Analysis using lipid measures from the same phase as the CIMT measures gave similar results (provided on request). Although the 2 studies differ in design, the consistency of genetic associations with lipid traits across studies of differing design has been a feature of numerous genome-wide association studies. Therefore, we present the effect estimates both separately and combined.

Often genes act on multiple pathways and may, therefore, be associated with multiple intermediate phenotypes, especially those that act as transcription factors for other genes. Some SNPs included in the score may be independently associated with other cardiovascular risk factors, and, therefore, individually they would not be valid instruments. By combining these multiple SNPs into 1 score, the problem of pleiotropy can be reduced. An association with 1 polymorphism could have arisen by chance or confounding, but associations with >1 polymorphism in different genes marking the same exposure are unlikely unless the exposure is causal.\(^3\) Given the large number of lipid genetic variants that have been identified by different studies, it is possible to generate many independent combinations of such variants and from these many independent instrumental variable estimates of the causal effect of exposure of interest on outcome. It is important to note that instrument strength of the Cardiochip genetic scores in WHII are likely to be inflated because of discovery bias. However, both the Cardiochip and GLGC genetic scores, which used only partially overlapping SNP sets, supported the causal association of LDL-C with CIMT in each study. Using 2 different scores containing only partially overlapping SNP sets gives us confidence that the results are not biased by the SNP set used, as the 2 instruments are unlikely to be influenced by the same pleiotropy or linkage disequilibrium-induced confounding, and their consistency provides strong evidence against the notion that reintroduced confounding is generating the effect.\(^3\)

Both Cardiochip and GLGC LDL genetic scores were stronger instruments than the HDL and TG genetic scores in WHII, and it remains to be seen whether the addition of more SNPs that increase the HDL and TG instrument strength will alter the conclusions based on this analysis. However, it is important to note that all genetic scores had comparable instrument strength in the IMPROVE study, and despite the HDL genetic scores being the strongest instruments in this cohort, causality was only observed for LDL-C.

Our method for generating genetic scores makes several assumptions: additive effects of alleles, no gene–gene interactions, and a linear effect of lipids on CIMT. Although not explored in this work, if these assumptions did not hold, it would be possible to incorporate such knowledge into the model. An alternative to using a composite genetic score as an instrument is to use the SNPs as multiple instruments. Although this may improve the power, the large number of SNPs may potentially create a weak instrumental variable problem.\(^4\) A comparison of the different methodologies would be worthwhile but is beyond the scope of this report.

**Conclusion**

An MR analysis, using the instrumental variable regression approach, supports a causal association between LDL-C and CIMT, indicating CIMT to be a useful surrogate end point in clinical trials of LDL-lowering medications. Whether HDL-C
or TGs are causally associated with CIMT is uncertain. Thus, we conclude that, at present, the suitability of CIMT as a surrogate marker in trials of therapies targeting these lipid fractions is questionable and requires further study.

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Disclosures

Dr Casas reports being a coapplicant on a BHF grant of Genetics and holding membership on speaker’s bureaus for Novartis, Roche, AstraZeneca, Roche, and Danone. Dr Humphries reports receiving payment for speaker’s bureau at the Genzyme Meeting on Familial Hypercholesterolemia and being on the advisory board for Storegene, a genetic testing UCL spin-off company for determining CHD risk. All authors declare no other relationships or activities that could appear to have influenced the submitted work. Dr Holmes reports receiving support from an MRC Population Health Scientist Fellowship (G0802432). Dr Kivimäki reports receiving National Heart, Lung, and Blood Institute and MRC grants. Drs Hingorani reports receiving BHF program and project grants. Drs. Baldassarre and Tremoli report receiving funding from the European Commission (contract number QLG1-CT-2002-00896). The other authors report no conflicts.

References


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**CLINICAL PERSPECTIVE**

Carotid intima–media thickness (CIMT) (assessed by ultrasound) is a noninvasive measure of atherosclerosis associated with a higher risk of stroke and coronary heart disease events in prospective observational studies. CIMT is also widely used as a surrogate outcome measure in clinical trials. Statins reduce progression or induce regression of CIMT in proportion to the degree of low-density lipoprotein cholesterol lowering, an effect consistent with the reduction in risk of coronary heart disease events. However, the extent of any causal association between high-density lipoprotein cholesterol or triglycerides and CIMT and coronary heart disease events is less clear. This mendelian randomization analysis using common variants in genes primarily associated with low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, or triglycerides provided evidence for a causal association between low-density lipoprotein cholesterol and CIMT, but not between high-density lipoprotein cholesterol or triglycerides and CIMT. The findings support the use of CIMT as a surrogate outcome measure in trials of low-density lipoprotein cholesterol-lowering drugs. CIMT may be a less useful surrogate end point in clinical trials of primarily high-density lipoprotein cholesterol– or triglyceride-modifying therapies.
Causal Relevance of Blood Lipid Fractions in the Development of Carotid Atherosclerosis: Mendelian Randomization Analysis

Sonia Shah, Juan-Pablo Casas, Fotios Drenos, John Whittaker, John Deanfield, Daniel I. Swerdlow, Michael V. Holmes, Mika Kivimaki, Claudia Langenberg, Nick Wareham, Karl Gertow, Bengt Sennblad, Rona J. Strawbridge, Damiano Baldassarre, Fabrizio Veglia, Elena Tremoli, Bruna Gigante, Ulf de Faire, Meena Kumari, Philippa J. Talmud, Anders Hamsten, Steve E. Humphries and Aroon D. Hingorani

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SUPPLEMENTAL MATERIAL

Causal relevance of blood lipid fractions in the development of carotid atherosclerosis: Mendelian randomization analysis

Shah et al

Methods

Lipid and carotid intima-media thickness measurements

In WHII, serum for lipid analyses was refrigerated at -4°C and assayed within 72 hours. Cholesterol and triglycerides were measured with the use of a Cobas Fara centrifugal analyzer (Roche Diagnostics System, Nutley, NJ). HDL-cholesterol was measured by precipitating non-HDL cholesterol with dextran sulfate-magnesium chloride with the use of a centrifuge and measuring cholesterol in the supernatant fluid (1). LDL-C concentration was calculated using the Friedewald formula (2). Phase 3 provided the first comprehensive dataset for lipid measurements and was considered the baseline phase. Ultrasound vascular measurements were taken at Phase 7 (2003–2004). Participants were examined in a supine position, with the head turned to a 45 degree angle away from the side to be scanned. CIMT was measured in the right and left common carotid arteries. Longitudinal images of the common carotid artery, triggered on the R-wave of the ECG, were magnified and recorded in DICOM format as a cine loop, on the hard drive of the ultrasound machine for later analysis. The common CIMT was measured at its thickest part 1 cm proximal to the bifurcation. A measurement was taken between the leading edge of the intima and the media adventitia on 3 separate images on each side using electronic callipers and the mean of the 6 measures
was used for analysis. The overall coefficient of variation for repeated measures of CIMT was 4.7% (N = 89) (1).

In IMPROVE, blood sampling for laboratory tests was performed after an overnight fast. Serum was frozen at –80°C prior to shipment for centralized biochemical analyses and biobanking in Stockholm (Karolinska Institute Stockholm, Sweden). Serum concentrations of total, HDL cholesterol and triglycerides were analyzed in a centralized laboratory with the use of LX Beckman instruments. Cholesterol and triglycerides were measured with enzymatic methods. LDL-C concentration was calculated using the Friedewald (2). The CIMT variable selected for this analysis was the maximum IMT of the CC which was measured as follows: The far walls of the left and right common carotid (CC) artery were visualized in anterior, lateral, and posterior projections and recorded on sVHS videotapes. Measurements were taken at the thickest part of common carotids 1 cm proximal to the bifurcation. The far walls of the CCs in their entire length were measured in at least three different images on each side using dedicated software able to automatically recognize the leading edge of the intima and the media adventitia. For each segment the mean of the 6 measures was used in this analysis. The overall coefficient of variation for repeated measures of CIMT was 3.9% (N = 121) (3).

Genotyping and quality control

For WHII participants, DNA was extracted from blood samples (via magnetic bead technology; Medical Solutions, Nottingham, UK) and normalized to a concentration of 50 ng/μl. 5592 samples were genotyped using the Illumina Human Cardiochip(4) and 3413 of these were also genotyped using the Illumina CardioMetabochip (also referred to as the
Illumina Metabochip) (5). Genotyping of the two APOE snps (rs7412 and rs429358) was previously carried out using a universal heteroduplex generator (6).

In the IMPROVE study, several biological samples were kept in a biobank. Specifically, the biobank contains 14 aliquots of 0.5 mL EDTA plasma and 8 aliquots of 0.5 mL serum for each subject. In addition, for each subject, $2 \times 5$ mL whole blood was stored for DNA extraction. DNA was purified (in the Atherosclerosis Research Unit, Karolinska Institute Stockholm, Sweden) from all patients who signed informed consent for genetic studies. In total 3695 samples were genotyped using the Illumina CardioMetabochip.

References


### Supplementary Table 1: LDL Gene Scores

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Gene</th>
<th>In Cardiochip Score</th>
<th>In GLGC Score</th>
<th>GLGC Risk Allele</th>
<th>Univariate Association in WHII</th>
<th>Univariate Association in IMPROVE</th>
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*NA – SNP not present in dataset*
### Supplementary Table 2: HDL Gene Score

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## Supplementary Table 3: Triglyceride Gene Score

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<th>Present in GLGC Score</th>
<th>Risk Allele</th>
<th>Univariate Association in WHII</th>
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Total: 13 15
Supplementary figure 1: Strength and specificity of GLGC gene scores inWHII

The figure compares the strength and specificity of single SNPs versus gene scores as instruments for lipid fractions. The proportion of variance in observed LDL-C, HDL-C and triglyceride levels that is explained by each genetic instrument (R^2 derived from the regression of observed lipid levels with the genetic instrument) is shown as a measure of the strength of the instrument for that lipid fraction. Specificity of each instrument is alluded by the comparison of R^2 for a lipid-fraction-specific instrument with other lipid fractions e.g. R^2 derived from the regression of LDL score with LDL-C levels compared to R^2 derived from the regression of LDL score with HDL-C or triglyceride levels.