Relationship Between Nonalcoholic Fatty Liver Disease Susceptibility Genes and Coronary Artery Disease

Martijn C.G.J. Brouwers,1,2 Nynke Simons,1,2,3 Coen D.A. Stehouwer,2,4 Ger H. Koek,5-7 Nicolaas C. Schaper,1,2 and Aaron Isaacs2,8,9

Coronary artery disease (CAD) is the principal cause of death in patients with nonalcoholic fatty liver disease (NAFLD). The aim of the present study was to investigate whether NAFLD is causally involved in the pathogenesis of CAD. For this, previously reported NAFLD susceptibility genes were clustered and tested for an association with CAD in the Coronary Artery Disease Genome-Wide Replication and Meta-Analysis plus the Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) Consortium data set. The role of plasma lipids as a potential mediator was explored by using data from the Global Lipids Genetics Consortium. Statistical analyses revealed that the combination of 12 NAFLD genes was not associated with CAD in 60,801 CAD cases and 123,504 controls (odds ratio [OR] per NAFLD risk allele, 1.0; 95% confidence interval [CI], 0.99-1.00). In a subsequent sensitivity analysis, a positive relationship was observed after exclusion of gene variants that are implicated in NAFLD through impaired very low-density lipoprotein secretion (i.e., microsomal triglyceride transfer protein [MTTP], patatin-like phospholipase domain containing 3 [PNPLA3], phosphatidylethanolamine N-methyltransferase [PEMT], and transmembrane 6 superfamily member 2 [TM6SF2]) (OR, 1.01; 95% CI, 1.00-1.02). Clustering of the excluded genes showed a significant negative relationship with CAD (OR, 0.97; 95% CI, 0.96-0.99). A substantial proportion of the observed heterogeneity between the individual NAFLD genes in relation to CAD could be explained by plasma lipids, as reflected by a strong relationship between plasma lipids and CAD risk conferred by the NAFLD susceptibility genes ($r = 0.76; P = 0.004$ for low-density lipoprotein cholesterol). Conclusion: NAFLD susceptibility genes do not cause CAD per se. The relationship between these genes and CAD appears to depend to a large extent on plasma lipids. These observations strongly suggest taking plasma lipids into account when designing a new drug to target NAFLD. (Hepatology Communications 2019;3:587-596).

Nonalcoholic fatty liver disease (NAFLD) is an emerging cause of progressive liver inflammation, fibrosis, cirrhosis, end-stage liver failure, and hepatocellular carcinoma in Western society. It has been estimated that NAFLD will be the principal cause of end-stage liver disease requiring liver transplantation by 2025.1 However, despite these alarming data concerning liver-related outcomes, most patients with NAFLD eventually die of cardiovascular complications.2 In

Abbreviations: ADIPOQ, adiponectin; C1Q and collagen domain containing; CAD, coronary artery disease; CARDIoGRAMplusC4D, Coronary Artery Disease Genome-Wide Replication and Meta-Analysis plus the Coronary Artery Disease Genetics; CI, confidence interval; CVD, cardiovascular disease; ERLIN1-CHUK-CWF19L, endoplasmic reticulum lipid raft associated 1-conserved helix-loop-helix ubiquituous kinase-CWF19-like cell cycle control factor 1; FADS, fatty acid desaturase; GCKR, glucokinase regulator; GWAS, genome-wide association study; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LYPLAL1, lysophospholipase-like 1; MBOAT7, membrane bound O-acyltransferase domain containing 7; MTTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; PEMT, phosphatidylethanolamine N-methyltransferase; PNPLA3, patatin-like phospholipase domain containing 3; PPP1R3B, protein phosphatase 1 regulatory subunit 3B; SNP, single-nucleotide polymorphism; TM6SF2, transmembrane 6 superfamily member 2; TRIB1, tribbles pseudokinase 1; VLDL, very low-density lipoprotein.

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their meta-analysis, Targher and colleagues recently showed that NAFLD is indeed associated with cardiovascular disease (CVD). These studies were, by design, not capable of designating NAFLD as either a causal factor or an innocent bystander. Because many patients with NAFLD display features of the metabolic syndrome, other factors, such as dyslipidemia, hypertension, and hyperglycemia, could account for the relationship between NAFLD and CVD. On the other hand, it has been suggested that NAFLD, in particular nonalcoholic steatohepatitis, is by itself linked to CVD by proinflammatory cytokines.

Genetic epidemiology may provide a solution to this conundrum. Because an individual is “randomized” at birth to receive either a gene variant that protects from or predisposes to a particular trait (in this case NAFLD), the association between this gene variant and CVD will provide an answer as to whether or not that trait is causally involved in the pathophysiologic process. Such a Mendelian randomization approach has already identified plasma low-density lipoprotein (LDL) cholesterol, triglycerides, and (postprandial) glucose levels as causal factors, whereas the inflammatory factor C-reactive protein has been ruled out.

To date, data on NAFLD genes in relation to CVD have been scarce. Previous studies on the transmembrane 6 superfamily member 2 (TM6SF2) gene have convincingly shown that variants in this gene predispose to all stages of NAFLD but protect from CVD. We recently reported a similar disentanglement for the patatin-like phospholipase domain containing 3 (PNPLA3) gene, the most replicated NAFLD gene. These findings question the presence of a causal, positive relationship between NAFLD/nonalcoholic steatohepatitis and CVD, although both PNPLA3 and TM6SF2 are also associated with lower secretion of very low-density lipoprotein (VLDL) particles, which may account for the protection from CVD.

In the current study, we sought to gain more insight into the causal relationship between NAFLD and coronary artery disease (CAD). For this, we clustered previously reported common NAFLD gene variants and tested for an association with CAD. Given the complex relationship between NAFLD and dyslipidemia, which is a major cardiovascular risk factor by itself, we additionally studied the association of these variants with plasma lipids and their role in explaining the relationship with CAD.

**Materials and Methods**

**SELECTION OF NAFLD SUSCEPTIBILITY GENES**

Selection of NAFLD susceptibility genes started with recent concise overviews on this topic and...
was subsequently expanded by a Medline search for “NAFLD” and “gene.” Because some genetic association studies have been conducted in relatively small populations and are therefore more likely to be underpowered, a stringent selection procedure was applied. A NAFLD gene was included in the present study when one of the following criteria was fulfilled: (1) The gene was identified by genome-wide association studies (GWASs) for NAFLD, as diagnosed by either histology or imaging; (2) the gene was identified by GWASs for a NAFLD-related trait, e.g., liver enzymes or alcoholic fatty liver disease (AFLD), and subsequently confirmed in a NAFLD cohort; or (3) the gene was reported by multiple case-control studies in NAFLD cohorts and subsequently confirmed by meta-analysis.

ASSOCIATION OF NAFLD SUSCEPTIBILITY GENES WITH CAD

The association between the NAFLD genes and CAD was assessed under the assumption of an additive mode of inheritance in the Coronary Artery Disease Genome-Wide Replication and Meta-Analysis plus the Coronary Artery Disease Genetics (CARDIoGRAMplusC4D) Consortium cohort. This cohort contains 8.6 million genetic variants from 48 GWASs, either case-control studies (~75%) or (population-based) prospective cohort studies. The median number of participants per study was 2,874 (interquartile range, 1,557-5,291). The overall data set includes 60,801 cases with CAD and 123,504 controls of predominantly European and Asian ancestry. Although the definition of CAD differed to some extent between the studies that were part of the CARDIoGRAMplusC4D cohort, it generally included myocardial infarction, acute coronary syndrome, coronary artery bypass grafting, coronary angioplasty, (un)stable angina, and/or coronary stenosis >50%. Approximately 70% of the CAD cases had a reported history of myocardial infarction, which was generally defined as the combination of symptoms, typical abnormalities on electrocardiogram, and elevation of cardiac enzymes.

The association between NAFLD susceptibility genes and myocardial infarction was studied as a secondary outcome.

The summarized data on CAD and myocardial infarction were contributed by CARDIoGRAMplusC4D investigators and downloaded from www.cardiogram-plusc4d.org (data set “CARDIoGRAMplusC4D 1000 Genomes-based GWAS”).

ASSOCIATION OF NAFLD SUSCEPTIBILITY GENES WITH PLASMA LIPIDS

Because plasma lipid levels were not available in CARDIoGRAMplusC4D, the relationships between the NAFLD-associated polymorphisms and plasma lipids, i.e., total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides, were studied by using the published joint Metabochip and GWAS data from the Global Lipids Genetics Consortium (n = 188,577 European-ancestry individuals).

In case a genetic variant was not present in this database, the analysis was conducted with a neighboring variant that is in strong linkage disequilibrium ($r^2 > 0.8$).

STATISTICAL ANALYSES

Clustering of the NAFLD susceptibility genes to test for an association with CAD and myocardial infarction was done by constructing a weighted fixed-effects model, which is similar to an inverse-variance weighted meta-analysis (see Supporting Materials for more details). The effect sizes and variances of the individual genes were derived from the CARDIoGRAMplusC4D data set. The final weighted-fixed effects model provides an estimate of the weighted average of the true effects in the set of genes. Because an additive model was used, this weighted average should be interpreted as the CAD risk conferred by one (average) NAFLD risk allele. To study the impact of genes that affect NAFLD risk by impairment of VLDL secretion (and thereby decreased plasma lipid levels), we additionally conducted a sensitivity analysis after exclusion of these genes.

Because NAFLD status was not present in CARDIoGRAMplusC4D and previous genetic studies have assessed NAFLD by different methods (e.g., biopsy, computed tomography, ultrasound) and expressed NAFLD in different ways (e.g., dichotomous, ordinal, and continuous), an analysis in which
the gene-exposure relationship is regressed against the
gene-outcome relationship was deemed unreliable.
Finally, the contribution of plasma lipids in explain-
ing the observed heterogeneity was evaluated with
meta-regression. All analyses were conducted with R
statistical software using the metafor package.(19)

Results

SELECTION OF NAFLD SUSCEPTIBILITY GENES

The literature search resulted in 12 NAFLD susceptibility variants, of which five were selected
based on previous GWASs for NAFLD (lysophos-
pholipase-like 1 \([LYPLAL1]\), glucokinase regulator,
\([GCKR]\), protein phosphatase 1 regulatory subunit
3B \([PPP1R3B]\), PNPLA3, and TM6SF2),\(^{(8,10,20)}\)
four from previous GWASs for NAFLD-related
traits combined with at least one case-control study
in NAFLD (tribbles pseudokinase 1 \([TRIB1]\), fatty
acid desaturase \([FADS1-2-3]\), endoplasmic reticu-



| Chr | Gene       | Variant\(^*\) | A1/A2\(^*\) | Effect Allele (A1) Frequency\(^*\) | GWAS for NAFLD | GWAS for Related Trait\(^\dagger\) | Meta-Analysis on NAFLD |
|-----|------------|---------------|-------------|---------------------------------|----------------|-------------------------------|----------------------|
| 1   | LYPLAL1    | rs12137855    | C/T         | 0.80                            | +              | -                            | -                    |
| 2   | GCKR       | rs1260326     | T/C         | 0.39                            | +              | -                            | -                    |
| 3   | ADIPOQ     | rs2241766     | G/T         | 0.12                            | -              | +                            | +                    |
| 4   | MTTP       | rs1800591     | T/G         | 0.26                            | -              | +                            | -                    |
| 8   | PPP1R3B    | rs4240624     | A/G         | 0.87                            | +              | -                            | -                    |
| 8   | TRIB1      | rs2954021     | A/G         | 0.50                            | -              | +                            | -                    |
| 8   | ERLIN1–CHUK–WF19L1 | rs11597086 | A/C         | 0.64                            | -              | +                            | -                    |
| 11  | FADS1      | rs174546      | T/C         | 0.31                            | -              | +                            | -                    |
| 17  | PEMT       | rs7946        | A/G         | 0.65                            | -              | -                            | +                    |
| 19  | MBOAT7     | rs641738      | T/C         | 0.43                            | -              | +                            | -                    |
| 19  | TM6SF2     | rs58542926    | T/C         | 0.07                            | +              | -                            | -                    |
| 22  | PNPLA3     | rs738409      | G/C         | 0.24                            | +              | -                            | -                    |

\(^*\) In CARDIoGRAMplusC4D.

\(^\dagger\) Effect allele (A1) refers to the allele that predisposes to NAFLD.

\(^\dagger\) In combination with a genetic association study for NAFLD (see Materials and Methods).
Table S1; Supporting Fig. S3). As expected, clustering of the four excluded genes resulted in a negative relationship with CAD (OR, 0.97; 95% CI, 0.96-0.99; 

\[ P < 0.0001 \]) (Supporting Fig. S3).

**TABLE 2. NAFLD SUSCEPTIBILITY SNPS IN RELATION TO PLASMA LIPIDS**

| Gene              | Odds Ratio [95% CI] |  |
|-------------------|---------------------|---|
| LYPLAL1           | 1.02 [1.00, 1.04]   |  |
| GCKR              | 1.00 [0.98, 1.02]   |  |
| ADIPOQ            | 1.01 [0.98, 1.04]   |  |
| MTP               | 0.96 [0.94, 0.98]   |  |
| PPP1R3B           | 1.00 [0.97, 1.03]   |  |
| TRIB1             | 1.05 [1.03, 1.06]   |  |
| ERLL1–CHUK–CWF19L1| 1.00 [0.98, 1.02]   |  |
| FADS1             | 0.98 [0.96, 1.00]   |  |
| PEMT              | 0.99 [0.97, 1.01]   |  |
| MBOAT7            | 1.02 [0.99, 1.04]   |  |
| TM6SF2            | 0.95 [0.92, 0.98]   |  |
| PNPLA3            | 0.98 [0.96, 1.00]   |  |

FIG. 1. Relationship between NAFLD susceptibility genes and CAD.

**TABLE 2. NAFLD SUSCEPTIBILITY SNPS IN RELATION TO PLASMA LIPIDS**

| Gene              | Total Cholesterol | LDL Cholesterol | HDL Cholesterol | Triglycerides |
|-------------------|-------------------|-----------------|-----------------|--------------|
|                  | \( \beta \) (SE)  | \( P \) Value   | \( \beta \) (SE) | \( P \) Value |
| LYPLAL1 rs12137855| 0.022 [0.009]     | 3 × 10–4        | 0.022 [0.009]   | 3 × 10–4     |
| GCKR rs1260326    | 0.018 [0.007]     | 1 × 10–3        | 0.018 [0.007]   | 1 × 10–3     |
| ADIPOQ rs266729   | 0.012 [0.006]     | 1 × 10–2        | 0.012 [0.006]   | 1 × 10–2     |
| MTP rs3816873     | 0.008 [0.004]     | 5 × 10–3        | 0.008 [0.004]   | 5 × 10–3     |
| PPP1R3B rs4240624 | 0.006 [0.003]     | 3 × 10–2        | 0.006 [0.003]   | 3 × 10–2     |
| TRIB1 rs2954029   | 0.004 [0.002]     | 4 × 10–2        | 0.004 [0.002]   | 4 × 10–2     |
| ERLL1–CHUK–CWF19L1| 0.003 [0.001]     | 1 × 10–1        | 0.003 [0.001]   | 1 × 10–1     |
| FADS1 rs174546    | 0.002 [0.001]     | 7 × 10–2        | 0.002 [0.001]   | 7 × 10–2     |
| PEMT rs7946       | 0.001 [0.001]     | 2 × 10–1        | 0.001 [0.001]   | 2 × 10–1     |
| MBOAT7 rs641738   | 0.0005 [0.0004]  | 1 × 10–3        | 0.0005 [0.0004] | 1 × 10–3     |
| TM6SF2 rs58842926| 0.0003 [0.0002]  | 1 × 10–3        | 0.0003 [0.0002] | 1 × 10–3     |
| PNPLA3 rs3869409  | 0.0001 [0.0001]  | 2 × 10–5        | 0.0001 [0.0001] | 2 × 10–5     |

*Data derived from the Global Lipids Genetics Consortium (see Materials and Methods).

Data includes individuals for the analysis with LDL cholesterol (an additional for the analysis with LDL cholesterol); for the other lipid traits generally include more individuals.*

**RELATIONSHIP BETWEEN NAFLD GENE VARIANTS AND PLASMA LIPIDS**

The association of the individual NAFLD SNPs with plasma lipids was subsequently assessed to evaluate the impact of plasma lipids on the observed relationships with CAD. Results for total cholesterol, LDL cholesterol, HDL cholesterol, and triglyceride levels were obtained from the Global Lipids Genetics Consortium and listed in Table 2. The effects of the individual NAFLD SNPs on plasma lipids were subsequently plotted (on the x axis) versus their effects on CAD risk (on the y axis) (Fig. 2). A strong correlation was observed for both total cholesterol and
LDL cholesterol with CAD \( (r = 0.73 \text{ and } P = 0.008; \ r = 0.76 \text{ and } P = 0.004; \text{Fig. } 2, \text{ panels A and B, respectively) but not for plasma triglycerides and HDL cholesterol} \ (r = 0.57 \text{ and } P = 0.05; \ r = -0.14 \text{ and } P = 0.66; \text{Fig. } 2, \text{ panels C and D, respectively). Similar results were observed when myocardial infarction was used as an outcome (Supporting Fig. S4).}

**Discussion**

The Mendelian randomization approach has demonstrated its utility in identifying causal factors in the pathogenesis of CAD not only for plasma biomarkers (e.g., LDL cholesterol) but also for traits such as body mass index and waist-to-hip ratio.\(^{31}\) The
current study shows that NAFLD susceptibility genes do not cause CAD per se.

One of the key assumptions of the Mendelian randomization approach is that the gene variants that affect the exposure of interest do not exert pleiotropic effects. The risk of pleiotropy increases when the exposure of interest is a complex, nonprotein phenotype,\(^{(5)}\) as is the case for NAFLD. Given the central role the liver plays in (systemic) metabolic processes, it is difficult to identify genes that only affect NAFLD risk. In this respect, a distinction should be made between vertical and horizontal pleiotropy (Supporting Fig. S5). Pleiotropy is vertical when the gene variant is associated with a trait or biomarker that is downstream in the pathway that relates the exposure of interest with the clinical endpoint (CAD). Because this is a mediating effect, it does not invalidate the Mendelian randomization assumptions. In contrast, pleiotropy is horizontal when the gene variant affects pathways other than the exposure of interest.\(^{(31)}\) The complex intertwined relationship between NAFLD genes and plasma lipids probably results in both horizontal and vertical pleiotropy in the present study. Stable isotope studies have demonstrated that an increased flux of fatty acids and \textit{de novo} lipogenesis contribute to the development of hepatic fat accumulation and consequently drive VLDL production.\(^{(32)}\) The observed associations of SNPs affecting fatty acid flux and \textit{de novo} lipogenesis with plasma lipids (e.g., \textit{TRIB1} and \textit{GCKR}) are therefore likely downstream effects of NAFLD and hence an example of vertical pleiotropy. Those SNPs that predispose to NAFLD through impaired VLDL secretion (i.e., \textit{MTTP}, \textit{PEMT}, \textit{PNPLA3}, and \textit{TM6SF2}) simultaneously decrease plasma lipids. Because NAFLD does in this case not precede the effect on plasma lipids, it could be viewed as an example of horizontal pleiotropy (Supporting Fig. S5). Of interest, when we conducted a sensitivity analysis without the latter genes, a positive relationship with CAD was observed.

Exclusion of those SNPs that affect NAFLD through impaired VLDL secretion does not necessarily eliminate all horizontal pleiotropy. Many SNPs were not specifically expressed in the liver (Supporting Table S1). Furthermore, the neutral effect of one liver-specific gene (\textit{GCKR}) on CAD may be explained by reported protective effects on kidney function.\(^{(33)}\) The use of a polygenic risk score, as done in the present study, may “balance out” horizontal pleiotropy.\(^{(31)}\) This can be formally tested by using Mendelian randomization–Egger regression or a weighted median method. Both methods require both gene-exposure and gene-outcome data.\(^{(34,35)}\) Unfortunately, gene-exposure data were not available for reasons outlined in the Materials and Methods section. This should be regarded as a limitation of this study.

The putative mechanism linking NAFLD to CAD has been a topic of interest in comprehensive reviews. Although low-grade inflammation has often been suggested as the principal mediator,\(^{(36)}\) the present study strongly suggests that plasma lipids are more important. Many of the NAFLD genes that were included in the present study not only predispose to hepatic fat accumulation but also to steatohepatitis.\(^{(8,10,20,22,26,37)}\) Nevertheless, there was a strong relationship between plasma lipids and CAD, as conferred by the 12 NAFLD susceptibility SNPs, which is very much in line with a previous Mendelian randomization study in which 185 lipid-susceptibility variants from these same cohorts were used to examine the causal relation between plasma lipids and CAD.\(^{(38)}\) Moreover, the intercepts of these relationships included zero (Fig. 2), which implicates that when the NAFLD susceptibility SNPs do not have any effect on plasma lipids, there is also no effect on CAD. This suggests that other vertical pleiotropic effects (such as low-grade inflammation) or unbalanced horizontal pleiotropy do not play a major role in our analyses because they would have resulted in an intercept deviating from zero. Unfortunately, we did not dispose of a set of inflammatory markers to formally investigate the mediating role of inflammation on the relationship between NAFLD and CAD.

The current observations have important implications for future therapies that target NAFLD. Genetic studies have been instructive in predicting pharmacologic effects. An illustrative example is proprotein convertase subtilisin kexin type 9. Studies demonstrating that lipid-lowering variants in the proprotein convertase subtilisin kexin type 9 gene protected from CAD preceded the recently reported results from phase 3 clinical trials.\(^{(39-42)}\) The strong relationship between plasma lipids and CAD risk conferred by the NAFLD susceptibility variants strongly suggests that the design of an anti-NAFLD drug should take into account any effects on plasma lipids. Otherwise, the beneficial effects on NAFLD may be offset by an increased risk to develop CAD. Genes in the right
upper quadrant (Fig. 2A–C) therefore appear to be the best therapeutic candidates.

This study has some strengths and limitations besides the previously mentioned issues regarding (horizontal) pleiotropy. The CARDIoGRAMplusC4D data set with more than 180,000 individuals genotyped for common and low-frequency variants allowed us to study the relationship between reported NAFLD susceptibility genes and CAD with sufficient statistical power. Because this data set contains summarized data, it was not possible to perform subgroup analyses stratified for sex or ethnicity.

Common gene variants generally have small effect sizes, which probably explains the modest CAD risks conferred by the NAFLD genes. Of interest, gene variants in TM6SF2 have been shown to confer a large effect on NAFLD, plasma lipids, and CAD risk. In contrast, previous studies have reported a large effect size for the association between PNPLA3 and NAFLD, whereas a small effect was observed for the association with plasma lipids and CAD in the current study. These data corroborate the suggestion that the effect size of NAFLD gene variants on CAD risk is related to their effects on plasma lipids and not on liver disease per se, which was indeed confirmed in a recent Mendelian randomization study in which PNPLA3 was used as an instrument.

PNPLA3 and TM6SF2 as well as MBOAT7 have not only been associated with NAFLD but also with other liver diseases, such as alcoholic fatty liver disease (AFLD). Although NAFLD is currently the most common liver disease in Western society, it is likely that patients with AFLD have also been included in CARDIoGRAMplusC4D. The conclusions drawn in the present study are therefore probably not confined to NAFLD but to hepatic fat accumulation in general.

Unfortunately, data on plasma lipids or other potential mediators/confounders of the relationship between NAFLD and CAD, such as type 2 diabetes, were not present in the CARDIoGRAMplusC4D data set. Previous GWASs have shown that variants in PNPLA3 and TM6SF2 are also associated with type 2 diabetes. We tried to resolve this, at least for plasma lipids, by using data from the well-recognized Global Lipids Genetics Consortium, in line with previous reports. Although, the consequential strong relationship between plasma lipids and CAD, as conferred by the NAFLD susceptibility genes (Fig. 2), suggests an active role of plasma lipids, the lack of individual lipid data in CARDIoGRAMplusC4D limits strong inferences on the role of plasma lipids as a mediator of the relationship between NAFLD and CAD.

We applied stringent selection criteria for the NAFLD susceptibility genes, which makes the inclusion of false-positive NAFLD variants unlikely. A disadvantage is that the number of genes included in the analyses was relatively low, which may have reduced statistical power. Finally, these gene variants can be in linkage disequilibrium with a variant in another gene, which is actually responsible for the observed association.

In summary, the present study nuances previous epidemiologic studies by demonstrating that NAFLD susceptibility genes do not unequivocally cause CAD. Further studies that focus on the mediating effects of plasma lipids are warranted because plasma lipids accounted for a substantial degree of the observed heterogeneity. These observations have important implications for future therapeutic strategies against NAFLD.

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Author names in bold designate shared co-first authorship.

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