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Relationship between NAFLD and coronary artery disease: A Mendelian randomization study

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Abstract

Background and Aims: There is an ongoing debate on whether NAFLD is an active contributor or an innocent bystander in the pathogenesis of coronary artery disease (CAD). The aim of the present study was to assess the causal relationship between NAFLD and CAD.

Approach and Results: We performed two-sample Mendelian randomization (MR) analyses using summary-level data to assess the association between genetically predicted NAFLD (i.e., chronically elevated serum alanine aminotransferase levels [cALT], imaging-based and biopsy-confirmed NAFLD) and risk of CAD. Analyses were repeated after exclusion of NAFLD susceptibility genes that are associated with impaired VLDL secretion. Inverse-variance weighted MR analyses showed a statistically significant association between genetically predicted cALT and risk of CAD (OR: 1.116, 95% CI: 1.039, 1.199), but not for the other NAFLD-related traits (OR: 1.046, 95% CI: 0.764, 1.433 and OR: 1.014, 95% CI: 0.968, 1.062 for imaging-based and biopsy-confirmed NAFLD, respectively). MR-Egger regression revealed a statistically significant intercept, indicative of directional pleiotropy, for all traits. Repeat analyses after exclusion of genes associated with impaired VLDL secretion showed consistent associations between genetically predicted NAFLD and CAD for all traits (i.e., cALT [OR: 1.203, 95% CI: 1.113, 1.300]), imaging-based (OR: 2.149, 95% CI: 1.276, 3.620) and biopsy-confirmed NAFLD (OR: 1.113, 95% CI: 1.041, 1.189), which persisted when more stringent biopsy-confirmed NAFLD criteria were used (OR: 1.154, 95% CI: 1.043, 1.278) or when more stringent MR methods were applied. MR-Egger regression did not show a statistically significant intercept.

Abbreviations: ALT, alanine transaminase; CAD, coronary artery disease; cALT, chronically elevated serum ALT levels; GWAS, genome-wide association study; IVW, inverse variance weighted; LD, linkage disequilibrium; MR, Mendelian randomization; PNPLA3, patatin-like phospholipase domain containing protein 3; PRESSO, pleiotropy residual sum and outlier; SNP, single nucleotide polymorphism.

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Conclusion: The two-sample MR analyses showed a robust association between genetically predicted NAFLD and CAD after exclusion of genetic variants that are implicated in impaired VLDL secretion.

INTRODUCTION

NAFLD has emerged as the leading cause of chronic liver disease worldwide.[1] It is a histological spectrum consisting of simple steatosis, NASH, fibrosis and cirrhosis,[2] which can progress to liver failure and HCC.[3] NAFLD is currently the second most common reason for liver transplantation in the United States.[4] Although these liver-related complications contribute to increased mortality rates, cardiovascular disease (CVD) is the leading cause of death among patients with NAFLD.[5]

Despite the strong epidemiological evidence on the association between NAFLD and CVD,[6] there is an ongoing discussion on whether NAFLD actively contributes to CVD or is just an innocent bystander. NAFLD is closely related with cardiometabolic risk factors, such as obesity and type 2 diabetes,[7] which could be confounders in the relationship between NAFLD and CVD.

Mendelian randomization (MR) can help to infer causality. As individuals are randomized at conception to receive genetic variants that either predispose to or protect from the exposure of interest (i.e., NAFLD), these variants can be used as instruments to study for a causal relationship with a clinically relevant outcome (i.e., CVD).[8,9] To date, the performance of MR studies on the relationship between NAFLD and CVD has been limited by the absence of extensive gene-exposure data sets. Lauridsen et al. previously used a common variant in the patatin-like phospholipase domain containing protein 3 (*PNPLA3*) gene in the first MR study and did not find an association.[10] This absent association may be explained by the mechanism by which this common variant in *PNPLA3* affects NAFLD risk (i.e., disturbed lipid remodeling and impaired VLDL secretion).[11] Indeed, it has been shown that gene variants that are associated with impaired VLDL secretion (including *PNPLA3*) not only predispose to NAFLD, but are also related with lower serum triglycerides and a reduced risk of coronary artery disease (CAD).[12] It can be questioned whether this pathway represents the "average" NAFLD phenotype that is characterized by an increased, rather than a decreased, VLDL secretion.[13]

Recently, the results from a large-scale genome-wide association study (GWAS) for chronically elevated serum alanine aminotransferase levels (cALT) (n = 218,595), intrahepatic lipid content assessed by imaging (n = 44,289), and biopsy-confirmed NAFLD (n = 63,969) were reported.[14] The availability of these data allows the performance of further MR analyses. In the present study, therefore, we conducted two-sample MR analyses to test for an association between these three NAFLD-related traits and CAD. For this, we used two different sets of instrumental variables: (1) all NAFLD susceptibility genes, regardless of their function, and (2) only those NAFLD susceptibility genes that are *not* implicated in impaired VLDL secretion.

METHODS

We performed two-sample MR analysis with publicly available summary-level data, which were derived from several large-scale cohorts[14,15]. Declaration of Helsinki statement and informed consent procedure have been described in the original publications of these cohorts.

NAFLD

Gene-exposure data were derived from a recently published GWAS for cALT, in which NAFLD was defined as an elevated alanine transaminase (ALT) > 40 U/L for men or >30U/L for women during at least two time points at least 6 months apart within a 2-year period, after exclusion of other liver diseases.[14] The study reported 77 independent, genome-wide significant ($p < 5\times10^{-8}$) single nucleotide polymorphisms (SNPs) in the discovery cohort (the Million Veteran Program) including 90,408 cALT cases and 128,187 controls of four ancestral groups (namely European-Americans, African-Americans, Hispanic-Americans, and Asian-Americans). Of these, 22 and 36 SNPs were subsequently replicated in two external validation cohorts: liver fat quantified by imaging (either computed tomography or magnetic resonance imaging [n = 44,289]) and biopsy-confirmed NAFLD (7,397 cases and 56,785 controls, respectively). Overall, 17 of 77 cALT SNPs were directionally concordant and nominally significant in both the imaging and biopsy cohorts.

The following sets of instrumental variables were used in the current MR study: (1) all cALT-associated SNPs (n = 77); (2) cALT-associated SNPs with nominal significance and directional concordance in the imaging cohorts (n = 22) (the effect estimates for the imaging data [expressed as Z-scores] were used for the analyses); (3) cALT-associated SNPs with nominal

significance and directional concordance in the biopsy cohorts ($n = 36$) (the effect estimates for the biopsy data [NAFLD yes/no] were used for the analyses); (4) cALT-associated SNPs with nominal significance and directional concordance in both the imaging and biopsy cohorts ($n = 17$) (the effect estimates for the biopsy data [NAFLD yes/no] were used for the analyses), further referred to as stringent criteria (Table 1 and Table S1). All analyses were subsequently repeated in all aforementioned data sets after exclusion of those NAFLD susceptibility genes that are implicated in impaired VLDL secretion (based on Genecards and Pubmed search) (Table S2).

SNPs were excluded if they were in linkage disequilibrium (LD) ($r^2 > 0.1$, the SNP with the largest absolute effect estimate was retained) or were palindromic (with a minor allele frequency > 0.42).

CAD

Gene-outcome data were retrieved from the Coronary Artery Disease Genome-Wide Replication and Meta-analysis plus the Coronary Artery Disease (CARDIoGRAMplusC4D) Consortium cohort. This cohort assembled 60,801 cases and 123,504 controls from 48 studies, of whom 77% of the participants were of European ancestry, 19% were of south and east Asian ancestry, and a small proportion were Hispanic and African Americans.[15] CAD cases were defined as an inclusive diagnosis of myocardial infarction, acute coronary syndrome, chronic stable angina, or coronary stenosis $> 50\%$[15] (Table 1). Missing genes were replaced with SNPs that were in high LD ($r^2 > 0.7$).

Statistical analyses

The inverse variance weighted (IVW) MR analysis with a random-effect model was used as the primary analysis for all four instrumental variable sets. Cochran's Q statistic was calculated to quantify heterogeneity. In addition, we conducted the following analyses with more stringent assumptions: (1) the simple median method, which provides effect estimates even when 50% of the genetic instrument are invalid[16]; (2) the penalized weighted median method, which reduces the contribution of genetic variants with heterogenous effect estimates and therefore is less affected by outliers[16]; and (3) contamination mixture analyses, which is based on the assumption that the true effect estimate is represented by the largest group of genetic variants with similar effect estimates, given that there is no larger group of invalid genetic variants with similar estimates. In this case, the true effect estimate can be represented by the largest number of genetic instruments.[17,18] The strength of the selected genetic instrument was assessed using F statistics, with a mean F-statistic < 10 regarded as a weak set of instrumental variables.[19]

The MR-Egger method was used to assess potential directional pleiotropy. A statistically significant intercept suggests directional pleiotropy, violating the instrumental variable assumptions.[20] In addition, the MR pleiotropy residual sum and outlier (PRESSO) method was performed, which attempts to reduce heterogeneity in the estimate of the causal effect by removing SNPs that contribute to the heterogeneity disproportionately more than expected (NbDistribution = 1,500).[21] Finally, Steiger-filtering analyses were adopted to identify and exclude genetic variants that have a stronger association with the outcome than with the exposure, suggestive of reverse causality.[22]

All analyses were performed using the R statistical software version 4.0.1 with the TwoSampleMR and MedelianRandomization packages.[23,24]

RESULTS

Association between genetically predicted NAFLD and CAD

The GWAS identified 77 cALT-associated SNPs (Table S3), of which six genes were missing in the CARDIoGRAMplusC4D data set. Four of these (rs574044675,

TABLE 1 Overview of databases used for gene-exposure and gene-outcome data

GWAS data set	Phenotype	Sample size	Ethnicity
Vujkovic et al.[14]	cALT (yes/no)	90,408 cases and 128,187 controls	European-American, African-American, Hispanic-American, and Asian-American
	Imaging-based NAFLD (CT/MRI) (Z-scores)	44,289	European-American, African-American, and Hispanic American
	NAFLD-confirmed biopsy (yes/no)	7,397 cases and 56,785 controls	European-American and Hispanic American
Nikpay et al.[15]	CAD (yes/no)	60,801 cases and 123,504 controls	European and Asian

Abbreviations: CT, computed tomography; GWAS, genome-wide association study; MRI, magnetic resonance imaging.

rs138033684, rs115038698, and rs14150524) could not be replaced by SNPs that are in high LD. Six SNPs were excluded because they were in LD (rs2207132, rs28929474, and rs60315134) or palindromic (rs4711750, rs4782568, and rs7041363), resulting in 67 independent SNPs that were used as genetic instruments (Tables S1 and S3), with a mean F statistic of 83.6.

IVW MR analysis with a random-effects model showed a statistically significant association between genetically predicted cALT and risk of CAD (OR: 1.116, 95% CI: 1.039, 1.199; Q: 231.982; Figure 1). Similar associations were observed with the simple median and contamination mixture methods (OR: 1.197, 95% CI: 1.105, 1.298; and OR: 1.216, 95% CI: 1.063, 1.316; respectively), but not with the penalized weighted median method (OR: 1.001, 95% CI: 0.929, 1.079) (Figure 1, Figure S1). MR-Egger regression analysis showed a significant intercept ($p < 0.001$), indicating horizontal pleiotropy. Furthermore, the MR-PRESSO method identified seven outliers, although exclusion of outliers did not substantially affect the results (OR: 1.156, 95% CI: 1.148, 1.164).

IVW MR analyses for the other NAFLD-related traits (i.e., imaging-based [19 SNPs; Tables S1 and S4] and biopsy-confirmed NAFLD [32 SNPs; Table S1 and S5]) were both nonsignificant with high heterogeneity (Q: 106.057 and 128.371, respectively) (Figure 1). Furthermore, the other MR methods showed inconsistent results (Figure 1, Figures S2 and S3). Similar inconsistent results were observed when only biopsy-confirmed NAFLD SNPs, which were nominally significant and directionally concordant with both biopsy and imaging (stringent criteria; 15 SNPs; Tables S1 and S6), were used (Figures 1 and 2). MR-Egger regression showed a statistically significant intercept for all traits ($p < 0.01$). MR-PRESSO identified several outliers for all traits, but there was no significant difference in the causal estimates before and after correction for outliers (p value for the distortion test > 0.05).

Association between genetically predicted NAFLD and CAD after exclusion of VLDL secretion–associated genes

We subsequently repeated the analyses after excluding genes that are associated with impaired VLDL secretion (*PNPLA3*, transmembrane 6 superfamily member 2 (*TM6SF2*), microsomal triglyceride transfer protein (*MTTP*), *fatty acid desaturase 2*, *apolipoprotein E*, and *MLX interacting protein like*[11,25–29]) (Table S2). IVW MR analysis with 61 SNPs (Table S1, F statistic 63.5) showed a statistically significant association between genetically predicted cALT and risk of CAD (OR: 1.203, 95% CI: 1.113, 1.300; Q: 171.139; Figure 3). Similar associations were found when the simple median, penalized weighted median, and contamination mixture methods were applied (Figure 3, Figure S4). MR-Egger regression analysis showed a nonsignificant intercept ($p = 0.144$). MR-PRESSO method identified four outliers, and exclusion of these SNPs did not substantially affect the results (OR: 1.177, 95% CI: 1.169, 1.186).

IVW MR analysis for the imaging data, including 15 SNPs (Table S1, F statistic: 16.7) showed a statistically significant association between genetically predicted imaging-based NAFLD and risk of CAD (OR: 2.149, 95% CI: 1.276, 3.620; Q: 51.334; Figure 3). Similar directional associations were found for the other methods, although the penalized weighted median was not statistically significant (OR: 1.317, 95% CI: 0.832, 2.086) (Figure 3, Figure S5). MR-Egger regression analysis showed a nonsignificant intercept ($p = 0.060$). The MR-PRESSO method identified two outliers, and exclusion of outliers did not substantially affect the results (OR: 2.301, 95% CI: 2.176, 2.427).

IVW MR analysis for the biopsy-confirmed NAFLD, including 28 SNPs (Table S1, F statistic: 14.5), showed a statistically significant association between biopsy-confirmed NAFLD and CAD (OR: 1.113, 95% CI: 1.041, 1.189; Q: 76.924), again with consistent results for the other MR methods (Figure 3, Figure S6). MR-Egger regression analysis showed a nonsignificant intercept ($p = 0.086$). The MR-PRESSO method identified four outliers, and exclusion of outliers did not substantially affect the results (OR: 1.167, 95% CI: 1.156, 1.179).

Finally, when only SNPs were included that are nominally significant and directionally concordant for all three traits (stringent criteria; $n = 11$; F statistic: 18.8; Table S1), a statistically significant association between genetically predicted biopsy-confirmed NAFLD and risk of CAD was found for all four MR methods (OR: 1.154, 95% CI: 1.043, 1.278; Q: 31.312 for IVW MR; Figures 3 and 4). MR-Egger regression analysis showed a nonsignificant intercept ($p = 0.376$). MR-PRESSO identified one outlier, and exclusion of this outlier did not substantially affect the results (OR: 1.192, 95% CI: 1.161, 1.222).

The Steiger-filtering method did not identify any genetic variants that explained significantly more of the variance in the outcome than any of the exposure traits.

DISCUSSION

The current MR study demonstrates that there is no consistent relationship between genetically predicted NAFLD and CAD when all NAFLD susceptibility genes are used as instrumental variables, regardless of their function. However, after exclusion of genes that have been implicated in impaired VLDL secretion, we found robust associations between genetically predicted NAFLD and CAD for all NAFLD-related traits, using different MR methods.

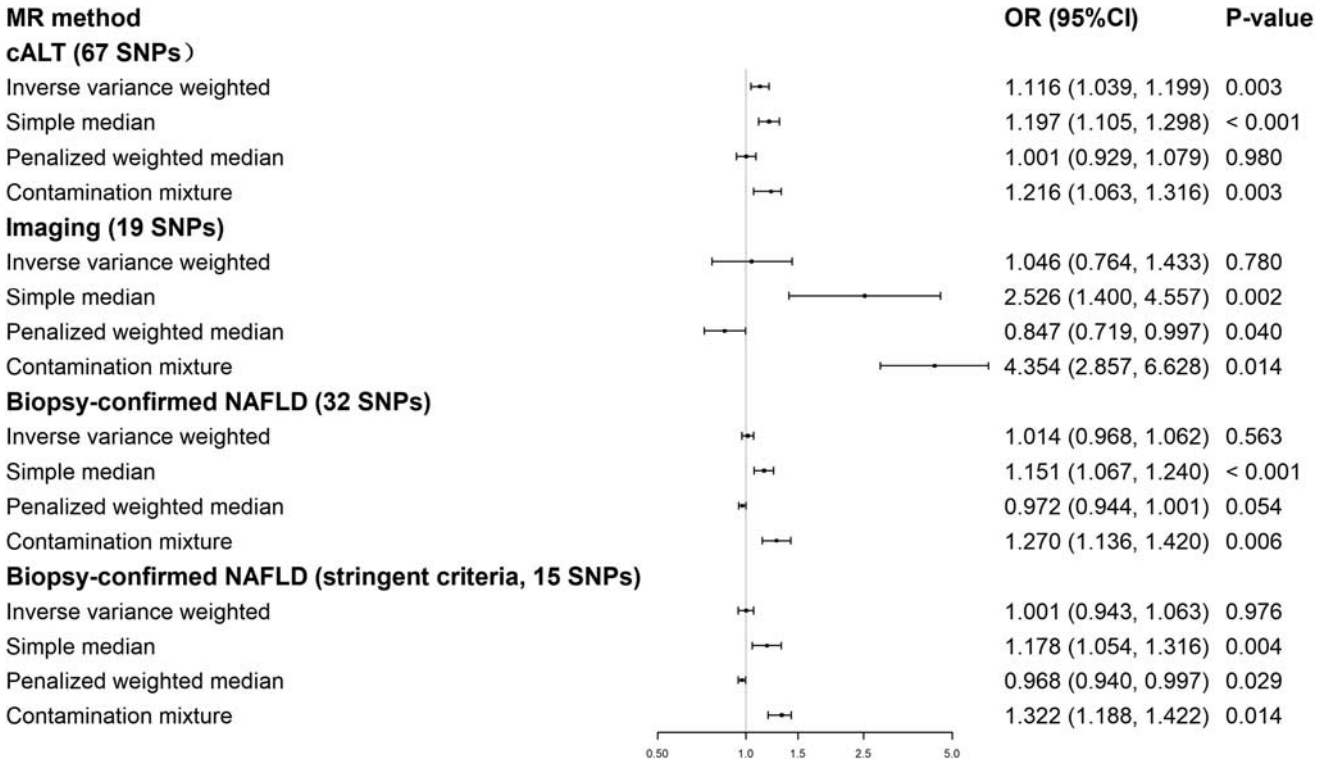

FIGURE 1 Association of genetically predicted chronically elevated serum alanine aminotransferase level (cALT), imaging-based and biopsy-confirmed NAFLD (using either general or stringent criteria, see Methods section) with risk of coronary artery disease (CAD), analyzed with four different Mendelian randomization (MR) methods. Effect estimates are presented as increase in odds of CAD per SD increase in imaging, or per unit increase in (log)odds of cALT or biopsy-confirmed NAFLD. SNP, single nucleotide polymorphism

The choice of an instrumental variable is critical to a valid MR study. One of the important assumptions of MR method is that the genetic variant should not have an effect on the outcome other than via a direct effect on the exposure (i.e., horizontal pleiotropy should be absent).[30] Because genetic variants that predispose

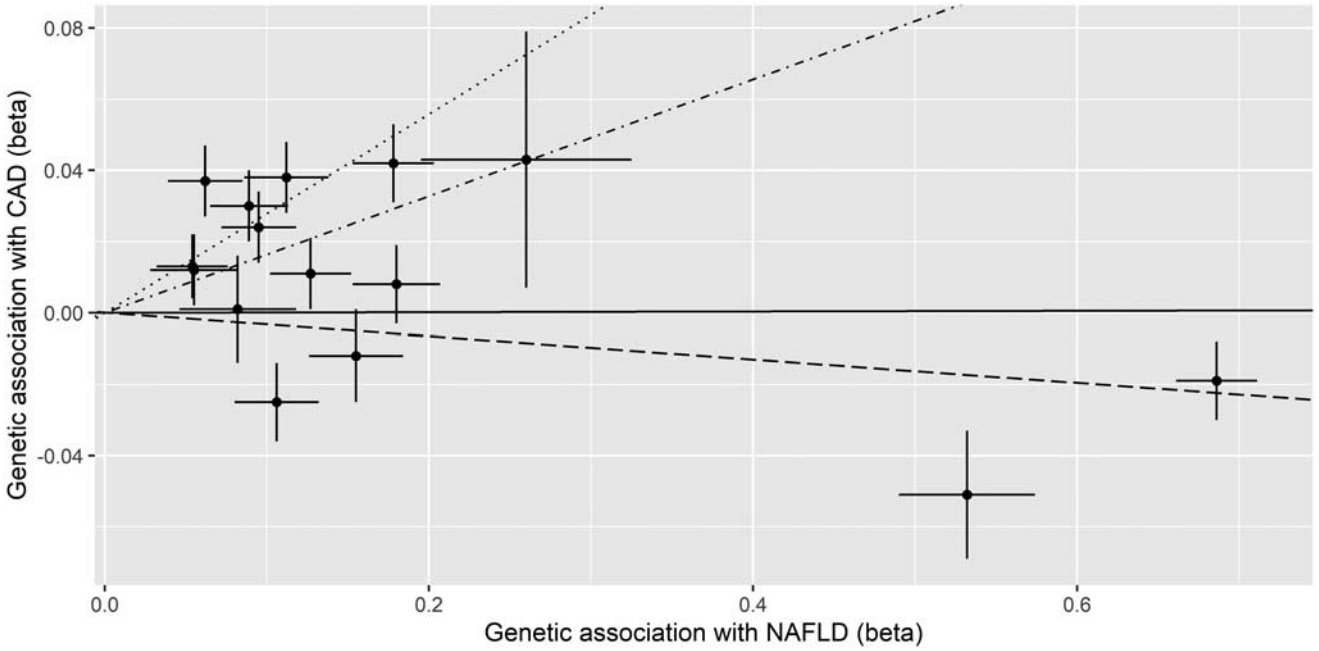

FIGURE 2 Relationship between genetically predicted biopsy-confirmed NAFLD (with stringent criteria) and CAD, using inverse variance–weighted method (solid line), simple median method (dashed-dotted line), penalized weighted–median method (dashed line), and contamination mixture method (dotted line)

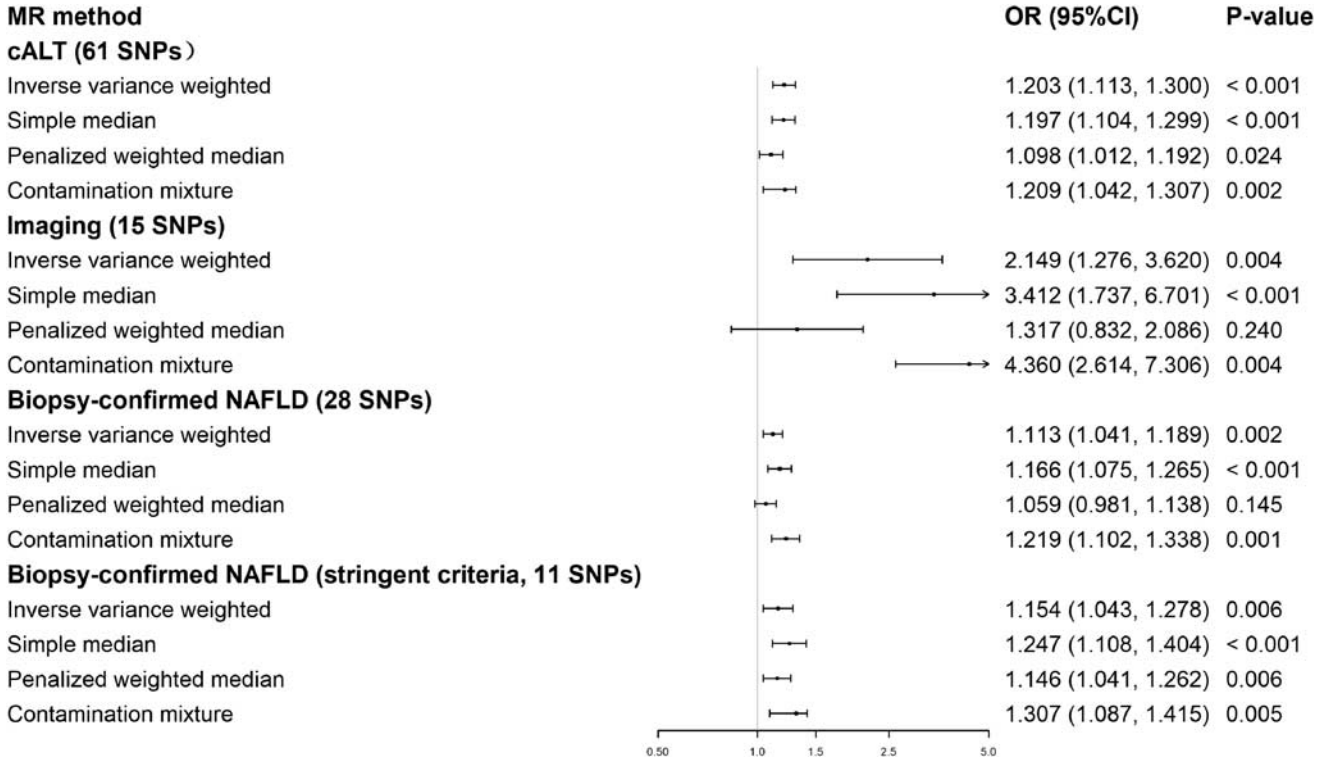

FIGURE 3 Association of genetically predicted cALT, imaging-based and biopsy-confirmed NAFLD (using either general or stringent criteria, see methods section) (after exclusion of genes associated with impaired VLDL secretion) with risk of CAD, analyzed with four different MR methods. Effect estimates are presented as increase in odds of CAD per SD increase in imaging, or per unit increase in (log)odds of cALT-based or biopsy-confirmed NAFLD

to NAFLD via impaired VLDL secretion also directly affect serum lipids,[12] a major cardiovascular risk factor, they are an example of horizontal pleiotropy (Figure S7).[9] Indeed, MR-Egger regression showed a statistically significant intercept when these variants were included. Exclusion of these genetic variants reduced

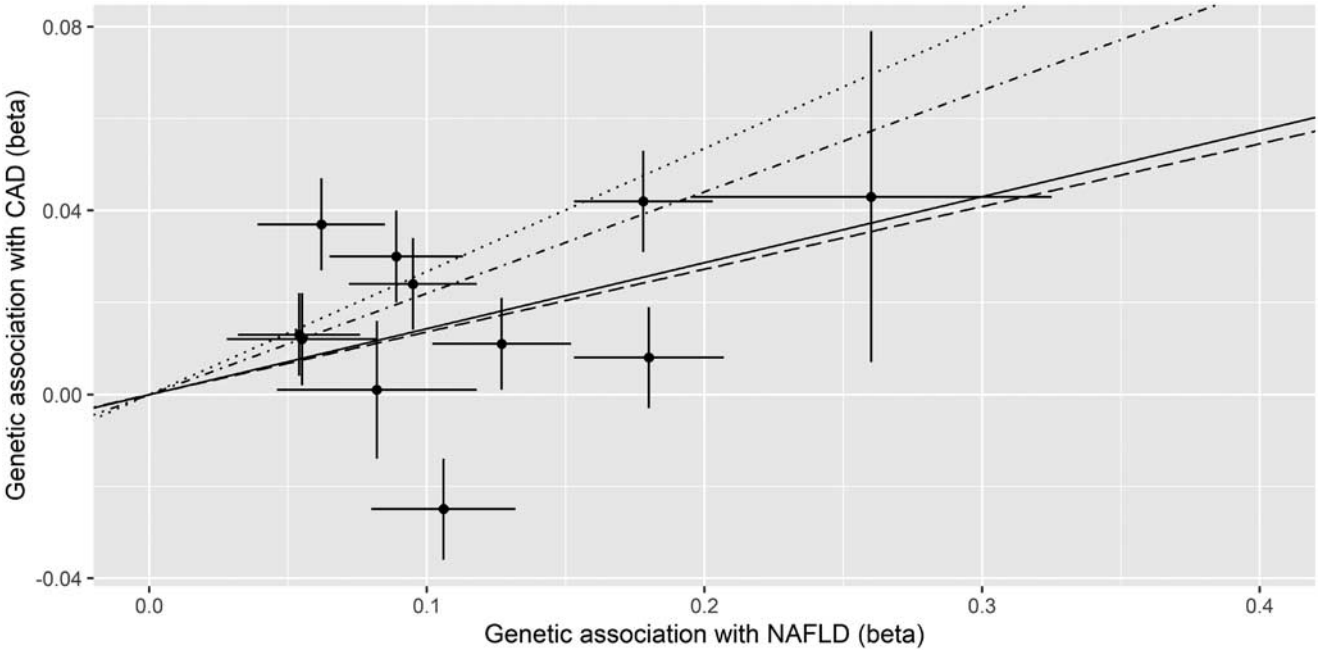

FIGURE 4 Relationship between genetically predicted biopsy-confirmed NAFLD (with stringent criteria, after exclusion of genes associated with impaired VLDL secretion) and CAD, using inverse variance–weighted method (solid line), simple median method (dashed-dotted line), penalized weighted– median method (dashed line), and contamination mixture method (dotted line)

heterogeneity and eliminated horizontal pleiotropy. Moreover, besides this methodological rationale for excluding genetic variants implicated in impaired VLDL secretion, there is also a biological argument in favor of exclusion. Stable isotopes studies have shown that an increased free fatty acid flux and greater rates of *de novo* lipogenesis are the principal causes of NAFLD.[31,32] Furthermore, patients with NAFLD are characterized by an up-regulated, rather than an impaired, VLDL secretion pathway.[13]

Our results are in line with our previous study showing that NAFLD susceptibility genes are associated with CAD after exclusion of VLDL secretion genes.[33] However, that study was limited by the relatively small number of NAFLD susceptibility genes ($n = 12$), and more importantly, the absence of a gene-exposure data set. Therefore, it was not possible to conduct formal MR analyses and to draw conclusions on the strength of the relationship (and, hence, the clinical relevance) between NAFLD and CAD. The present study shows that (biopsy-confirmed) NAFLD increases CAD risk with 15% per unit increase in odds of NAFLD. The strength of this association is lower in comparison to a previous meta-analysis for the epidemiological relationship between NAFLD and CVD (HR: 1.45, 95% CI:1.31–1.61),[34] which is more prone to confounding. Of interest, when compared with other MR studies, the strength of the currently observed relationship is in the same order of magnitude as has been found for type 2 diabetes in relation to CAD.[35]

This study has several strengths and limitations. First, as mentioned previously, by using large-scale, summary-level data, there was sufficient instrumental variable strength to demonstrate a causal effect of NAFLD on CAD. Second, in comparison to previous MR studies using surrogate markers of NAFLD,[36,37] we used gene-exposure data of three different NAFLD-related traits, including biopsy-confirmed NAFLD, which—in combination with the use of different MR methods—contribute to the robustness and validity of our findings. Of note, the methodological approach of the original GWAS (i.e., identification of SNPs based on GWAS for cALT and subsequent replication in imaging and biopsy cohorts[14]) has resulted in the selection of NAFLD genes that are associated with cALT. Because ALT is not a perfect biomarker of NAFLD,[38] it is likely that NAFLD susceptibility genes that are not associated with serum ALT levels have not been included in the present MR study. On the other hand, we are confident that the imaging-based and biopsy-confirmed NAFLD susceptibility genes are truly NAFLD susceptibility genes, as they have not only been associated with cALT, but also with imaging and/or biopsy.[14] Furthermore, many of the biopsy-confirmed NAFLD SNPs included in this study have been reported before, including *PNPLA3*, *TM6SF2, HSD17B13* and *MTTP*,[29,39–41] illustrating the validity of the current approach. Another limitation is that the original GWAS was not distinct between the different histological stages of NAFLD, which is of importance as fibrosis has specifically been associated with cardiovascular mortality.[42] Third, exclusion of those genes affecting NAFLD through impaired VLDL secretion does not necessarily eliminate all potential horizontal pleiotropy, as many SNPs were not only expressed in the liver (Table S2). It is, however, expected that the impact on the currently observed outcomes is marginal, given the nonsignificant intercepts after MR-Egger regression. Finally, by using summary-level data, we were not able to perform subgroup analyses, such as stratified by sex or ethnicity.

In conclusion, in this two-sample MR study, we observed a robust association between genetically predicted NAFLD and CAD after exclusion of genetic variants that are implicated in impaired VLDL secretion.

AUTHOR CONTRIBUTIONS

Study concept and research elaboration: Martijn C. G. J. Brouwers, Pomme I. H. G. Simons, and Zhewen Ren. *Main data analysis and manuscript draft:* Zhewen Ren. *Data analysis:* Pomme I. H. G. Simons. *Manuscript revisions:* Pomme I. H. G. Simons, Anke Wesselius, and Coen D. A. Stehouwer. *Manuscript review:* Anke Wesselius and Coen D. A. Stehouwer. *Study supervision and data analysis:* Martijn C. G. J. Brouwers. All authors gave consent to the publication of this study.

CONFLICTS OF INTEREST

The authors declare there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data is available in Suppl Tables 1–6.

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