Relationship between NAFLD and coronary artery disease

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Relationship between non-alcoholic fatty liver disease and coronary artery disease: A Mendelian randomization study

Short title: NAFLD and CAD

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Abbreviations

ALT

alanine transaminase

CAD

Coronary artery disease

cALT

chronically-elevated serum alanine aminotransferase levels

GWAS

genome-wide association study

IVW

inverse variance weighted

LD

linkage disequilibrium

MR

Mendelian randomization

MR-Egger

Mendelian randomization-Egger

MR-PRESSO

Mendelian Randomization Pleiotropy Residual Sum and Outlier

NAFLD

Non-alcoholic fatty liver disease

PNPLA3

patatin-like phospholipase domain containing protein 3

SNPs

single nucleotide polymorphisms

VLDL

very low-density lipoprotein

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Abstract

Background & Aims: There is an ongoing debate on whether non-alcoholic fatty liver disease (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 & 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 (IVW) MR analyses showed a statistically significant association

between genetically predicted cALT and risk of CAD (odds ratio [OR]:1.116, 95% confidence interval [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.

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

Non-alcoholic fatty liver disease (NAFLD) has emerged as the leading cause of chronic liver disease worldwide (1). It is a histological spectrum consisting of simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis (2), which can progress to liver failure and hepatocellular carcinoma (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), that 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 datasets. Lauridsen and colleagues 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 very low-density lipoprotein (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 new 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 and Materials

We performed two-sample MR analyses with summary-level data, which were derived from several large-scale cohorts.

Non-alcoholic fatty liver disease

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 two-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, i.e. 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 datasets 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).

Coronary artery disease

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 which 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); 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 Mendelian randomization-Egger (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 Mendelian Randomization Pleiotropy Residual Sum and Outlier (MR-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 6 genes were missing in the CARDIoGRAMplusC4D dataset. Four of these, i.e. rs574044675, rs138033684, rs115038698 and rs14150524, could not be replaced by SNPs that are in high LD. Six SNPs were excluded as they were in LD (rs2207132, rs28929474, rs60315134) or palindromic (rs4711750, rs4782568, rs7041363), resulting in 67 independent SNPs that were used as genetic instruments (Tabe 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 (odds ratio [OR]: 1.116, 95% confidence interval [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, Supplementary Figure 1). MR-Egger regression analysis showed a significant intercept ($p < 0.001$), indicating horizontal pleiotropy. Furthermore, the MR-PRESSO method identified 7 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, Table S1 and S4) and biopsy-confirmed NAFLD (32 SNPs, Table S1 and S5), were both non-significant with high heterogeneity (Q: 106.057 and 128.371, respectively) (Figure 1). Furthermore, the other MR methods showed inconsistent results (Figure 1, Supplementary Figures 2-3). 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, Table S1 and Table S6), were used (Figure 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*, *TM6SF2*, *MTTP*, *FADS2*, *APOE*, *MLXIPL* (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, Supplementary Figure 4). MR-Egger regression analysis showed a non-significant intercept (p = 0.144). MR-PRESSO method identified 4 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, Supplementary Figure 5). MR-Egger regression analysis showed a non-significant intercept (p = 0.060). The MR-PRESSO method identified 2 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), with again consistent results for the other MR methods (Figure 3, Supplementary Figure 6). MR-Egger regression analysis showed a non-significant intercept (p = 0.086). MR-PRESSO method identified 4 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; Figure 3 and 4). MR-Egger regression analysis showed a non-significant 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.

The choice of an instrumental variable is critical to a valid MR study. One of the important assumptions of Mendelian randomization 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). Since genetic variants that predispose to NAFLD via impaired VLDL secretion also directly affect serum lipids (12), a major cardiovascular risk factor, they are an example of horizontal pleiotropy (Supplementary Figure 7) (9). Indeed, MR-Egger regression showed a statistically significant intercept when these variants were included. Exclusion of these genetic variants reduced 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 upregulated, 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 dataset. It was, therefore, 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 to 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 before, 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. Since 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 did not distinct between the different histological stages of NAFLD, which is of importance as fibrosis has specifically been associated with cardiovascular mortality (42).Thirdly, exclusion of those genes affecting NAFLD through impaired VLDL secretion does not necessarily eliminate all potential horizontal pleiotropy, since 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 non-significant intercepts after MR-Egger regression. Finally, by using summary-level data, we were not able to perform subgroup analyses, e.g. 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.

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Author Contributions

Martijn C.G.J. Brouwers, Pomme I.H.G. Simons and Zhewen Ren proposed the idea and elaborated the research. Zhewen Ren performed the main data analysis and wrote the draft of the manuscript. Pomme I.H.G. Simons contributed to the data analysis and manuscript revision. Anke Wesselius and Coen D.A. Stehouwer reviewed and revised the manuscript. Martijn C.G.J. Brouwers supervised the whole research and is responsible for the integrity of data analysis. All authors have given consent to the publication of this study.

Conflicts of Interest

The authors declare there are no conflicts of interest

Figure legends

Figure 1. Association of genetically predicted cALT, imaging-based and biopsy-confirmed NAFLD (using either general or stringent criteria, see methods section) with risk of CAD, analyzed with four different MR methods. Effect estimates are presented as increase in odds of CAD per standard deviation increase in imaging, or per unit increase in (log)odds of cALT or biopsy-confirmed NAFLD.

Abbreviations: cALT, chronically-elevated serum alanine aminotransferase levels; CI, confidence interval; MR, mendelian randomization; NAFLD, Non-alcoholic fatty liver disease; OR, odds ratio; SNPs, single nucleotide polymorphisms.

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).

Abbreviations: CAD, Coronary artery disease; MR, mendelian randomization; NAFLD, Non-alcoholic fatty liver disease.

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 standard deviation increase in imaging, or per unit increase in (log)odds of cALT-based or biopsy-confirmed NAFLD.

Abbreviations: cALT, chronically-elevated serum alanine aminotransferase levels; CI, confidence

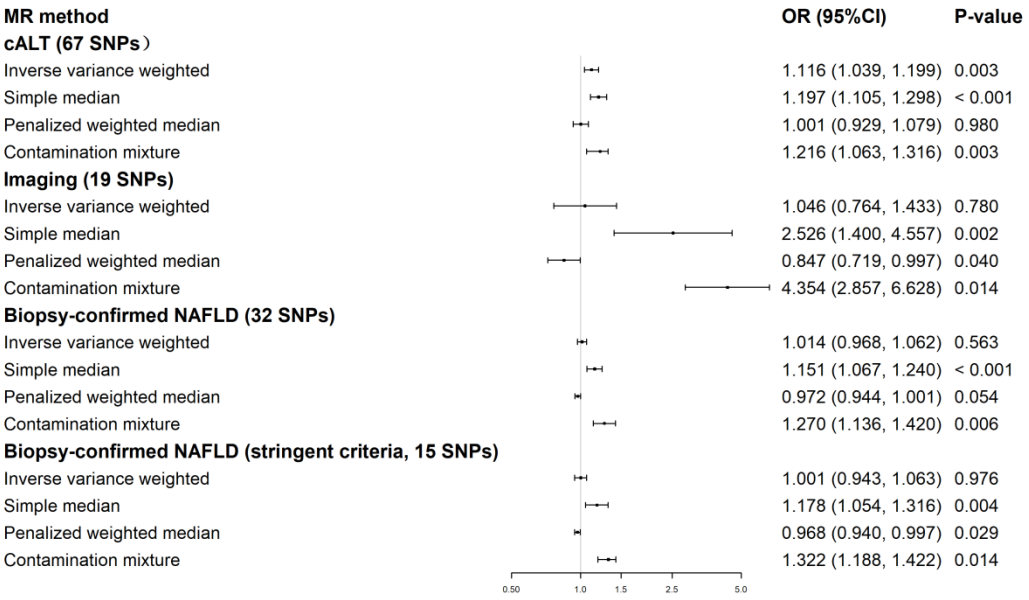
interval; MR, mendelian randomization; NAFLD, Non-alcoholic fatty liver disease; OR, odds ratio; SNPs, single nucleotide polymorphisms; VLDL, very low-density lipoprotein**.**

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). Abbreviations: CAD, Coronary artery disease; MR, mendelian randomization; NAFLD, Non-alcoholic fatty liver disease; VLDL, very low-density lipoprotein**.**

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

GWAS dataset	Phenotype	Sample size	Ethnicity
Vujkovic et al (13)	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 (14)	Coronary artery disease (yes/no)	60,801 cases and 123,504 controls	European and Asian

Abbreviations: cALT, chronically-elevated serum alanine aminotransferase levels; CT, computed tomography; GWAS, genome-wide association study; MRI, magnetic resonance imaging; NAFLD, non-alcoholic fatty liver disease.



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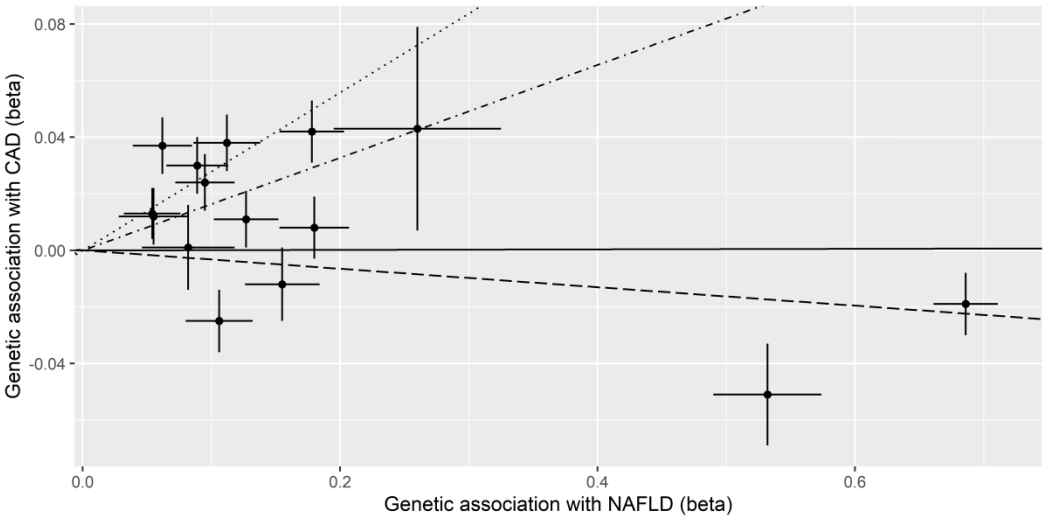

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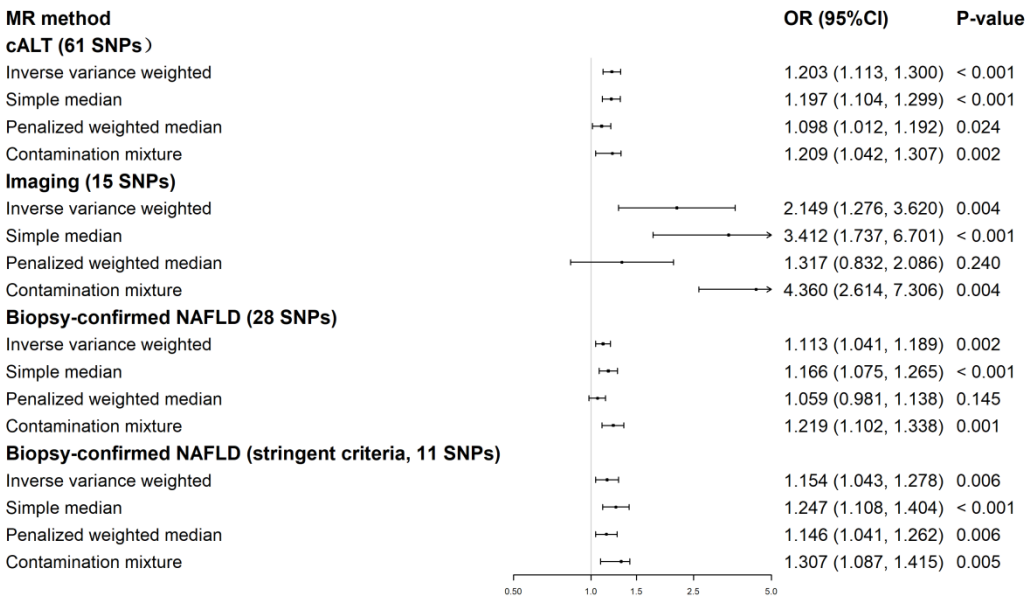

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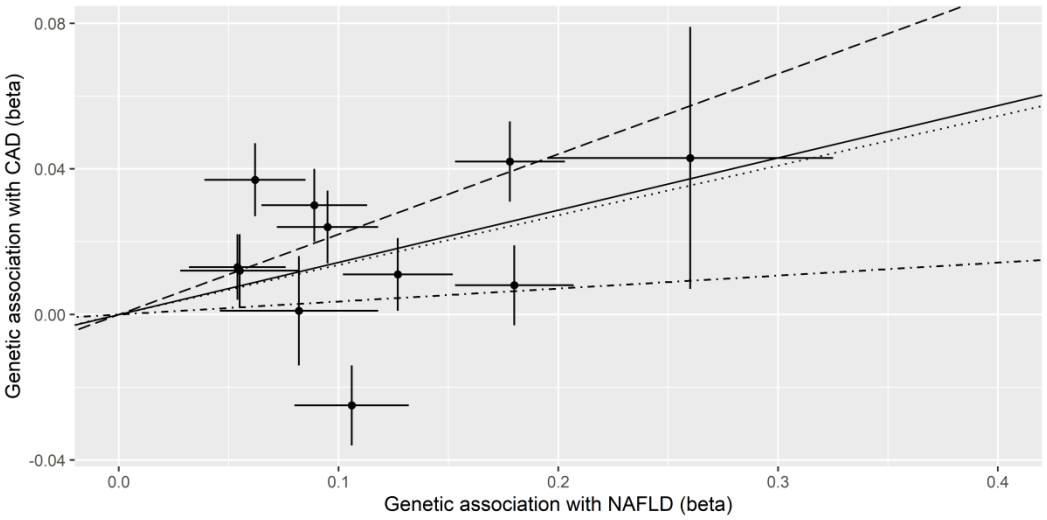

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