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Applied

Plasma phospholipid arachidonic acid in relation to non-alcoholic fatty liver disease: Mendelian randomization study

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ABSTRACT

Objectives: The role of plasma phospholipid arachidonic acid (AA) in the development of non-alcoholic fatty liver disease (NALFD), cirrhosis, and liver cancer remains unclear. This study aimed to determine the causality of the associations of plasma phospholipid AA with NALFD, cirrhosis, and liver cancer using Mendelian randomization analysis.

Methods: Nine independent single-nucleotide polymorphisms associated with plasma phospholipid AA at the genome-wide significance were used as instrumental variables. Summary-level data for three outcomes were obtained from 1) a genome-wide association study for NAFLD, 2) the UK Biobank study, and 3) the FinnGen study. The sensitivity analysis excluding the pleiotropic variant rs174547 in the *FADS1* gene was performed. Estimates from different sources were combined using the fixed-effects meta-analysis method.

Results: Per standard deviation increase in AA levels, the combined odds ratio was 1.06 (95% confidence interval, 1.02–1.11; $P = 0.008$) for NAFLD, 1.05 (95% confidence interval, 1.01–1.09; $P = 0.009$) for cirrhosis, and 0.99 (95% confidence interval, 0.94–1.05; $P = 0.765$) for liver cancer. The associations remained stable in the sensitivity analysis excluding rs174547.

Conclusions: This study suggests potential causal associations of high levels of plasma phospholipid AA with the risk of NAFLD and cirrhosis.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the major contributor to chronic liver disease in the Western world and causes a substantial health burden [1,2]. Approximately 5–10% of NAFLD patients develop irreversible cirrhosis [3], which, in 0.4–2.6% of those patients, eventually progresses to hepatocellular carcinoma [4]. NAFLD is usually defined 1) by evidence of hepatic steatosis that is detected by imaging or histology and 2) as without secondary causes of liver fat accumulation (e.g., alcohol abuse, steatogenic treatments, or inherited diseases) [5]. NAFLD is histologically further categorized into non-alcoholic fatty liver and non-alcoholic steatohepatitis [5]. The former is characterized by steatosis without evidence of hepatocellular injury, and the latter is defined as the presence of steatosis and inflammation with hepatocyte injury [5].

Dietary intervention is a practical and cost-effective strategy in NAFLD patients [6]. Randomized controlled trials in overweight or obese adults with NAFLD have shown that polyunsaturated fatty acid (PUFA) (mainly n-3 PUFAs) supplements (1–2 g/d for 12–16 wk) decrease blood triacylglycerol levels and the risk of liver steatosis and injury [7]. The n-6 PUFA arachidonic acid (AA; C20:4n-6) is a proinflammatory precursor that may be involved in the

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The data used in the present study are publicly available and accessible summary-level data with the relevant studies cited.

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pathogenesis of NAFLD [8]. However, evidence of this association is conflicting, with a null finding in a cohort study including 80 obese children [9] and a positive association in several population-based cross-sectional studies [10–12]. In addition, the observed associations in observational studies may be susceptible to confounding and reverse causation bias.

Mendelian randomization (MR) analysis uses genetic variants (i.e., single-nucleotide polymorphisms [SNPs]) strongly associated with an exposure (e.g., circulating levels of AA) as instrumental variables to strengthen the causal inference in an exposure-outcome association. Because genetic variants are randomly allocated at conception, MR analysis is by nature likely to reduce the potential of confounding. Additionally, because the fixed alleles are not modified by disease status, MR analysis can minimize reverse causation. Here, we assessed the association of genetically predicted plasma phospholipid AA levels with NAFLD using an MR analytical approach. We also examined the MR associations of AA levels with cirrhosis and liver cancer that are two common hepatic consequences of NAFLD.

Materials and methods

Study design

Three vital assumptions should be satisfied in MR analysis. First, selected genetic variants as instrumental variables should be strongly associated with the exposure. Second, used variants should not be associated with any important confounders. Third, the genetic variants should not affect the outcome directly or via other alternative pathways [13] (Fig. 1). We first assessed the associations of genetically predicted AA levels with NAFLD, cirrhosis, and liver cancer. Because the diagnosis of NAFLD usually follows the mildly raised levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [14] and a recent genome-wide association study (GWAS) study successfully constructed NAFLD phenotype based on chronic elevation of ALT levels, we used these two liver enzymes as secondary outcomes. Included studies had been approved by corresponding ethical review committees and all participants had provided consent forms. The present study was based on summary-level data and thus was exempt from ethical permits. Data used in the study are presented in Supplementary Table 1.

Genetic instrument selection

Genetic variants strongly ($P < 5 \times 10^{-8}$) associated with plasma phospholipid AA levels were obtained from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium, comprising 8631 participants of European ancestry with an average age of 60 y [15] (Supplementary Table 1). Nine SNPs used as instrumental variables were corroborated with linkage disequilibrium $r^2 < 0.01$ and clump distance > 10 000 kb. We assessed the strength of instrumental variables by the *F* statistic [16], and an *F* statistic > 10 indicated no weak instrument bias (Supplementary Table 2). SNPs were identified to have no known pleiotropic effects or associated with potential confounders (i.e., education, Townsend index, alcohol drinking, smoking, specific dietary patterns, and physical activity) via a search in PhenoScanner V2 (Supplementary Table 3) [17]. We identified one SNP rs174547 in the *FADS1* gene region that explains a considerable variance in plasma phospholipid AA levels (≤37.6%) [18] but has strong correlations with levels of other PUFAs, which might introduce horizontal pleiotropy. Thus, two sets of genetic variants were used as instrumental variables: all 9 SNPs and 8 SNPs after excluding rs174547. Detailed information on the SNPs is displayed in

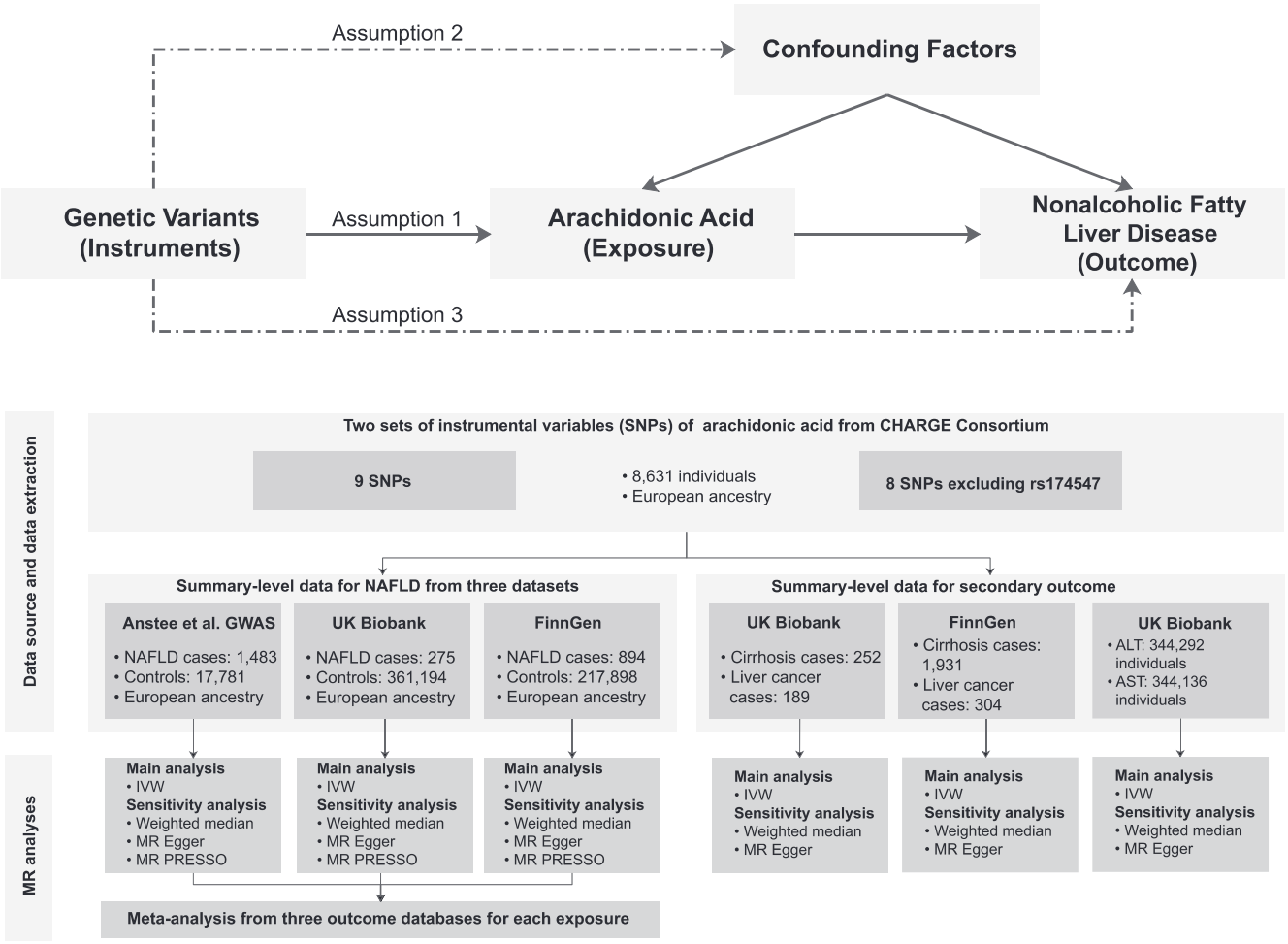

Fig. 1. Assumptions and schematic overview of the present study design. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CHARGE, Cohorts for Heart and Aging Research in Genomic Epidemiology; GWAS, genome-wide association study; IVW, inverse-variance weighted; MR, Mendelian randomization weighted; NAFLD, nonalcoholic fatty liver disease; SNPs, single nucleotide polymorphisms.

Supplementary Table 2. The genetic associations with plasma phospholipid AA levels were scaled as per SD (~1.96% of total fatty acids).

Data sources for NAFLD

Summary-level data for NAFLD were obtained from three data sets: 1) the GWAS by Anstee et al. [19], 2) the UK Biobank study [20], and 3) the FinnGen study [21] (Supplementary Table 1). There was no sample overlap across these data sets.

The GWAS conducted by Anstee et al. [19] included 1483 NAFLD cases and 17 781 controls. In this study, European-descent participants were diagnosed by histology at an average age of 50.1 ±13.0 y and with a median (interquartile range) body mass index (BMI) of 35.19 (29.1–39.7). The patients with NAFLD were diagnosed by a liver biopsy because of abnormal biochemical tests and/or ultrasonographic evidence or by having abnormal biochemical tests and macroscopic appearances of a steatosis liver at surgery [19]. Alternative diagnoses (i.e., excess alcohol intake, chronic viral hepatitis, and autoimmune liver diseases) were excluded. The UK Biobank is a large, multicenter, prospective cohort study of >500 000 participants ages between 40 and 69 y in 2006–2010 [20]. The UK Biobank linked to inpatient hospital episode records, primary care, cancer registries, and death registries [20]. We restricted our analyses to participants of European ancestry to minimize bias caused by population structure. Individuals with three degrees or higher-degree relatedness were excluded. The final sample size was 361 194 individuals, comprising 275 NAFLD cases (*International Classification of Diseases, Tenth Revision* [*ICD-10*]: K76.0) and 360 919 controls. The FinnGen study is a population-based cohort study combining genotype data from Finnish biobanks and health record data from Finnish health registries, and each end point was defined by the *ICD* codes [22]. Individuals with ambiguous gender, high genotype missingness (>5%), excess heterozygosity (± 4 SD), and non-Finnish ancestry were excluded [22]. This analysis was based on the FinnGen data freeze 5, including 894 cases of NAFLD (*ICD-10*: K76.0) and 217 898 controls.

Data sources for cirrhosis and liver cancer

Summary-level data for cirrhosis and liver cancer were obtained from the UK Biobank study, including 252 cases of cirrhosis (*ICD-10*: K74) and 360 942 controls, and 204 cases of liver cancer (*ICD-10*: C22) and 360 990 controls. Summary-level data were also obtained from the FinnGen study, including 1931 cirrhosis patients and 216 861 controls (broad definition in the FinnGen study [23]), and 304 liver cancer patients (*ICD-10*: C22) and 174 006 controls (Supplementary Table 1).

Data source for ALT and AST

Summary-level data for the associations between AA-associated SNPs and the two liver function markers were extracted from the UK Biobank study, including individuals with information on levels of ALT (n = 344 292) and AST (n = 344 136) (Supplementary Table 1).

Statistical analysis

The multiplicative random-effects inverse-variance weighted method was used as the principal analysis to obtain causal estimates. Ratio estimates were calculated for each SNP as the β coefficient for the SNP-NAFLD association divided by the β coefficient for the SNP-AA association. Estimates for one outcome from different sources were combined using the fixed-effects meta-analysis method. To examine the consistency of results and horizontal pleiotropy, we used the weighted median, MR-Egger, and MR pleiotropy residual sum and outlier (PRESSO) methods as sensitivity analyses. The weighted median method generates consistent estimates if ≥50% of the instrumental variables are valid [16]. MR-Egger regression allows for horizontal pleiotropic effects and provides unbiased causal effect estimates, although the estimates are usually underpowered [24]. MR-PRESSO can detect and correct for horizontal pleiotropic outliers [25]. Heterogeneity among estimates of SNPs was measured by the Cochran Q statistic. To explore whether the association of serum AA with NAFLD is independent of BMI, we performed a multivariable MR with adjustment for genetically predicted BMI. Genetic association with BMI was obtained from a publicly available GWAS [26]. All tests were two sided and the association with $P < 0.05$ was deemed significant. Analyses were performed using the TwoSampleMR [27], MendelianRandomization [28], and MRPRESSO [25] packages in R software 4.1.2.

Results

Plasma phospholipid arachidonic acid and NAFLD

A positive association between genetically predicted AA levels and NAFLD was observed (Fig. 2). Per SD increase in genetically predicted to lifelong higher levels of plasma phospholipid AA, the odds ratios (ORs) of NAFLD were 1.08 (95% confidence interval [CI], 1.01–1.16; P = 0.029) in the Anstee et al. [19] GWAS and 1.31 (95% CI, 1.12–1.53; P = 0.001) in the UK Biobank study. The association was not significant but directionally consistent in the FinnGen study (OR 1.01; 95% CI, 0.95–1.08; P = 0.725). The combined OR of NAFLD was 1.06 (95% CI, 1.02–1.11; P = 0.008). The association became stronger in the sensitivity analysis when excluding rs174547 (OR 1.08; 95% CI, 1.01–1.15; P = 0.022).

We observed no heterogeneity in any analysis (Supplementary Table 4). Results were consistent in the weighted median method and directionally stable in the MR-Egger regression analysis (Fig. 2). There was no horizontal pleiotropy detected by the MR-Egger intercept test, and no SNP outliers were detected by the MR-PRESSO analysis (Supplementary Table 4). After adjusting for genetically predicted BMI, the combined OR of NAFLD was 1.08 (95% CI, 1.01–1.15; P = 0.022) (Supplementary Table 5).

Plasma phospholipid arachidonic acid, cirrhosis, and liver cancer

Genetically predicted high levels of AA were associated with an increased risk of cirrhosis (OR 1.04; 95% CI, 1.00–1.09; P = 0.033) (Fig. 3), whereas genetically predicted AA levels were not associated with the risk of liver cancer (OR 0.99; 95% CI, 0.93–1.05; P = 0.717) (Fig. 4). The direction of the associations remained stable in sensitivity analysis after excluding rs174547 (Fig. 3 and 4).

Plasma phospholipid arachidonic acid and liver enzymes

Genetically predicted higher levels of AA were associated with increased levels of ALT (Fig. 5). Per SD increase in genetically predicted levels of AA, the change of ALT levels was 0.21 U/L (95% CI, 0.09–0.33; $P < 0.001$). There was no association between genetically predicted AA levels and AST levels (0.00U/L; 95% CI, −0.05–0.06; P = 0.905). Similarly, the association between genetically predicted AA level and ALT became stronger albeit with a larger CI in the sensitivity analysis after excluding rs174547 (0.39U/L; 95% CI, 0.09–0.70; P = 0.012) (Fig. 5 and Supplementary Table 6).

Discussion

In this MR study, we found that genetically predicted higher plasma phospholipid AA levels were associated with an increased risk of NAFLD and cirrhosis but not with liver cancer. There was a positive association between genetically predicted AA levels and ALT levels.

The association between AA and NAFLD has been explored in population-based studies [10–12,29,30]. A cross-sectional study comprising 161 NAFLD patients and 149 healthy participants found that increased serum AA levels were associated with NAFLD development in orthogonal projections to latent structures discriminant analysis (a regression modeling provides insights into separations between experimental groups) [10]. Similarly, another cross-sectional study, including 112 NAFLD patients and 112 controls, found that higher serum levels of AA were associated with NAFLD [12]. Results from a cross-sectional analysis including 59 Hispanic adolescents showed that increasing intake of AA was associated with cirrhosis in NAFLD (β = 1.14; P = 0.03) [11]. Another cross-sectional study with 77 elevated liver enzymes patients showed that plasma AA levels were higher in NAFLD patients relative to healthy controls in several phospholipid species, especially in phosphatidylserine [29]. In addition, certain plasma AA metabolites were observed to be significantly higher in NAFLD compared with healthy controls in a study with 19 biopsy-confirmed NAFLD patients [30]. Our results supported these studies and strengthened the causal potential of the association between AA levels and NAFLD. However, there were other studies with contradictory findings or an absence of an association between AA and NAFLD [9,31]. One prospective

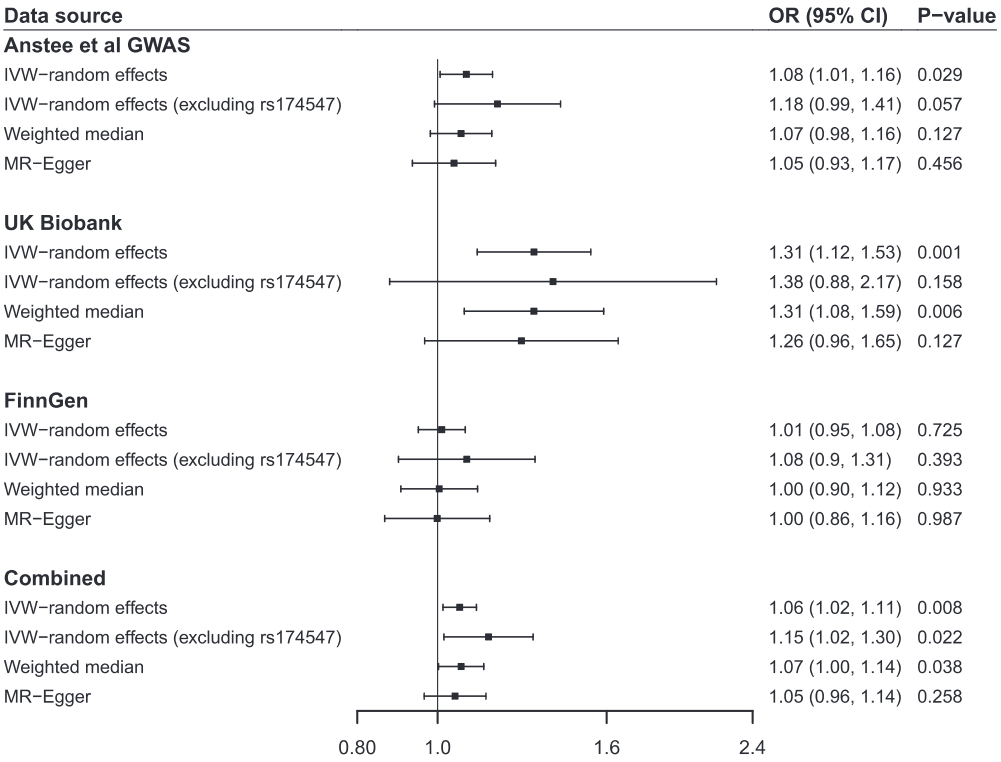

Fig. 2. Associations of plasma phospholipid AA levels with risk of non-alcoholic fatty liver disease (NAFLD) in MR analyses. The ORs of NAFLD were scaled to a per SD increase in AA level. AA, arachidonic acid; CI, confidence interval; IVW, inverse-variance weighted; MR, Mendelian randomization; OR, odds ratio; SD, standard deviation.

study involving 80 obese children found no association between circulating AA levels and NAFLD [9]. The discrepancy may be caused by the different population and the small sample size. Beyond that, observational studies are prone to be biased by reverse causation and residual confounding, which might also explain the discrepancy.

Previous cross-sectional studies found decreased AA levels in patients with liver cirrhosis compared with healthy controls [32,33]. However, the present MR investigation observed that genetically predicted high levels of AA were positively associated with cirrhosis risk. The potential residual confounders and reverse causality in cross-sectional studies may result in the different associations. Previous study also suggested that AA levels were higher in the liver cancer patients than that in the cancer-free controls [34,35], which was not observed in the present study. The null finding of our study might be caused by a relatively small sample size that led to limited statistic power to detect the weak association. Thus, the association between AA levels and liver cancer needs further verification.

Our novel albeit preliminary findings have important clinical implications. There are no proved medications for NAFLD. The primary therapeutic strategy is lifestyle and diet modification [36].

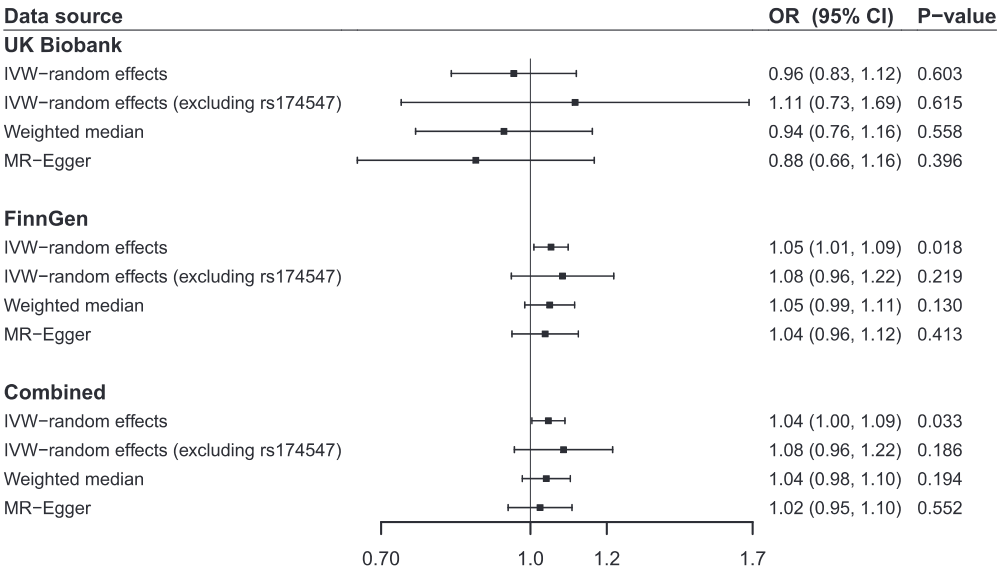

Fig. 3. Associations of genetically predicted per SD increase in AA with cirrhosis. The ORs of cirrhosis were scaled to a per SD increase in AA level. AA, arachidonic acid; CI, confidence interval; IVW, inverse-variance weighted; MR, Mendelian randomization; OR, odds ratio; SD, standard deviation.

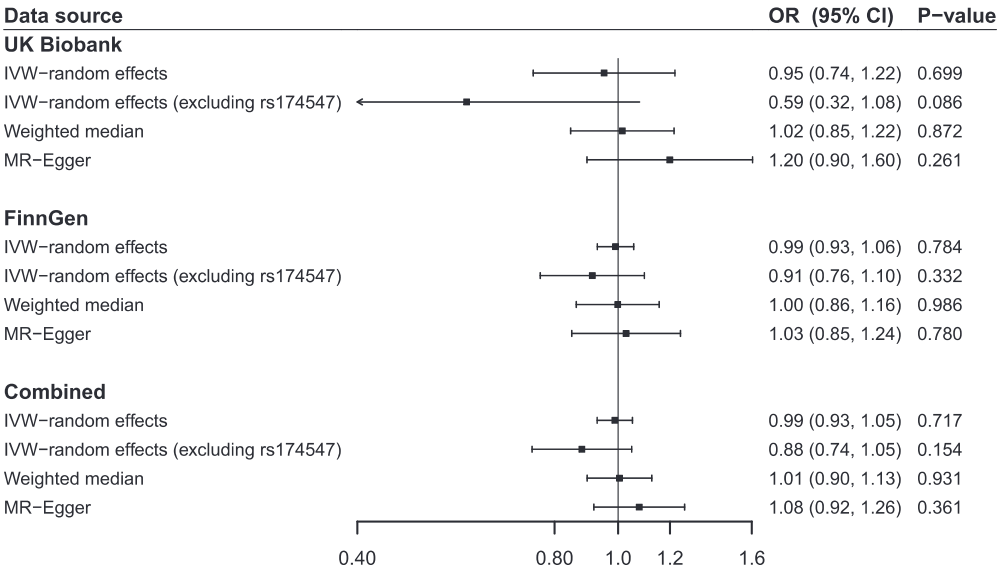

Fig. 4. Associations of genetically predicted per SD increase in AA with liver cancer. The ORs of liver cancer were scaled to a per SD increase in AA level. AA, arachidonic acid; CI, confidence interval; IVW, inverse-variance weighted; MR, Mendelian randomization; OR, odds ratio; SD, standard deviation.

The study found that lowering circulating levels of AA might reduce the risk developing NAFLD, which implies that recommending a diet with low AA intake may be a nutritional prevention strategy for NAFLD given that plasma AA composition relates to dietary or supplemental AA intake dose dependently over a wide range of AA intake (82−3600 mg/d) [37]. In addition, AA is a proinflammatory factor and its derived eicosanoids are associated with inflammation [38]. Thus, the positive association between AA and NAFLD partly suggests that inflammation may play a role in the development of NAFLD. Anti-inflammation therapies may benefit NAFLD prevention.

The correlations between AA and NAFLD have been observed in cell and animal studies. An in vitro study observed that in human hepatoma HepG2 cells, a high AA−to−docosahexaenoic acid (DHA) ratio reduced mitochondrial activity and increased triacylglycerol accumulation, which are critical features of NAFLD development [39]. An animal study revealed that a high-fat diet promoted an increase in liver and plasma AA levels, which paralleled the development of inflammation in NAFLD models [8]. Several underlying mechanisms have been proposed to explain the harmful effect of high plasma phospholipid AA levels on NAFLD risk. Animal studies have speculated that aggregatory mediators of AA (e.g., prostaglandin E2, thromboxane A2, and leukotriene B4) are predominantly proinflammatory, leading to chronic inflammation in pericentral hepatocytes [40,41]. High-level plasma AA may also promote lipotoxicity and progressive liver injury by increasing the risk of insulin resistance [42,43]. Furthermore, an accumulation of AA in the plasma and tissue might increase NAFLD risk by inhibiting the antiinflammatory effects of the n-3 PUFAs eicosapentaenoic acid and DHA [44]. As for liver enzymes, aggregatory mediators of AA may facilitate slight hepatocellular injury, causing the elevations of ALT rather than AST, which may be due to the former located in the cytoplasm and the latter in the mitochondria [45].

There are several strengths in the present study. The key strength is that the MR design diminishes reverse causality and confounding bias compared with observational studies. Additionally, similar results in three independent populations and consistent results from sensitivity analyses gave a boost to the reliability of our findings. We separated two sets of instrumental variables dependent or independent of rs174547, which also associates with other fatty acids, and distinguished AA's effects from this pleiotropic SNP. The consistent associations for the two sets of instrumental variables strengthened the validity of the observed relationship

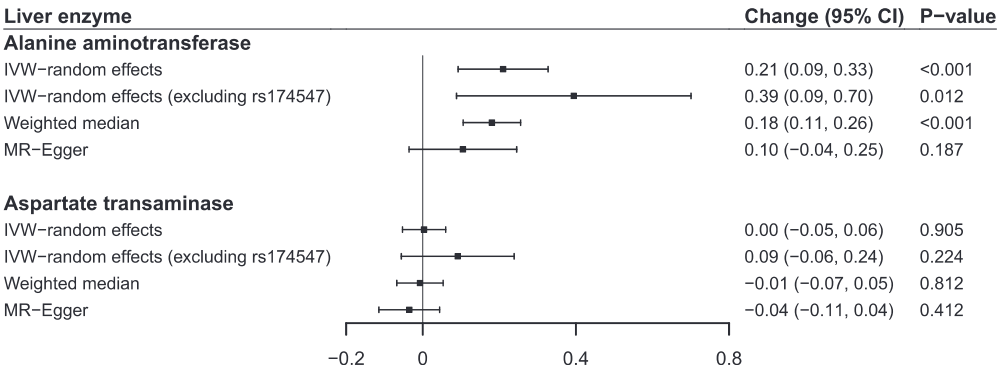

Fig. 5. Associations of plasma phospholipid AA levels with levels of the liver enzymes ALT and AST in MR analyses. The ORs of NAFLD were scaled to a per SD increase in AA level. AA, arachidonic acid; AL, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; IVW, inverse-variance weighted; MR, Mendelian randomization; OR, odds ratio; SD, standard deviation.

between AA and NAFLD. We used the liver function markers as secondary outcome measures to reflect liver dysfunction. The positive association between AA and ALT partly supported the associations of high AA levels with an increased risk of NAFLD.

We recognize several limitations. First, we cannot completely rule out that the AA-associated SNPs might affect NAFLD outcomes through other pathways (horizontal pleiotropy). In detail, except for rs174547, the genetic instruments for AA were not associated with any n-3 PUFA at the genome-wide significance. However, four variants (rs2903922, rs472031, rs760306, and rs1741) were associated with linoleic acid, and two SNPs (rs472031 and rs1741) were associated with dihomo-γ-linolenic acid at the genome-wide significance [46]. We obtained consistent results by excluding the pleiotropic SNP (rs174547) and did not detect any indications of horizontal pleiotropy in MR-Egger regression or MR-PRESSO analyses, which indicated a negligible distortion by potential pleiotropy. Second, the number of cases of NAFLD is relatively small because of the stringent definition in the UK Biobank and FinnGen studies, which might misclassify cases as non-cases and thus lead to underestimation. In addition, the small number of NAFLD cases might cause inadequate power for certain analyses, like the analysis in the FinnGen study with a positive trend, albeit non-significant. To increase the power, we conducted a meta-analysis of MR associations from three data sets and obtained a stable combined association. Another shortcoming is that the role of plasma phospholipid AA levels on the development of NAFLD might not correspond to that of exogenous AA supplementation. Despite this, plasma AA levels are dose-dependently correlated with dietary AA intake over a wide range of AA consumption [37]. Fourth, our findings might not be generalized to individuals of non-European ancestry or those with special diet preferences, like vegetarians. Finally, we could not assess the nonlinear association between AA levels and NAFLD risk in the present analysis based on summary-level data.

Conclusions

To summarize, our MR study found that genetically predicted higher levels of plasma phospholipid AA were associated with an increased risk of NAFLD and cirrhosis. More studies are needed to investigate potential mechanisms and corresponding therapeutic interventions.

CRediT authorship contribution statement

Jie Chen: Methodology, Software, Formal analysis, Investigation. **Shuai Yuan:** Conceptualization, Methodology, Software, Formal analysis, Investigation. **Susanna C. Larsson:** Conceptualization.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nut.2022.111910.

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