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Liver fat content, non-alcoholic fatty liver disease, and ischaemic heart disease: Mendelian randomization and meta-analysis of 279 013 individuals

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Aims In observational studies, non-alcoholic fatty liver disease (NAFLD) is associated with high risk of ischaemic heart disease (IHD). We tested the hypothesis that a high liver fat content or a diagnosis of NAFLD is a causal risk factor for IHD.

Methods and results In a cohort study of the Danish general population ($n = 94\,708$/IHD = 10 897), we first tested whether a high liver fat content or a diagnosis of NAFLD was associated observationally with IHD. Subsequently, using Mendelian randomization, we tested whether a genetic variant in the gene encoding the protein patatin-like phospholipase domain containing 3 protein (*PNPLA3*), I148M (rs738409), a strong and specific cause of high liver fat content and NAFLD, was causally associated with the risk of IHD. We found that the risk of IHD increased stepwise with increasing liver fat content (in quartiles) up to an odds ratio (OR) of 2.41 (1.28–4.51)(P-trend = 0.004). The corresponding OR for IHD in individuals with vs. without NAFLD was 1.65 (1.34–2.04)($P = 3\times10^{-6}$). *PNPLA3* I148M was associated with a stepwise increase in liver fat content of up to 28% in MM vs. II-homozygotes (P-trend = 0.0001) and with ORs of 2.03 (1.52–2.70) for NAFLD ($P = 3\times10^{-7}$), 3.28 (2.37–4.54) for cirrhosis ($P = 4\times10^{-12}$), and 0.95 (0.86–1.04) for IHD (P = 0.46). In agreement, in meta-analysis ($N = 279\,013$/IHD = 71 698), the OR for IHD was 0.98 (0.96–1.00) per M-allele vs. I-allele. The OR for IHD per M-allele higher genetically determined liver fat content was 0.98 (0.94–1.03) vs. an observational estimate of 1.05 (1.02–1.09)(P for comparison = 0.02).

Conclusion Despite confirming the known observational association of liver fat content and NAFLD with IHD, lifelong, genetically high liver fat content was not causally associated with risk of IHD. These results suggest that the observational association is due to confounding or reverse causation.

Keywords Cardiovascular disease • Causality • Epidemiology • Genetics • Liver disease

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Introduction

Non-alcoholic fatty liver disease (NAFLD) has reached epidemic proportions and now affects approximately 25% of the adult European population.[1,2] The disease begins with the accumulation of fat in the liver (steatosis) and may over time lead to inflammation of the liver (steatohepatitis) and ultimately to end-stage liver disease such as cirrhosis and hepatocellular carcinoma. Previously an uncommon disease, NAFLD is now the second most common indication for liver transplantation in the USA.[3]

In observational epidemiological studies, NAFLD has been associated with an increased risk of a range of extrahepatic disorders, including atherosclerosis and ischaemic heart disease (IHD),[4,5] leading to the hypothesis that NAFLD might be a causal risk factor for IHD. However, the association between NAFLD and IHD might also be due to shared underlying risk factors (confounding) or IHD might causally influence the development or progression of NAFLD (reverse causation).

One way to circumvent confounding and reverse causation is to use genetic variants as proxies for the exposure of interest (liver fat content and NAFLD), a method known as Mendelian randomization.[6] If liver fat content and NAFLD contributes causally to the development of IHD, genetic variants that cause a high liver fat content and high risk of NAFLD should also cause a high risk of IHD (*Take home figure*).[7]

A prerequisite for any Mendelian randomization study is the existence of genetic variation that robustly and specifically associates with the exposure of interest. A common variant in the gene encoding the protein patatin-like phospholipase domain containing 3 protein (*PNPLA3*), rs738409, is ideally suited as a proxy for liver fat content.[8] This variant is a cytosine to guanine substitution that changes codon 148 in PNPLA3 from isoleucine to methionine (I148M). Physiological expression of the M-allele is strongly associated with increased hepatic fat content, and with high risk of the entire spectrum of NAFLD, without being associated with other risk factors for IHD, including body mass index (BMI) and plasma lipid levels.[8,9] This is in contrast to a variant in *TM6SF2*, E167K, which associates robustly with NAFLD but, in addition, associates with a 13% reduction in plasma levels of both LDL cholesterol and triglycerides.[10] Because these reductions in well-known risk factors for IHD are likely to reduce risk of IHD *per se*, genetic variants in *TM6SF2* are problematic to use as instruments for Mendelian randomization studies addressing causality of NAFLD for IHD risk.

Using a Mendelian randomization design, we tested the hypothesis that lifelong high liver fat content or a diagnosis of NAFLD is a causal risk factor for IHD. First, we tested whether a high liver fat content or a diagnosis of NAFLD was associated observationally with increased risk of IHD; second, whether the M-allele of the *PNPLA3* I148M genotype was causally associated with high liver fat content and NAFLD as expected[8]; third, whether the M-allele was associated directly with a high risk of IHD in the Danish general population, and in meta-analysis of 279 013 individuals, including 71 698 cases with IHD. Furthermore, using instrumental variable analysis, we determined whether the causal effect of genetically high liver fat content on risk of IHD was consistent with the observational association between liver fat content and risk of IHD. Finally, in additional analyses, we examined the risk of IHD as a function of *TM6SF2* E167K genotype alone or combined with *PNPLA3* I148M.

Methods

For information on participants, diagnoses of IHD, NAFLD, and liver cirrhosis, measurement of liver fat content, genotyping, laboratory analyses, covariates, and statistical analyses, see Supplementary material online, Methods.

Results

A flowchart of the study design and the available data are shown in *Figure 1*. Baseline characteristics of study participants by IHD event and computed tomography (CT) scan status are shown in *Table 1* and Supplementary material online, *Table S1*, respectively. Most major risk factors for IHD were similarly distributed among *PNPLA3* I148M genotypes and were therefore unlikely to confound the results (see Supplementary material online, *Table S2*). *PNPLA3* I148M genotype was associated with a modest 0.02 mmol/L (1.3%) lower HDL cholesterol and a 2% lower frequency of lipid-lowering therapy in MM vs. II homozygotes ($P = 0.005$ and 0.02, respectively; see Supplementary material online, *Table S2*). I148M genotype did not differ from Hardy–Weinberg equilibrium ($P = 0.08$).

Liver fat content, non-alcoholic fatty liver disease, and risk of ischaemic heart disease: observational analyses

The distribution of CT liver attenuation measurements in the 1439 individuals in the CGPS was similar to that found in previous studies (see Supplementary material online, *Figure S1*). Increasing liver fat content causes a decrease in CT liver attenuation [i.e. decreased Hounsfield Units (HUs)].[11,12] We divided HUs into quartiles, with the 4th quartile of HUs (=lowest amount of hepatic fat) acting as the reference group. Mean HUs were 67.5 in the 4th quartile and 42.8 in the 1st quartile, corresponding to a liver fat content of 0.3% and 12.6%, respectively (percent liver fat content was -5× HUs + 340/10).[13]

Increasing liver fat content was associated with a stepwise increased risk of IHD, with multifactorially (age, gender, study, hypertension, smoking, and physical activity) adjusted odds ratios (ORs) of up to 2.41 (1.28–4.51) for the top vs. the lowest quartile (*Figure 2*, top left panel; $P = 0.004$). This association was similar after additional adjustment for alcohol consumption [OR = 2.39 (1.27–4.47)] (*Figure 2*, top middle panel; *P*-trend = 0.004) or diabetes [OR = 2.32 (1.23–4.37)] (*Figure 2*, top right panel; *P*-trend = 0.007). Adding lipid-lowering therapy or BMI to the multivariate model attenuated the association [ORs 1.81 (0.94–3.48) and 1.74 (0.85–3.57), respectively] (*Figure 2*, top 2nd and 4th panels from left; *P*-trend = 0.05 and 0.12), suggesting that BMI and lipid-lowering therapy were confounders of the observational estimate between liver fat content and risk of IHD. In agreement, a diagnosis of NAFLD among the 94 708 participants in the Copenhagen Studies was associated with an increased risk of IHD in the multifactorially adjusted model with an OR of 1.65 (1.34–2.04) (*Figure 2*, bottom left panel; $P = 3 \times 10^{-6}$). This association was similar

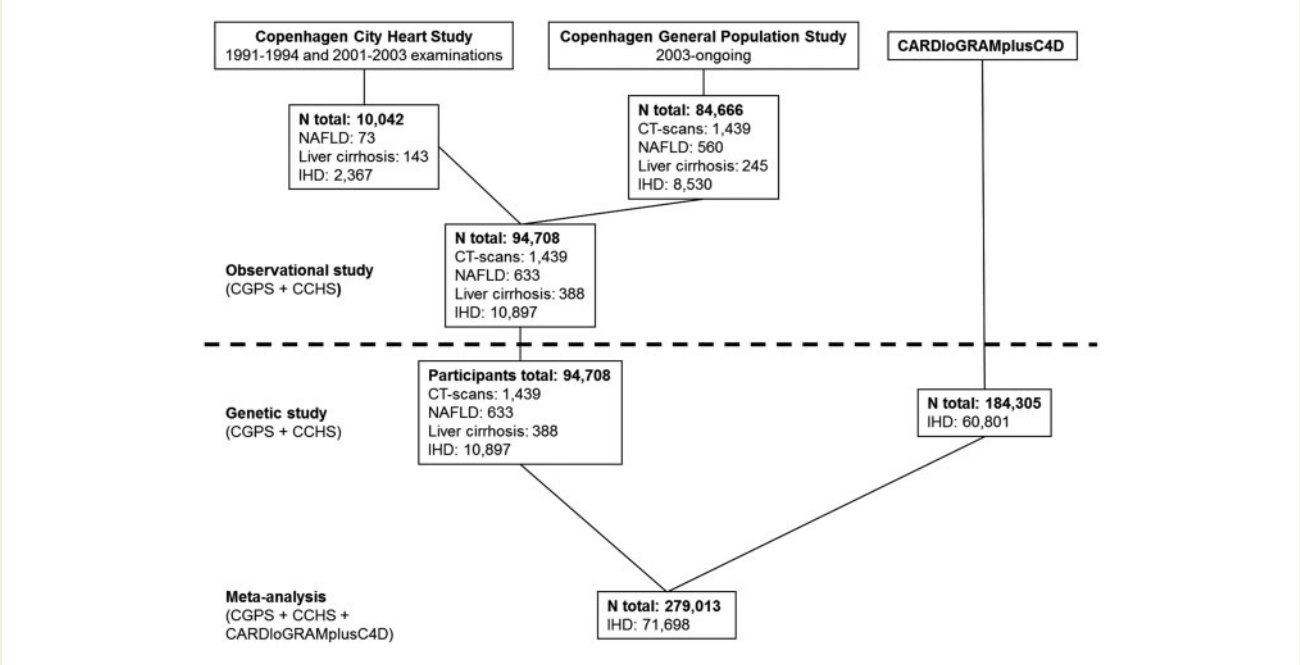


Figure 1 Flowchart of the study design and available information on participants in the CCHS, the CGPS, and the CARDIoGRAMplusC4D consortium. CT, computerized tomography scan; IHD, ischaemic heart disease; NAFLD, non-alcoholic fatty liver disease.

Table 1 Baseline characteristics of participants

	No event	Ischaemic heart disease
Number of individuals (%), *n* (%)	83 811 (88)	10 897 (12)
Age (years), median (IQR)	56 (46–65)	67 (59–74)[a]
Women, *n* (%)	47 587 (57)	4712 (43)[a]
Body mass index (kg/m^2), median (IQR)	25 (23–28)	27 (24–30)[a]
Hypertension, *n* (%)	46 954 (57)	8153 (76)[a]
Diabetes mellitus, *n* (%)	2640 (3)	1009 (9)[a]
Low physical activity, *n* (%)	41 195 (50)	6352 (60)[a]
Smoking, *n* (%)	17 348 (21)	2800 (26)[a]
Alcohol intake (units/week), median (IQR)	4 (2–7)	4 (2–8)[a]
Cholesterol (mmol/L), median (IQR)	5.70 (5.00–6.40)	5.88 (5.13–6.70)[a]
LDL cholesterol (mmol/L), median (IQR)	3.30 (2.70–4.00)	3.41 (2.80–4.17)[a]
HDL cholesterol (mmol/L), median (IQR)	1.57 (1.25–1.94)	1.44 (1.15–1.80)[a]
Triglycerides (mmol/L), median (IQR)	1.41 (0.98–2.10)	1.78 (1.24–2.60)[a]
Lipid-lowering therapy, *n* (%)	6316 (8)	3354 (31)[a]

P-values by the Mann–Whitney *U* test or the Pearson's χ^2 test.
[a]P<0.001: vs. individuals with no event.

when adding alcohol consumption to the model but was attenuated by adjustment for lipid-lowering therapy, BMI, or diabetes (*Figure 2*, bottom 2nd, 4th, and 5th panels from left; *P*-values: 4×10^{-5}, 9×10^{-5}, and 6×10^{-5}, respectively).

PNPLA3 I148M genotype and liver fat content, plasma markers of liver function, and inflammation

PNPLA3 I148M genotype was associated with relative increases in liver fat content of 18% in IM-heterozygotes and 28% in MM-homozygotes vs. II-homozygotes (absolute mean percent liver fat content: II = 5.1%, IM = 6.0%, and MM = 6.5%) (*Figure 3*, left panel; *P*-trend = 0.0001), and with corresponding increases in plasma levels of alanine aminotransferase, marking steatosis-mediated liver cell damage (see Supplementary material online, *Table S3*; *P*-trend = 3×10^{-40}). Genotype was not consistently associated with other liver parameters (alkaline phosphatase, gamma-glutamyltransferase, bilirubin, albumin, and coagulation factors II, VII, and X), or with markers of systemic inflammation (high sensitivity C-reactive protein; see Supplementary material online, *Table S3*).

PNPLA3 I148M genotype and risk of non-alcoholic fatty liver disease, cirrhosis, and ischaemic heart disease: genetic analyses

Among the 94 708 individuals in the Copenhagen Studies, 633 had a diagnosis of NAFLD, 388 had liver cirrhosis, and 10 897 had IHD. In individuals with the *PNLPA3* MM vs. II genotype, in whom there was a stepwise increase in liver fat across genotypes (*Figure 3*, left panel; P-trend = 0.0001), the age-, gender-, and study-adjusted ORs increased stepwise up to 2.03 (1.52–2.70) for NAFLD (*Figure 3*,

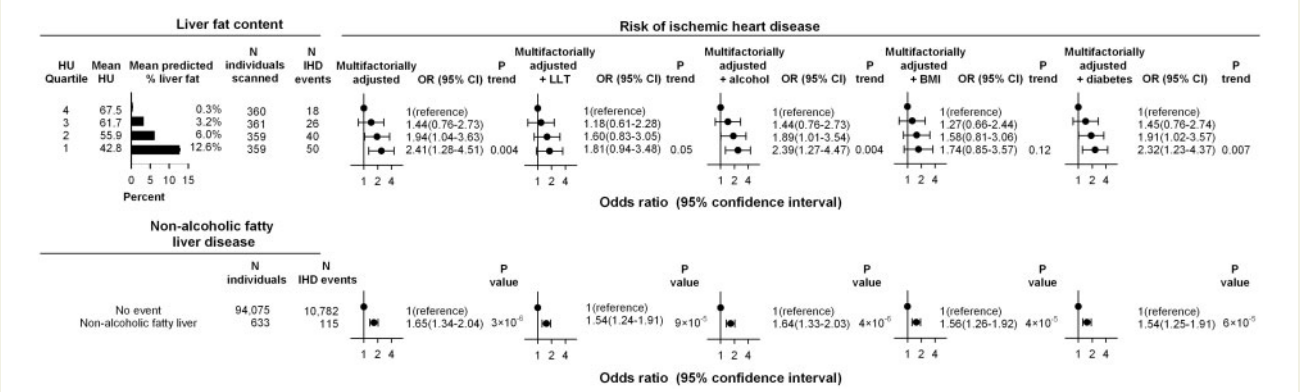


Figure 2 Risk of ischaemic heart disease (IHD) as a function of increasing liver fat content or a clinical diagnosis of non-alcoholic fatty liver disease (NAFLD). Top left: Liver fat content, measured as liver attenuation by computerized tomography scan in Hounsfield Units (HUs), in 1439 participants in the CGPS divided into quartiles, with decreasing quartiles (decreasing HUs) corresponding to increasing percent liver fat content. Mean percent liver fat was: (-5 × HUs + 340)/10 [total lipid (mg/g wet liver) × 10^{-3} × 100].[12] Error bars indicate standard error of the mean. Risk of IHD as a function of either liver fat content (top) or a clinical diagnosis of NAFLD (bottom) in 94 708 participants in the CCHS and the CGPS combined. Multifactorial adjustment was for age, gender, study, hypertension, smoking, and physical activity, and additionally for lipid-lowering therapy (LLT), alcohol consumption, body mass index (BMI), or diabetes. *N*, number; OR, odds ratio. *P*-values are for tests for trend or Wald test.

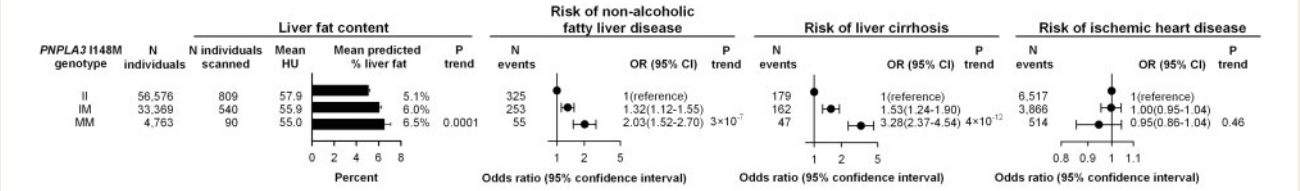


Figure 3 Liver fat content and risk of non-alcoholic fatty liver disease (NAFLD), liver cirrhosis, and ischaemic heart disease (IHD) as a function of *PNPLA3* I148M genotype. Left: Measurements of liver attenuation by computerized tomography scan in Hounsfield Units (HUs), and percent liver fat content in 1439 participants in the CGPS. Mean percent liver fat was: (-5 × HUs + 340)/10 [total lipid (mg/g wet liver) × 10^{-3} x 100].[13] Error bars indicate standard error of the mean. Middle and right: Risk of NAFLD, liver cirrhosis and IHD as a function of *PNPLA3* I148M genotype, adjusted for age, gender and study in the 94 708 participants in the CCHS and the CGPS combined. HU, Hounsfield Unit; *N*, number; OR, odds ratio. *P*-values are for tests for trend.

middle left panel; *P*-trend = 3×10^{-7}) and up to 3.28 (2.37–4.54) for liver cirrhosis (*Figure 3*, middle right panel; *P*-trend = 4×10^{-12}). In contrast, *PNPLA3* I148M genotype did not associate with risk of IHD; the corresponding ORs were 1.00 (0.95–1.04) for IM and 0.95 (0.86–1.04) for MM vs. II genotypes (*Figure 3*, right panel; *P*-trend = 0.46).

In sensitivity analyses, we additionally adjusted for factors that were modestly associated with *PNPLA3* genotype in the present or in previous studies (total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, lipid-lowering therapy, red blood cell traits: haemoglobin, mean cell volume (MCV), mean cell haemoglobin concentration (MCHC), erythrocyte count, erythrocyte volume fraction (haematocrit), diabetes, or alanine aminotransferase (see Supplementary material online, *Tables S2* and *S4*).[14–16] The results were largely similar in these analyses (see Supplementary material online, *Figure S2* and data not shown). We also tested for interaction between *PNPLA3* I148M genotype and major risk factors for IHD (age, gender, body mass index, hypertension, diabetes, physical activity, smoking, alcohol intake, total cholesterol, LDL cholesterol HDL cholesterol, triglycerides, or use of lipid-lowering therapy) on risk of IHD. There were no interactions (see Supplementary material online, *Figure S3*).

We tested whether potential confounding factors were associated with liver fat content, NAFLD, liver cirrhosis, IHD, and *PNPLA3* genotype. Potential confounders were dichotomized, and for each confounder, logistic regression analysis was used to calculate gender- and age-adjusted ORs and *P*-values for, respectively, a one-quartile increase in liver fat content, NAFLD vs. no event, liver cirrhosis vs. no event, IHD vs. no event, and a one-category (per M-allele) change in genotype (see Supplementary material online, *Figure S4*). Most or all of these risk factors for IHD were associated with increased liver fat, NAFLD, cirrhosis, and IHD and, therefore, constitute potential confounders for the observational associations between liver fat content

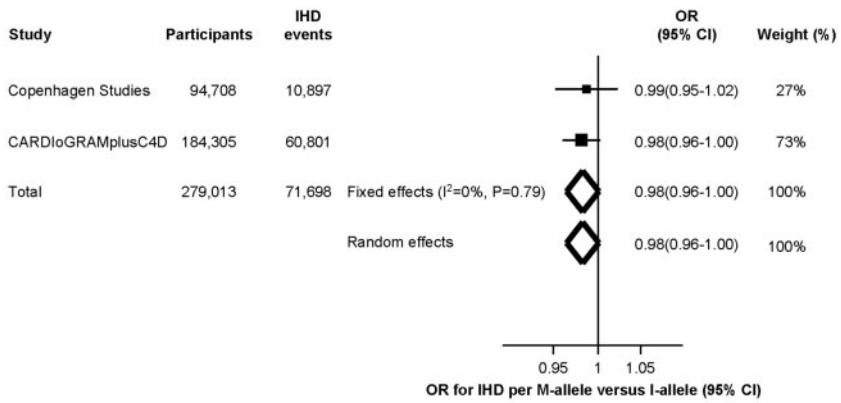


Figure 4 Meta-analysis of odds ratio for ischaemic heart disease per M-allele of the *PNPLA3* I148M genotype vs. I-allele. Analyses included 94 708 individuals from the CGPS and CCHS (=Copenhagen Studies), and genetic risk estimates on up to 184 305 individuals from the CARDIoGRAMplusC4D consortia at www.cardiogramplusc4d.org separately and combined into fixed-effects and random-effects models in meta-analysis. Odds ratios were adjusted for age, gender, and study in the Copenhagen Studies, and for study-specific covariates in CARDIoGRAMplusC4D. OR, odds ratio; I^2 = percent between study variability due to heterogeneity between studies; *P* = heterogeneity assessed by *Q* statistics.

and risk of NAFLD and IHD. In contrast, a one-category (per M-allele) change in genotype was not associated with most of these potential confounders, with the exception of modest associations with lower levels of HDL- and total cholesterol ($P = 0.005$ and 0.04), and with less use of lipid-lowering therapy ($P = 0.01$), suggesting that genotype could be used as a largely unconfounded proxy for the effect of liver fat content on risk of NAFLD, liver cirrhosis, and IHD.

To maximize the statistical power, we combined the estimates for the association between *PNPLA3* I148M and IHD in the Copenhagen Studies with publicly available data from the CARDIoGRAMplusC4D consortium,[17] yielding a total of 279 013 individuals of whom 71 698 had IHD. In a meta-analysis, ORs for IHD per *PNPLA3* I148M M-allele, associated with high liver fat, were 0.99 (0.95–1.02) in the Copenhagen Studies and 0.98 (0.96–1.00) in CARDIoGRAMplus C4D (*Figure 4*, top). The overall ORs for IHD per M-allele were 0.98 (0.96–1.00) using a fixed-effects model ($I^2 = 0\%$; $P = 0.79$) and 0.98 (0.96–1.00) using a random-effects model (*Figure 4*, bottom).

Finally, we tested the association of *PNPLA3* I148M genotype with risk of IHD using a recessive model (MM vs. II + IM genotype). In this model, ORs for IHD were 0.95 (0.86–1.04) in the Copenhagen Studies and 0.92 (0.87–0.97) in CARDIoGRAMplusC4D (see Supplementary material online, *Figure S5*). The overall OR for IHD for MM vs II + IM was 0.93 (0.88–0.97) in both fixed- and random-effects models ($I^2 = 0\%$; $P = 0.61$) (see Supplementary material online, *Figure S5*).

Liver fat content and risk of ischaemic heart disease: causal genetic estimates

Under the hypothesis that a high liver fat content causes IHD, a lifelong high liver fat content resulting from genetic variation should confer a similar high risk of IHD as that observed for a comparable higher liver fat content in the general population. We examined the potential causal effect of high liver fat content on risk of IHD using instrumental variable analysis, and as positive controls, we included the corresponding causal genetic estimates for NAFLD and liver cirrhosis.

The OR for a per M-allele increase in genetically determined liver fat content in the 94 708 participants in the Copenhagen Studies was 0.98 (0.94–1.03) (*Figure 5* bottom; $P = 0.47$). A similar increase in observationally determined liver fat content among the 1439 participants with CT scans available was associated with a multifactorially adjusted OR for IHD of 1.05 (1.02–1.09) (*Figure 5* top; $P = 0.004$), and this observational estimate differed from the causal genetic estimate (*Figure 5*; *P* for comparison = 0.02). In contrast, the causal genetic estimates for a similar increase in liver fat content were 1.23 (1.08–1.4) for NAFLD and 1.41 (1.15–1.72) for liver cirrhosis (data not shown), validating the genetic instrument. *PNPLA3* genotype explained 1.1% ($=R^2$) of the inter-individual variation in liver fat content, and the genetic instrument had an *F*-score of 15.

PNPLA3 I148M and *TM6SF2* E167K genotypes, liver fat content, and risk of non-alcoholic fatty liver disease, cirrhosis, and ischaemic heart disease

To increase the power of the genetic instrument, we combined the two strongest genetic risk factors for NAFLD, *PNPLA3* I148M and *TM6SF2* E167K, into an allele score. An increasing number of NAFLD-promoting alleles was associated with stepwise increases in liver fat content, and with increased risk of NAFLD and cirrhosis, but not with risk of IHD (see Supplementary material online, *Figure S6*). Compared to individuals with 0 NAFLD-promoting alleles, those carrying 3–4 NAFLD-promoting alleles had relative increases in liver fat content of 67% (absolute mean percent liver fat content: 0 alleles = 4.9%, 3–4 alleles = 8.2%) (see Supplementary material online, *Figure S6*, left panel; *P*-trend = 7×10^{-5}) and ORs for NAFLD, cirrhosis, and IHD of 2.89 (1.76–4.74), 5.03 (2.97–8.51), and 0.88 (0.71–1.09),

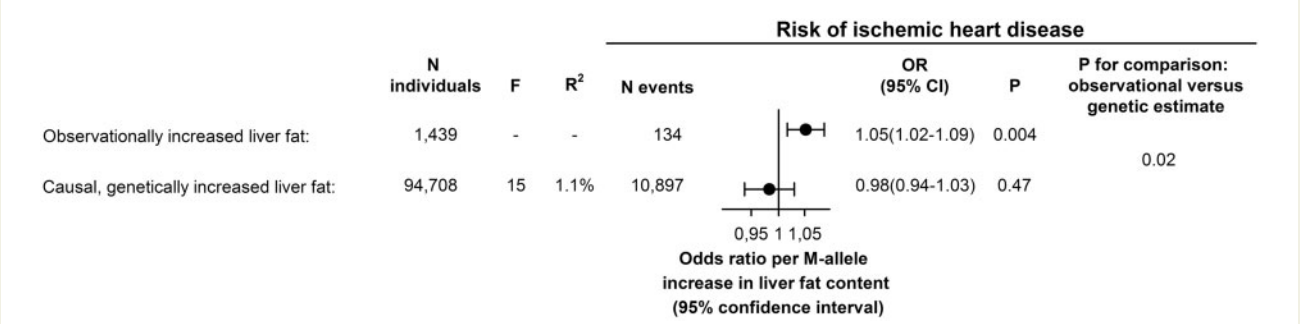


Figure 5 Risk of ischaemic heart disease (IHD) for a per M-allele increase in genetically determined liver fat content, and for a similar increase in observationally determined liver fat content using instrumental variable analysis. First, gender- and age-adjusted regression analysis was used to determine the increase in liver fat content per *PNPLA3* I148M M-allele in 1439 participants in the CGPS. Second, logistic regression was used to calculate the log odds for the per M-allele association with IHD, adjusted for age, gender, and study, among the 94 708 participants in the CGPS and CCHS combined. Finally, the causal, genetic effect of increased liver fat on IHD was determined as the exponentiated Wald estimate from the ratio between the second (IHD vs. genotype) and first (liver fat vs. genotype) regression. The exponentiated Wald estimate was compared to the multifactorially adjusted observational estimate of a similar increase in liver fat content using a generalized Hausman test. Strength of the genetic instrument (association of M-allele with liver fat content) was evaluated by *F*-statistics from the first-stage regression, whereas R^2 is used as a measure of percent contribution of genotype to the variation in liver fat content. Observational estimates were adjusted for age and gender, or multifactorially for age, gender, study, hypertension, smoking, and physical activity. The genetic estimate was adjusted for age, gender, and study.

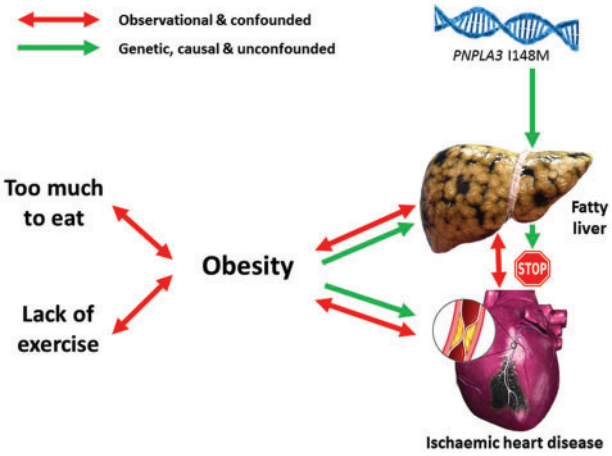
respectively (see Supplementary material online, *Figure S6*; *P* for trend: 2×10^{-8}, 1×10^{-11}, and 0.26).

The associations of *TM6SF2* E167K alone with risk of IHD in the Copenhagen Studies, in CARDIoGRAMplusC4D, and in a meta-analysis of these studies, are shown in Supplementary material online, *Figure S7*. The K-allele, which we have previously shown associates with up to 13% lower plasma levels of both LDL cholesterol and triglycerides,[10] was associated with ORs for IHD of 0.98 (0.93–1.03) in the Copenhagen Studies, 0.95 (0.92–0.98) in CARDIoGRAMplusC4D, and 0.96 (0.93–0.99) in the combined studies, in both fixed- and random-effects models ($I^2 = 0$; $P = 0.42$; see Supplementary material online, *Figure S7*).

Discussion

To our knowledge, this is the first study to examine the causal association of liver fat content with risk of IHD using a Mendelian randomization approach, with *simultaneous* assessment of liver fat content, the entire spectrum of NAFLD, IHD, and *PNPLA3* I148M genotype, a strong and specific genetic cause of high liver fat content and NAFLD. The main finding is that genetically high liver fat content is not associated with increased risk of IHD in the general population, despite causing a high risk of the entire spectrum of NAFLD. This implies that liver fat content and NAFLD are unlikely to be causal factors in the development of IHD (*Take home figure*). With up to 279 013 participants, including 71 698 cases with IHD, our study had sufficient statistical power to exclude even very small effects.

Observational epidemiological studies have consistently reported an association between high liver fat content and/or NAFLD and high risk of IHD.[4,5] These associations have led to the hypothesis that the entire spectrum of NAFLD (steatosis, steatohepatitis, and cirrhosis) may play a causal role in the development of atherosclerosis and related endpoints, including IHD. However, the association between NAFLD and IHD may have been influenced by confounding due to a number of common risk factors such as age, BMI, low physical activity, smoking, hypertension, and plasma levels of lipids and lipoproteins as demonstrated in this study, and/or due to reverse causation, inherent limitations of observational epidemiology. To avoid these limitations, we used Mendelian randomization, a method that can be likened to the randomized clinical trial, and takes advantage of the random assortment of alleles at conception, and the fact that genotypes remain constant throughout life. We used the genetic variant *PNPLA3* I148M as an unconfounded and lifelong proxy for liver fat content. The variant causes a substitution of methionine for isoleucine at amino acid residue 148 in PNPLA3, a lipid-droplet-associated protein expressed in the liver and adipose tissue.[9] Mice genetically engineered to express the 148 M isoform of PNPLA3 develop hepatic steatosis, similar to humans, demonstrating that the M-allele is the causal variant underlying the NAFLD phenotype.[9] In humans, the M-allele has a strong effect on the entire spectrum of NAFLD, with a two- to four-fold increased risk of liver steatosis, steatohepatitis, and cirrhosis for MM-homozygotes vs. II-homozygotes, and effects of IM-heterozygotes between that of MM and II, consistent with a co-dominant model of inheritance.[8] The steatogenic effect of the M-variant is amplified by obesity (BMI $\geq 30\,kg/m^2$), an example of gene × environment interaction.[18] The effect of the M-variant has been observed in multiple cohorts, in both adults and children, and in different ethnicities.[8,19] If increased liver fat content causally contributes to IHD, one would expect the M-allele of *PNPLA3* I148M to be associated with an increased risk of IHD. Counter to this, and despite demonstrating the expected genetically high risk of NAFLD and liver cirrhosis, we found that I148M was not associated with IHD either among 94 708 participants from the Danish general population including 10 897 with IHD, among 184 305 participants in the largest



Take home figure A high caloric intake combined with lack of excercise associates with (=leads to) obesity which observationally is associated with both non-alcoholic fatty liver disease (NAFLD) and ischaemic heart disease (IHD). Moreover, NAFLD is observationally associated with IHD. However, all these associations likely are confounded by common risk factors and may also be influenced by reverse causation. The most likely confounder of the association between NAFLD and IHD is obesity and causes of obesity such as a high caloric intake combined with lack of exercise. To circumvent confounding and reverse causation, we used a genetic variant in *PNPLA3*, I148M, as a proxy for liver fat content and NAFLD in a Mendelian randomization (MR) design (a so-called single locus *cis*-MR analysis of the patatin-like phospholipase domain containing 3 protein encoded by *PNPLA3*).[7] Here, we show that *PNPLA3* I148M genotype, a robust and specific cause of NAFLD, is not associated with risk of IHD. In other words, a high liver fat content or NAFLD is unlikely to cause IHD.

IHD GWAS, CARDIoGRAMplusC4D, including 60 801 IHD cases, or in meta-analysis of the combined studies totaling 279 013 participants of whom 71 698 had IHD. The interpretation of this lack of association is that high liver fat content is unlikely to cause high risk of IHD (*Take home figure*). In CARDIoGRAMplusC4D, MM-homozygotes even had a slightly lower risk of IHD than II-homozygotes and IM-heterozygotes combined.[20] However, this recessive effect was not replicated in the Copenhagen Studies.

The clinical implication of these data is that reducing liver fat content *per se* is unlikely to protect against IHD. However, it is worth noting that the lifestyle interventions currently recommended for the prevention or treatment of NAFLD (e.g. physical activity and restriction of caloric intake) are likely to also have beneficial effects on the risk of IHD.[21] Most individuals with NAFLD have increased risk of IHD, because they are obese and also have high levels of triglyceride-rich remnant lipoproteins in plasma. These lipoproteins are causally associated with increased risk of IHD through elevated remnant cholesterol.[22–24] This emphasizes the need to lower not only LDL cholesterol but also the cholesterol content of triglyceride-rich lipoproteins,[25,26] particularly in patients with NAFLD at high risk of IHD.

Several studies have found an association between the K-allele of *TM6SF2* E167K, another genetic variant robustly associated with NAFLD, and low risk of cardiovascular endpoints (i.e. the opposite of what would be expected from the association with high liver fat). However, in the Copenhagen Studies[10] and in other large studies,[27] the K-allele of *TM6SF2* E167K was associated with up to 13% lower plasma levels of both LDL cholesterol and triglycerides, likely explaining the lower risk of cardiovascular disease observed by us and others.[28]

The strengths of our study include the large sample size of individual participant data, the *simultaneous* assessment of liver fat content, NAFLD, IHD, and *PNPLA3* I148M genotype, the independent replication of the null finding in data from the largest published IHD GWAS, and the use of a strong and largely unconfounded genetic proxy for liver fat content. Another strength is that we looked at the entire spectrum of NAFLD. Because *PNPLA3* I148M associated strongly with high liver fat content, as well as with clinically diagnosed NAFLD and liver cirrhosis, we could also rule out that these individual components of NAFLD cause IHD. Finally, in the instrumental variable analysis, we included the causal, genetic estimates for NAFLD and liver cirrhosis as positive controls.

There are limitations to our study that deserve mentioning. Measurements of liver fat content were only available on 1439 individuals, a small subset of the total cohort. Due to the modest risk of radiation these individuals were older (inclusion criteria >40 years) than those not CT scanned. However, the association between *PNPLA3* I148M and liver fat content is firmly established for all age groups, and age-related modifications of its effect size have not been reported. In agreement, age was not a confounder of the per M-allele change in *PNPLA3* genotype in this study. An assumption of our study is that the steatogenic effect of the *PNPLA3* I148M variant applies to the entire cohort and to the participants from the IHD GWAS. This is likely a reasonable assumption, given the strong phenotypic effect of the variant. Only 633 (0.7% of the cohort) in the Danish studies had International Classification of Diseases (ICD)-defined NAFLD. There are several potential explanations for this low prevalence. First, the registry-based method used to define the endpoint (ICD code received in a hospital setting) likely means that we mainly captured the subset of symptomatic NAFLD patients. Second, until

recently, NAFLD was not widely recognized clinically, likely causing the disease to be underdiagnosed. Third, the median BMI in the Danish cohorts was 26 kg/m^2, lower than what is typically seen in American cohorts.[8] Supporting the validity of the ICD-based NAFLD endpoint used here is the fact that *PNPLA3* I148M was strongly associated with the endpoint, with odds ratios of comparable magnitude to those reported in previous studies that used imaging to define NAFLD.[19]

We mainly studied individuals of European descent, potentially reducing the generalizability of our findings. However, *PNPLA3* I148M is known to associate strongly with high liver fat content in individuals of non-European descent, including African Americans, Hispanics, and Asians.[19] There have been several GWAS of IHD conducted in individuals of non-European descent. The fact that *PNPLA3* I148M has not been detected in any of these GWAS supports that NAFLD is unlikely to cause IHD, regardless of ethnicity.

We used only one functional variant as a genetic instrument for liver fat. Confirming the null association with IHD for NAFLD-associated variants in other genes without effects on lipids and lipoproteins or other risk factors for IHD would be ideal. However, *PNPLA3* I148M is so far the strongest, and the only widely replicated genetic risk factor for NAFLD. No strong associations with *PNPLA3* I148M have been reported for other traits, although we could confirm marginal associations with HDL cholesterol and with red blood cell traits.[14–16] ICAM1 was not measured in this study.[29] Nevertheless, we cannot entirely rule out associations with factors that are not routinely measured (e.g. metabolomic traits). Importantly, any such associations would likely be directly caused by the M-allele, the so-called vertical pleiotropy, which is less problematic than horizontal pleiotropy in an MR setting.[7] The above-mentioned pleiotropic *TM6SF2* E167K variant and a common variant near the *MBOAT7-TMC4* genes have also been associated with risk of NAFLD.[10,30] The *MBOAT7-TMC4* SNP (rs641738) had a modest effect on hepatic steatosis,[30] and was not associated with risk of IHD in the CARDIoGRAMplusC4D GWAS ($P = 0.15$).[17] It is likely that additional genetic associations with NAFLD will be discovered in the future, allowing for new Mendelian randomization studies to further confirm the null association reported here.

In conclusion, despite confirming the known observational association of liver fat content and NAFLD with IHD, lifelong, genetically high liver fat content was not associated with a high risk of IHD. These results suggest that the observational association between liver fat content, NAFLD, and IHD is likely due to confounding or reverse causation.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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